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Tissue-Specific Protein Expression in Plant Mitochondria

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

Section of Genetics and Development, Biotechnology Building, Cornell University, Ithaca, New York 14853–2703

Although the physiological role of plant mitochondria is thought to vary in different tissues at progressive stages of development, there has been little documentation that the complement of mitochondrial proteins is altered in different plant organs. Because the phenomenon of cytoplasmic male sterility suggests an unusual function for mitochondria in floral buds, we examined the tissue-specific expression of mitochondrial proteins in petunia buds at several stages of development, using both fertile and cytoplasmic male sterile plants. On tissue prints of cryostat-sectioned buds, antibodies recognizing subunit A of the mitochondrial ATPase (ATPA) localized very differently from antibodies recognizing subunit II of the cytochrome oxidase (COXII), which indicated that mitochondria in the same tissue could differentially express mitochondrially encoded proteins. The petunia cytoplasmic male sterility-associated fused (*pcf*) gene encodes a protein that colocalized with ATPA and the nuclear-encoded mitochondrial alternative oxidase (AOA) in sporogenous tissues, where little COXII protein was found. These overlapping and differential localization patterns may provide clues to the molecular mechanism of cytoplasmic male sterility.

INTRODUCTION

The mitochondria of higher plants have numerous functions, from respiration and energy production to the creation of carbon skeletons (Dennis, 1987). This diversity of physiological function may be reflected in a diverse protein composition for mitochondria from different tissues at progressive stages of development; however, little information is available concerning the spatial and temporal regulation of expression for specific nuclear- or organellar-encoded mitochondrial proteins. It is known that alterations in mitochondrial protein synthesis are associated with aberrations in floral development of *Nicotiana* (Bonnett et al., 1991; Spangenberg et al., 1992) and that the alternative oxidase increases in abundance during the development of voodoo lily spadices (Elthon and McIntosh, 1989a).

A special role for mitochondria during anther development is suggested by the phenomenon of cytoplasmic male sterility (CMS), in which mitochondrial defects specifically disrupt pollen formation (Hanson and Conde, 1985; Hanson, 1991). In most cases, CMS plants appear morphologically normal, although viable pollen is not produced (Kaul, 1988). When the flowers of various CMS species are examined under the microscope, developmental aberrations are found to begin at any time from before meiosis through pollen maturation (Kaul, 1988).

In the petunia CMS system we have studied, degeneration of sporogenous tissue is first apparent in late meiosis, and several abnormalities are present by the stage at which fertile plants are producing tetrads (Bino, 1985a, 1985b; Conley et al., 1994). We correlated the CMS phenotype with the expression of the unique mitochondrial petunia CMS-associated fused gene (*pcf*), consisting of parts of the coding region of subunit 9 of the mitochondrial ATPase (*atp9*), cytochrome oxidase subunit II (*coxII*), and an unidentified reading frame called *urfS* (Young and Hanson, 1987; Nivison and Hanson, 1989; Hanson, 1991; Hanson et al., 1994). Expression of the *pcf* gene results in the accumulation of a 25-kD product that is post-translationally processed from a 43-kD precursor protein (Nivison et al., 1994). Plants that carry a dominant nuclear fertility-restoration gene and the CMS cytoplasm exhibit reduced levels of both the PCF protein and some *pcf* transcripts relative to sterile plants (Nivison and Hanson, 1989; Pruitt and Hanson, 1991).

Because mitochondria appear to have unique functions during pollen production, we selected developing anthers as the best organs to examine for possible tissue specificity of mitochondrial protein expression. We analyzed two pairs of isonuclear petunia lines, in which one member of each pair carries the CMS-encoding mitochondrial genome and is sterile, while the other member of the pair carries normal mitochondria and is fertile. One isonuclear pair contains a *P. hybrida* nuclear genome, while the other contains a *P. parodii* nuclear genome. We examined anthers from CMS and fertile petunia plants of both isonuclear pairs to determine whether differences in mitochondrial protein levels might suggest mechanisms for the molecular action of the developmental disruption. We analyzed tissue prints of anthers by probing with antibodies that recognized several nuclear and mitochondrially encoded proteins present in both CMS and

[1] To whom correspondence should be addressed.

wild-type plants, as well as an antibody to the unique CMS-associated 25-kD PCF protein. In this study, we provide direct evidence of differential localization of mitochondrially encoded proteins in situ at the tissue level.

RESULTS

Specificity of Antibody Probes

Using gel electrophoresis and immunoblotting, we examined the specificity of antibodies to the mitochondrially encoded proteins PCF, COXII, and subunit A of the mitochondrial ATPase (ATPA), and to the nuclear-encoded mitochondrial protein alternative oxidase (AOA). Figure 1 shows representative blots probed with a monoclonal antibody (MAb) recognizing ATPA and a purified antibody recognizing the 25-kD PCF protein. The purified antibody to PCF also recognized a faint higher molecular weight band that is present in both fertile and sterile plants (data not shown). All localization experiments using this antibody were performed simultaneously on isonuclear fertile and sterile buds of the same stage to control for possible detection of this nonspecific band. No signal was ever seen in fertile tissue. The MAbs to AOA and COXII are highly specific (Nivison and Hanson, 1987, 1989; Elthon et al., 1989b; Nivison et al., 1994; data not shown).

Floral Development in Petunia

Figure 2 shows representative buds of stages 1 to 6 and mature flowers from both *Petunia hybrida* and *P. parodii.* Anthers of stages 1 and 2 are premeiotic; stage 3 is just after meiosis of the fertile sporogenous tissue, as the tetrads are forming; and stages 4, 5, and 6 are progressively later during floral development (Conley et al., 1994). After anthesis, anthers of both fertile and male-sterile flowers dehisced similarly.

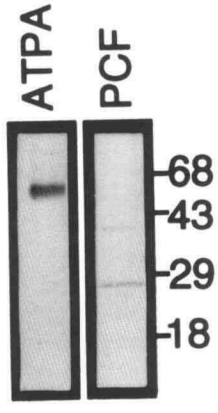

Figure 1. Specificity of Antibodies.

Immunoblots of total floral tip protein from sterile plants were probed with antibodies specific to the PCF protein and ATPA. The faint higher molecular mass band in the lane containing PCF is also present in fertile plants, although the 25-kD band is not. Molecular mass markers at right are given in kilodaltons.

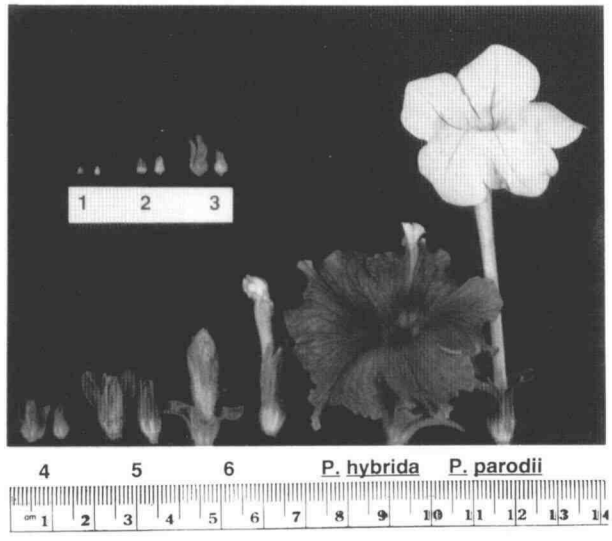

Figure 2. Petunia Floral Development.

Six stages in the development of the petunia bud are shown. Meiosis has occurred by stage 3. A mature flower from *P. hybrida* is on the left; a mature flower from *P. parodii* is on the right. The scale is in centimeters.

Localization of Mitochondrial Proteins on Anther Tissue Prints

We utilized a cryostat tissue printing technique to deliver a consistent amount of material to nitrocellulose membrane and to retain the relative orientation of floral organs in cross-sections of whole buds. Because of the small size and softness of developing bud tissues, the quality of the cryostat sections is far superior to hand-sectioned material as used in the classical tissue printing technique (Ye and Varner, 1991) (data not shown). By comparing cryostat sections, before or after tissue print localization, with fixed and stained embedded sections (Conley et al., 1994), we were able to identify the single layer of large cells that makes up the tapetum at stage 3 or later. In cases where a specific identification was not possible, we use the term "sporogenous/tapetal" to indicate both tissues. As an aid in the interpretation of tissue prints, Figure 3 illustrates the anatomy of the anthers and ovary with diagrams of cross-sections through a stage 3 petunia single anther (Figure 3A) and bud (Figure 3B).

Each antibody discussed showed a similar pattern of localization for all sterile members of the isonuclear pairs, and a different pattern that was similar between all fertile members.

This indicates that the petunia nuclear genome did not influence the expression we observed and thus is irrelevant to our conclusions. Figure 4 shows tissue prints of isonuclear fertile and sterile buds at two developmental stages probed with the anti-PCF antibody. According to immunoblot analysis, the 25-kD PCF protein recognized by this antibody is present only in CMS plants (Nivison and Hanson, 1989). In the fertile buds (Figures 4A and 4C) little or no antibody binding is seen; only some tissue remnants are visible. Anthers of the sterile bud at stage 3 (Figure 4B) show strong signals in both the sporogenous tissue, tapetal layer, and the vascular bundle, as well as in the ovules of the ovary. At stage 5 (Figure 4D), the sporogenous/tapetal tissue of the sterile anthers is much less strongly labeled, although the vascular bundle still shows intense localization. Figure 4 demonstrates the specificity of the tissue print technique in that the tissue that did not contain the antigen did not stain, although both blots were probed simultaneously.

Next we probed a number of stages of fertile and sterile buds with a MAb to the mitochondrially encoded ATPA protein. Figure 5 shows two stages of sterile and fertile buds probed for ATPA. The localization pattern in both the fertile and the sterile buds at all stages is similar, excluding morphological differences caused by pollen abortion. In both stages of fertile buds, the highest signal is found in the tapetal layer and vascular bundle. The fertile stage 3 sporogenous tissue and ovary show intermediate levels of expression (Figure 5A), which are reduced by stage 4 (Figure 5C). The sterile stage 3 anther (Figure 5B) does not contain normal sporogenous tissue, and the degenerating sporogenous/tapetal tissue expresses ATPA at high levels. By stage 4 (Figure 5D), the localization pattern seen in the sterile anther is similar to that of the fertile anther. Thus, at least to the level of resolution attainable by tissue prints, both sterile and fertile buds showed ATPA localization patterns that were similar to that of the PCF protein found in sterile anthers.

In contrast, the localization of the mitochondrially encoded COXII protein differed significantly from that of PCF and ATPA. Figure 6 shows fertile and sterile anthers probed with the anti-COXII MAb. At all stages in both fertile and sterile anthers the localization is very strong in the outermost cell layers of the anther. The vascular bundle does not express COXII at high

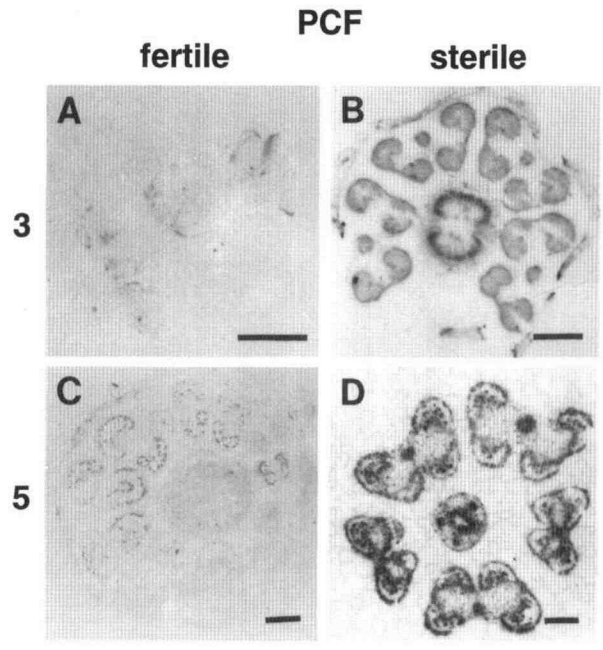

Figure 4. Localization of PCF.

Tissue prints of fertile and sterile buds from *P. parodii* were probed with the anti-PCF antibody. Faint imprints from the fertile buds show the outline of anther tissues, but no enzyme reaction product is visible. Bars = 200 μm.

(A) Stage 3 fertile bud.
(B) Stage 3 sterile bud.
(C) Stage 5 fertile bud.
(D) Stage 5 sterile bud.

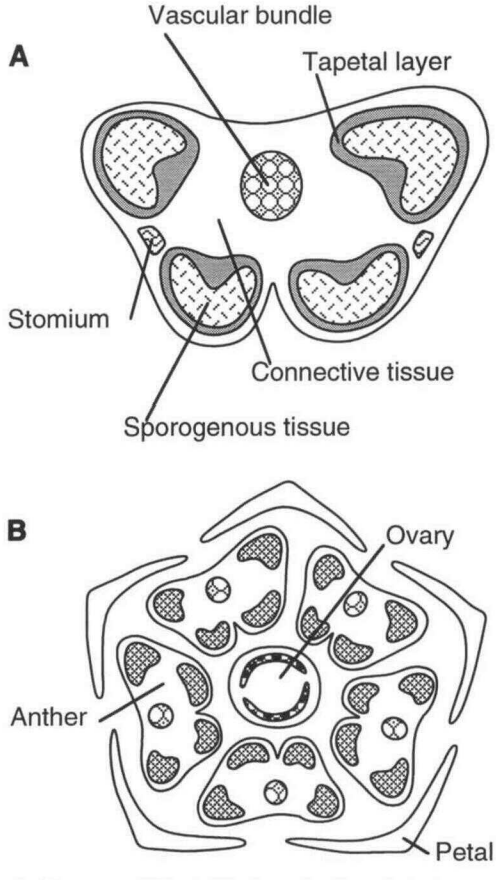

Figure 3. Diagrams of Cross-Sections of a Petunia Anther and Bud.

(A) Anatomy of anther tissues.
(B) Arrangement of floral organs in a bud.

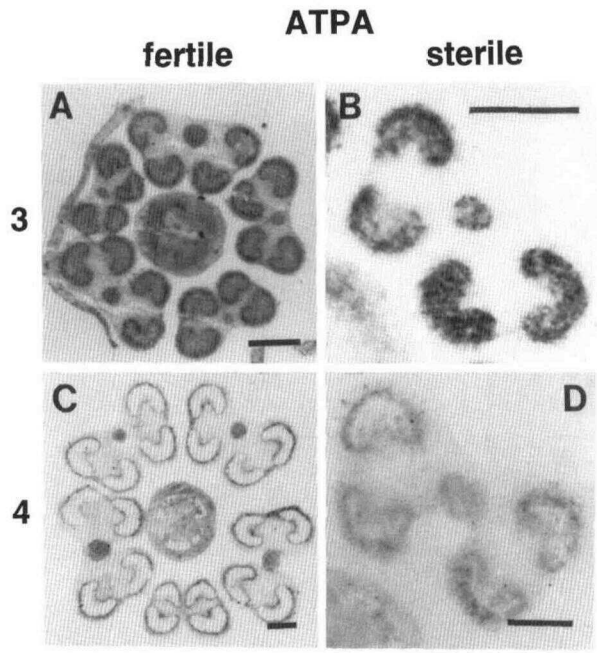

Figure 5. Localization of ATPA.

Tissue prints of stage 3 and stage 4 fertile and sterile buds from *P. parodii* probed with the ATPA monoclonal antibody are shown. Bars = 200 μm.

(A) Stage 3 fertile bud.
(B) Stage 3 sterile anther.
(C) Stage 4 fertile bud.
(D) Stage 4 sterile anther.

levels, nor does the sporogenous tissue. In the stage 3 fertile anther (Figure 6A), the cytoplasm of the tetrads shows some signal, as does the tapetal layer (some individual cells are visible). In the stage 5 fertile anther (Figure 6C), the tapetal localization is somewhat reduced relative to the surrounding tissue. In sterile anthers at both stages, expression in the sporogenous/tapetal tissue is at relatively low levels (Figures 6B and 6D).

When tissue prints of fertile and sterile stage 3 were probed with the AOA MAb, yet a third pattern was observed, as shown in Figure 7. The tapetal layer of the fertile anther (Figure 7A) is darkly stained (note individual cells), but no other parental tissues show high levels of antigen. Developing pollen grains occupy most of the locule space and show localization only in the cytoplasm of the tetrads. The sterile anther (Figure 7B) contains degenerating sporogenous/tapetal cells that show high levels of antigen, but all other tissues show weak localization.

A side-by-side comparison of tissue prints from stage 3 buds reacted with our antibody probes readily demonstrates the differences in expression of mitochondrial proteins that we detected. Tissue prints of stage 3 sterile buds probed with all four antibodies are shown in Figure 8. PCF (Figure 8A) and ATPA (Figure 8B) are both strongly localized to the degenerating sporogenous tissue/tapetal layer and the vascular bundle, AOA signal (Figure 8C) is strong in the sporogenous tissue only, and COXII (Figure 8D) is highly expressed in the subepidermal layers, moderately expressed in the connective tissue, and not detectable in the sporogenous tissue.

DISCUSSION

Previously, organ-specific expression of mitochondrial proteins has been analyzed by the presence of bands as visualized by one- or two-dimensional gel electrophoresis. In some species (e.g., rice and oilseed rape), mitochondrial proteins synthesized in organello do not differ between control plants and plants given dark or cold treatments (Hahn and Walbot, 1989; Hansen et al., 1992; Dai et al., 1993). In contrast, light treatment leading to floral induction of morning glory results in three additional mitochondrially synthesized bands relative to noninduced tissues (Li and Tan, 1990). Mitochondrial proteins synthesized in organello differ among leaves, roots, and

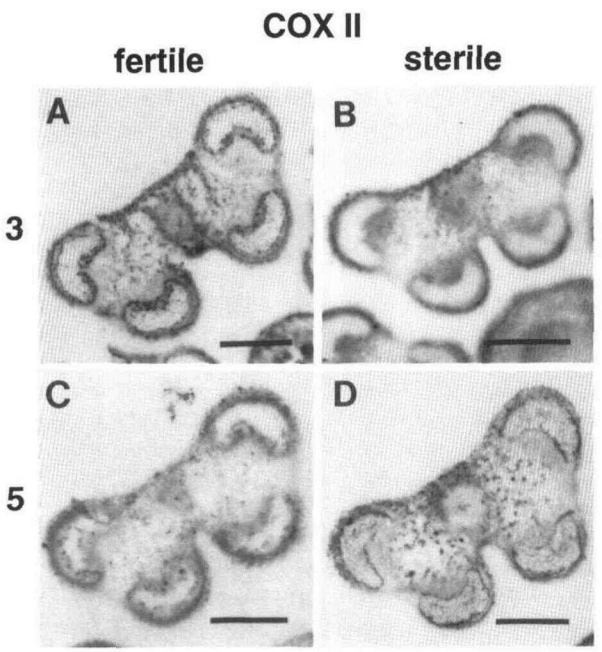

Figure 6. Localization of COXII.

Tissue prints of stage 3 and stage 5 fertile and sterile anthers from *P. hybrida* probed with COXII monoclonal antibody are shown. Bars = 200 μm.

(A) Stage 3 fertile anther.
(B) Stage 3 sterile anther.
(C) Stage 5 fertile anther.
(D) Stage 5 sterile anther.

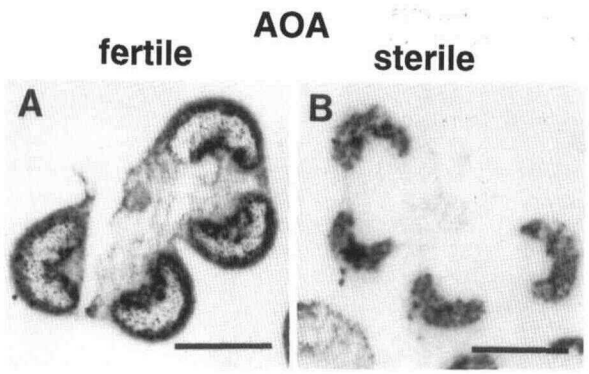

Figure 7. Localization of AOA.

Tissue prints of fertile and sterile stage 3 anthers from *P. hybrida* probed with a monoclonal antibody to AOA are shown. Bars = 200 μm.
(A) Fertile anther.
(B) Sterile anther.

flowers of sugar beet (Lind et al., 1991), shoots, cobs, tassels, and scutella of maize (Newton and Walbot, 1985), and leaves or developing pollen of *N. sylvestris* (De Paepe et al., 1993). Variant mitochondrial proteins are synthesized in plants with differing nuclear backgrounds; these plants include *Nicotiana* spp (Hakansson et al., 1988; De Paepe et al., 1990; Bonnett et al., 1991; and Spangenberg et al., 1992), sorghum (Bailey-Serres et al., 1987), and *Zea luxurians* (Cooper and Newton, 1989). Although these studies reveal that isolated mitochondria from different tissues or genotypes vary in the complement of proteins they synthesize, no conclusions can be drawn concerning the possible physiological or biochemical roles these unidentified proteins may play.

The alternative oxidase, in contrast, is a nuclear-encoded mitochondrial protein with a defined biochemical activity. Increases in levels of the alternative oxidase are associated with heat production in voodoo lily spadices (Elthon et al., 1989a) and aging in potato tubers (Hiser and McIntosh, 1990). It is found at higher levels in soybean roots and shoots relative to nodules (Kearns et al., 1992) and increases during early growth of soybean shoots (Obenland et al., 1990). Levels of alternative oxidase are increased in cold-grown plants of both maize (Stewart et al., 1990) and tobacco (van Lerberghe and McIntosh, 1992). However, the demonstration that mitochondrial proteins show altered abundance at the whole organ or plant level does not reveal which tissues are involved.

We report the localization of specific mitochondrial proteins to tissues of petunia buds at several stages of development. Using antibodies to four mitochondrial proteins, encoded by either the nuclear or the mitochondrial genome, we found several different patterns of localization. The nuclear-encoded AOA protein was found at high levels predominantly in the young sporogenous tissue/tapetal layer. The mitochondrially encoded COXII protein was localized differently than ATPA, a mitochondrially encoded subunit of the mitochondrial ATPase. The localization pattern displayed by ATPA resembled that of the PCF protein, which is associated with CMS in petunia. The similarity is not entirely surprising, because the PCF protein is encoded by a fused gene that has the upstream sequences and 5′ coding region of a gene encoding another member of the ATPase complex, ATP9 (Hanson et al., 1988).

If the various localization patterns seen in anthers reflect the activity of the proteins localized, then in fertile plants both the cytochrome oxidase and the alternative oxidase are active in tetrads and younger tapetal cells, the cytochrome oxidase but not the alternative oxidase is more active in the connective tissue and subepidermal layers, and the alternative oxidase but not the cytochrome oxidase is more active in the degenerating sterile sporogenous tissue and tapetal layer. The elevated level of alternative oxidase in the tapetal layer of fertile anthers may support the hypothesis described by Dennis (1987) that the alternative oxidase is needed for regeneration of NAD^+ in tissues with high biosynthetic activity, because the tapetal layer is thought to be the site of synthesis for many nutrients required by developing microspores (Kaul, 1988). In sterile plants, the alternative oxidase is present at high levels in the

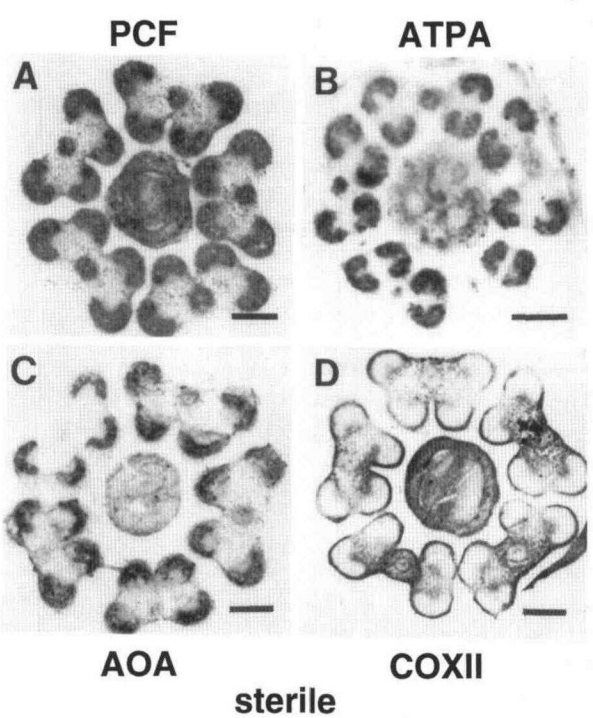

Figure 8. Localization of Several Mitochondrial Proteins.

Tissue prints of sterile stage 3 buds showing the different patterns of localization given by antibodies to PCF (*P. hybrida*), ATPA (*P. parodii*), AOA (*P. hybrida*), and COXII (*P. hybrida*) are shown. Bars = 200 μm.
(A) PCF localization.
(B) ATPA localization.
(C) AOA localization.
(D) COXII localization.

degenerating sporogenous tissue/tapetum, but the cytochrome oxidase is much reduced. A high level of ATPase in the sporogenous tissue, tapetal layer, and vascular bundle of both fertile and sterile plants is consistent with the high metabolic activity of these tissues. The PCF protein localizes to the vascular bundle in addition to the sporogenous/tapetal tissue of sterile anthers, which may implicate the CMS-associated mitochondria in the lack of ovoid bodies we reported in vascular bundles of sterile anthers viewed in the scanning electron microscope (Conley et al., 1994).

The different patterns of localization we observed in anther tissues for the various mitochondrial proteins demonstrate that the expression of two specific mitochondrially encoded proteins was regulated differentially in the same anther tissues. The COXII protein was localized at high levels in the connective and subepidermal tissues and at relatively low levels in the sporogenous tissue and vascular bundle, whereas the ATPA protein was found at high levels in the sporogenous tissue and vascular bundle and at relatively low levels in the connective and subepidermal tissues. Both proteins were found at fairly high levels in the tapetum. These data strongly suggest that plant cells exert control over the tissue-specific expression of individual mitochondrial proteins, and that different patterns of localization are not solely a result of varying quantities of mitochondria between distinct anther tissues.

The location of PCF in relation to other mitochondrial proteins in anther tissues may hint at the molecular mechanism of CMS. The similar localization patterns of PCF and ATPA may indicate that PCF could interfere with the functioning of the mitochondrial ATPase in a tissue undergoing the very energetically demanding processes of meiosis and microsporogenesis. Inadequate synthesis or utilization of ATP could directly disrupt development. Alternatively, there may be significance to the overlapping localization of AOA and PCF. Previous work from our laboratory has shown a correlation between expression of the PCF protein and altered partitioning of reducing equivalents between the cytochrome oxidase and alternative oxidase pathways (Connett and Hanson, 1990; A.L. Moore, D.C. Ruth, and M.R. Hanson, manuscript submitted). The low COXII content in tissues in which PCF is highly expressed could lead to low but critical cytochrome oxidase activity that is more readily disrupted by the PCF protein than in other tissues where COXII is more highly expressed. Perhaps more likely is the hypothesis that the PCF protein disrupts the alternative oxidase in sporogenous tissue where both PCF and AOA are present in abundance. Optimal alternative oxidase activity may be essential for NAD^+ regeneration and carbon skeleton biosynthesis during pollen development.

METHODS

Plant Materials

All plants were grown inside at 26°C under 16-hr light and 8-hr dark. *Petunia hybrida* isonuclear lines 3704 (fertile) and 11127 (sterile) and *P. parodii* isonuclear lines 3699 (fertile) and 3688 (sterile) were obtained from Shamay Izhar (Volcani Institute, Bet Dagan, Israel).

Antibody Preparation

The polyclonal anti-URFS antibody was purified from antiserum as described by Nivison et al. (1994). The ATPase subunit A monoclonal antibody (ATPA MAb) was obtained from the maize mitochondrial antibody kit (GTMA; Lincoln, NE), the cytochrome oxidase subunit II (COXII) MAb was a gift from T. Mason (University of Massachusetts, Amherst), and the alternative oxidase (AOA) MAb was a gift from T. Elthon (University of Nebraska, Lincoln).

Protein Preparation and Protein Gel Blotting

Flowering tips from *P. parodii* sterile line 3688 were ground under liquid nitrogen with a mortar and pestle. Powdered material was suspended in LDS sample buffer (Nivison and Hanson, 1989) and filtered through several layers of Miracloth (Calbiochem). Filtrate was spun for 10 min in a microcentrifuge, and the supernatant was retained as total floral tip protein. Denaturing gel electrophoresis and electroblotting were performed according to the procedure of Nivison and Hanson (1989). Immunoblotting was performed with the same solutions used in tissue printing.

Tissue Printing

Plant material was frozen, and 8-mm sections were cut on a cryostat (Conley et al., 1991). Probing was essentially as described by Ye and Varner (1991), using dilutions of 1:50 for the COXII, ATPA, and AOA MAbs, and 1:10 for the purified anti-URFS antibody. Secondary antibodies conjugated to alkaline phosphatase (Kirkegaard and Perry, Gaithersburg, MD) were diluted 1:1000, and binding was monitored using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Amresco, Solon, OH) as substrates.

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