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Cell-Specific Expression of Mitochondrial Transcripts in Maize Seedlings

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Although mitochondria are thought to assume crucial and possibly novel physiological functions during male gametogenesis, it is not known to what extent mitochondrial function is necessary for other aspects of plant development or to what degree the expression of plant mitochondrial genes is subject to cell-specific regulation, particularly during vegetative growth. We have used in situ hybridization to show that extensive differences exist in the levels of mitochondrial RNAs (mtRNAs) among different tissues and among different individual cell types within the same organ of maize seedlings. The expression of all examined mtRNAs is enhanced in vascular bundles, particularly in procambium- and xylem-forming cells. Mitochondrial transcript levels correlated highly with cell division activity. For example, in roots, the transcripts are abundant in the dividing cells of the meristem but drop to very low levels in the nondividing cells of the root cap and the meristem quiescent center. By comparison, levels of functional mitochondria, as assessed by rhodamine-123 fluorescence, did not vary greatly among the same group of cells. In shoots, in situ hybridization and blot hybridization revealed differences in the patterns of localization among different mtRNAs. The results indicate that during vegetative growth, mitochondrial gene expression at the transcript level is subject to an unexpected degree of cell-specific regulation and that different controls may operate on different transcripts.

INTRODUCTION

The key function of mitochondria is the production of ATP through oxidative phosphorylation. Oxidative phosphorylation activity in different types of plant cells is expected to vary, depending on the cell's physiological function, its photosynthetic competence, and its access to oxygen, among other factors (Dennis, 1987). This variation is reflected, to some extent, in the numbers of mitochondria in different types of plant cells, as estimated from ultrastructural studies. For example, up to 20-fold differences in the number of mitochondria per cell have been observed among different cell types in maize (Clowes and Juniper, 1964; Juniper and Clowes, 1965), timothy grass (Avers, 1962), and cucurbits (Bendich and Gauriloff, 1984), and an ~40-fold increase in mitochondrial number accompanies the formation of the tapetal cell layer in maize anthers (Lee and Warmke, 1979).

Literature pertaining to the relationships between the number of mitochondria per cell and relevant biogenetic parameters, such as mitochondrial DNA (mtDNA) copy number and RNA transcript levels, is limited. The amount of mtDNA per cell appears to be less variable than the mitochondrial number because only a fivefold difference was observed among different cells in pea (Lamppa and Bendich, 1984). Several studies have reported quantitative changes in mitochondrial transcript abundance during development. For example, the cellular levels of several mitochondrial transcripts were shown to decrease as cells acquired photosynthetic competence during the development of wheat leaves (Topping and Leaver, 1990), whereas mitochondrial transcript abundance was found to increase during the formation of microspores in maize (Monéger et al., 1992). Recently, Huang et al. (1994) showed that levels of transcripts of the nuclear genes encoding the tobacco mitochondrial Rieske non-heme iron protein are 10-fold higher in flowers than in leaves. Flower and leaf mitochondria contain similar amounts of the protein, and the elevated transcript levels were attributed to a higher overall amount of mitochondria in floral tissues.

Several lines of evidence suggest that not only does the number of mitochondria differ among different types of plant cells, but qualitative differences among the mitochondria exist as well. Differences in the relative levels of specific mitochondrial transcripts among different plant organs have been observed in petunia (Young and Hanson, 1987), Brassica (Singh and Brown, 1993), and sunflower (Monéger et al., 1994; Smart et al., 1994). Differences in the numbers and types of polypeptides synthesized by isolated mitochondria have been observed in maize (Newton and Walbot, 1985), sugar beet (Lind et al., 1991), and tobacco (De Paepe et al., 1993), and differences in polypeptide composition have been detected between

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mitochondria from photosynthetic and nonphotosynthetic tissue (Day et al., 1985; Walker and Oliver, 1986).

Most previous studies of tissue-specific or developmental regulation of mitochondrial gene expression have relied on the analysis of preparations of mitochondrial DNA, RNA, and proteins isolated from different plant organs. These studies have thus identified only overall differences between nucleic acid or protein preparations, and differences within the specialized cell types of specific organs and tissues could not be detected. Recently, examples of localized expression of mitochondrial genes in specific anther cell types have been observed. Conley and Hanson (1994) found that antibodies raised against several different mitochondrial proteins showed distinct patterns of localization in developing petunia anthers. Smart et al. (1994) used in situ hybridization to show that transcripts of several different mitochondrial genes were all expressed at elevated levels, specifically in microsporocytes, during male meiosis, and in developing tapetal tissue.

The trait of cytoplasmic male sterility (CMS), in which expression of abnormal mitochondrial genes leads to a specific defect in pollen production, suggests that mitochondria assume crucial and possibly novel physiological functions during male gametogenesis (Hanson, 1991; Bonen and Brown, 1993). It is not known to what extent mitochondrial function is crucial for other aspects of plant development or to what degree the expression of plant mitochondrial genes is subject to cell-specific regulation during vegetative growth. The maize seedling has been used in many studies of mitochondrial function and biogenesis, and the morphological changes accompanying its development and germination are relatively well characterized (Abbe and Stein, 1954; Stein and Quastler, 1963). We have used in situ hybridization to investigate the expression of several mitochondrial RNAs (mtRNAs) at an early stage in maize seedling growth. We show that extensive differences exist in levels of expression of mitochondrial genes among different tissues and among individual cell types within the same organ. We also show that smaller differences exist in the relative levels of expression of different mitochondrial genes within cells of the same type.

RESULTS

Anatomy of Maize Seedlings

The major morphological features of maize seedlings 2 to 3 days after germination are schematically depicted in Figure 1. The organ systems of the seedling are defined during embryogenesis (Abbe and Stein, 1954). Longitudinal sections (left) of the young maize shoot reveal four or five foliage leaves that are completely surrounded by the coleoptile, a protective sheathlike structure consisting largely of parenchyma tissue. Cross-sections taken slightly above the shoot apex (C1) reveal two opposing vascular bundles that run most of the length of the coleoptile. The establishment of procambial strands is visible in the outer (older) leaves in cross-sections passing just above or through the shoot apex. Germination is accompanied by the resumption of cell division, first in the root and later in the mesocotyl, coleoptile, and leaves, and by differentiation of the vascular system in the root, leaves, and coleoptile (Stein and Quastler, 1963). By 60 hr postgermination, meristematic activity extends over the shoot apex and foliage leaves but is partially confined to the lower third of the coleoptile (Stein and Quastler, 1963).

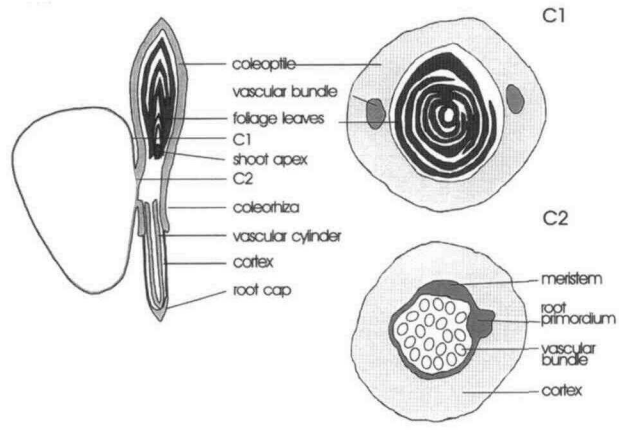

Figure 1. Anatomy of Germinating Maize Seedlings.

At left are illustrated the major structures evident in longitudinal sections through the seedling shoot and root. Features evident in cross-sections of the shoot (C1) and mesocotyl (C2) are illustrated at right.

The seedling primary root, like the shoot, is also surrounded by a protective sheath, in this case termed the coleorhiza, that is physically continuous with the coleoptile. The coleorhiza terminates just below the point at which the seedling emerges from the seed and ruptures within 50 hr after the onset of germination. The gross morphological features evident in the primary root are a central vascular cylinder surrounded by the root cortex. Not depicted in Figure 1 are the endodermis, which is located between the vascular cylinder and the cortex, and the epidermis, which forms the outermost layer of the root. The frequency of mitosis is low in a region just above the root cap termed the quiescent center (Clowes, 1976), then increases dramatically, and subsequently decreases as the distance from the root apex increases. Cells of both the cortex and the vascular cylinder are mitotically active in the region above the quiescent center.

Cross-sections through the mesocotyl (C2), the region of the seedling below the shoot apex but above the root, reveal a core of relatively undifferentiated vascular tissue surrounded, in succession, by a zone of meristematic activity and the cortex. The primordium of the forming adventitious root is visible in many of these sections.

Distribution of *atp6* and *rrn26* Transcripts and RNA*b* in the Seedling Shoot and Mesocotyl

To investigate the distribution of different mitochondrial transcripts within the maize seedling, we employed in situ hybridization, initially using four different complementary RNA probes. Each of these RNAs represented a different transcript type: a mitochondrial mRNA (transcripts of the *atp6* gene, encoding ATPase subunit 6), a mitochondrial rRNA (26S rRNA, encoded by the *rrn26* gene), a cytoplasmic rRNA (17S rRNA), and an autonomously replicating mtRNA plasmid, RNA*b* (Schuster et al., 1983; Finnegan and Brown, 1986; Zhang and Brown, 1993). RNA probes were labeled by incorporation of sulfur-35 during in vitro transcription of the corresponding DNA templates and hybridized with longitudinal or cross-sections of maize seedlings taken 60 hr after germination. The sections were subsequently counterstained with toluidine blue. The silver grains resulting from hybridization in the in situ autoradiographs were visualized as bright red dots under dark-field illumination through a red filter that was concurrent with bright-field illumination. The intensity of the red color therefore corresponds to the local concentration of the respective RNAs, whereas the blue corresponds to the toluidine blue–stained cellular material.

Figure 2 shows the distribution of the different RNAs in cross-sections taken through the lower portion (Figure 1, section C1) of the shoot and through the mesocotyl (Figure 1, section C2). In the lower shoot cross-sections, the *atp6* and RNA*b* (Figures 2A and 2B) transcripts show a similar pattern of distribution in that levels of both RNAs are considerably higher in the leaves than in most of the cells of the coleoptile. Surprisingly, the 26S rRNA shows a different distribution pattern: in general, its level is higher throughout the coleoptile than in the leaves, especially the younger, innermost leaves. This indicates that different mtRNAs may be expressed at different levels in different vegetative cell types and suggests that the observed patterns of localization of the different mtRNAs do not simply reflect the abundance of mitochondria in the various cells. Levels of all of the mtRNAs are higher in the vasculature of the coleoptile than in the surrounding parenchyma tissue. Enhanced levels of the 26S rRNA also are evident in the developing vascular bundles visible in Figure 2C of the older, outermost leaves. Levels of RNA*b* and the mitochondrial rRNA both appear to be more highly localized in the epidermis than in the parenchyma of the coleoptile. The cytoplasmic rRNA (Figure 2D) is the least highly localized of the RNAs examined and is present at similar levels in all of the tissues of the shoot. This last observation suggests that the localized distribution of the mtRNAs does not simply reflect the density of cytoplasm in the various cell types.

The distribution of the four RNAs in the mesocotyl (Figure 1, section C2), as deduced from the examination of in situ autoradiographs of tissue cross-sections (Figures 2E to 2H), differed in several ways from the patterns seen in shoots. Whereas the *atp6* and RNA*b* transcripts were similarly localized in shoot sections, in the mesocotyl, *atp6* transcripts were barely detectable and localized only over the meristem and the primordium of the adventitious root. RNA*b* was present at high levels in the meristem, root primordium, vascular tissue, and epidermis. The mitochondrial 26S rRNA signals were largely confined to the mesocotyl epidermis and adjacent cortex tissue, and the cytoplasmic rRNA showed a similar pattern of localization. Thus, in mesocotyl tissue, the three different mtRNAs showed different distribution patterns.

Additional aspects of mitochondrial transcript localization are evident in longitudinal sections that include upper (Figures 3A to 3D) and lower (Figures 3E and 3F) regions of the foliage leaves of the shoot. (The middle portions of the shoot were not analyzed.) The *atp6* transcripts (Figures 3A and 3E) are abundant throughout the foliage leaves, shoot apex, and subtending cells and present only at very low levels in the surrounding coleoptilar tissue, consistent with the distribution pattern observed in shoot cross-sections. RNA*b* (Figures 3B and 3F) is similarly localized, except that the labeling is particularly strong in the extreme lower parts of the youngest (inner) leaf cells and in the shoot apex. Interestingly, these are the last tissues in which cell division begins to take place after germination (Stein and Quastler, 1963). In the lower portion of the shoot, the distribution of the mitochondrial 26S rRNA (Figure 3G) differs from that of the other two mtRNAs in that it appears equally as abundant in the coleoptile and outer leaves and possibly more abundant in the coleoptile than in the inner leaves. This distribution is again consistent with that seen in shoot cross-sections. In the upper region of the leaves, however, the distribution of the 26S rRNA (Figure 3C) is more similar to that of the other mtRNAs in that its levels in leaf tissue are higher than in the coleoptile. As with the shoot and mesocotyl cross-sections, the cytoplasmic rRNA (Figures 3D and 3H) does not appear to be specifically localized.

RNA Dot Blot Analysis of Tissue-Specific Differences in Transcript Abundance

The finding that various mtRNAs differed from one another in abundance in shoot tissues was unexpected. Therefore, we sought to confirm, by an alternative experimental approach, that these differences were a true reflection of the RNA abundance in the different tissues and not an artifact of the in situ hybridization technique employed. We chose quantitative RNA dot blot hybridization, as employed by Meagher and co-workers (Hightower and Meagher, 1985; Shirley and Meagher, 1990), as an alternative approach for estimating the relative abundance of the different RNAs. When this method is used, different amounts of RNA from the tissues being compared are applied directly to hybridization membranes; the relative abundance of specific transcripts in the different tissues is estimated from the amounts of RNA required to produce signals of comparable intensity upon hybridization with a labeled complementary DNA probe. The method is highly reliable in that

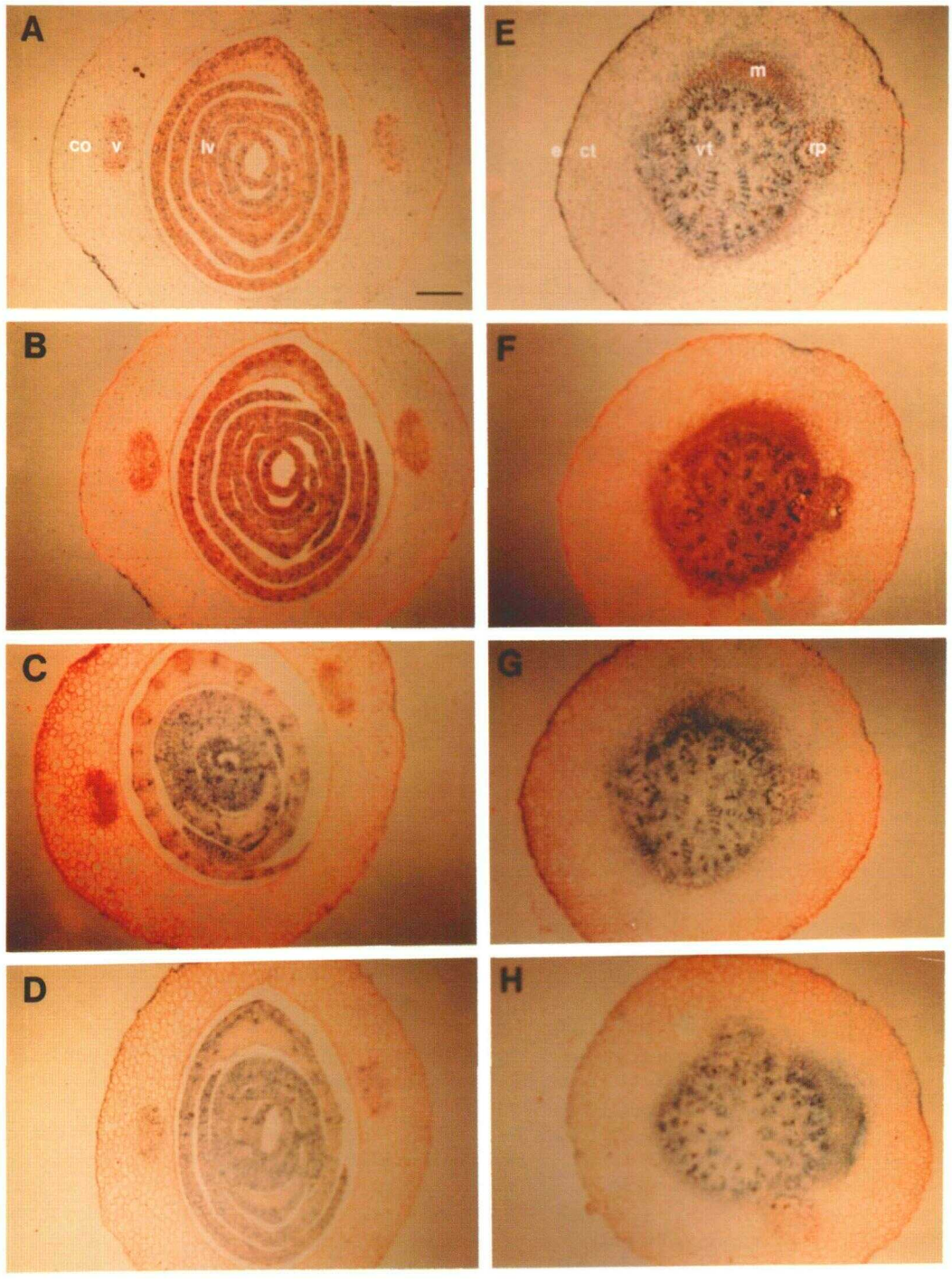

Figure 2. Transcript Localization in Cross-Sections of the Seedling Shoot and Mesocotyl.

Sections from the same seedling corresponding to positions C1 (**[A]** to **[D]**) or C2 (**[E]** to **[H]**) of Figure 1 were stained with toluidine blue after in situ hybridization with antisense RNA probes and visualized by bright-field and concomitant dark-field illumination with use of a red filter. Silver grains resulting from hybridization appear red; the blue corresponds to toluidine blue–stained cellular material. co and ct, coleoptile; e, epithelium; lv, leaves; m, meristem; rp, adventitious root primordium; v, vascular bundle; vt, vascular tissue.

(A) and **(E)** Mitochondrial *atp6* RNA probe.

(B) and **(F)** Mitochondrial RNA*b* RNA probe.

(C) and **(G)** Mitochondrial 26S rRNA (*rrn26*) probe.

(D) and **(H)** Cytoplasmic 17S rRNA probe.

Bar in **(A)** = 0.2 mm for **(A)** to **(H)**.

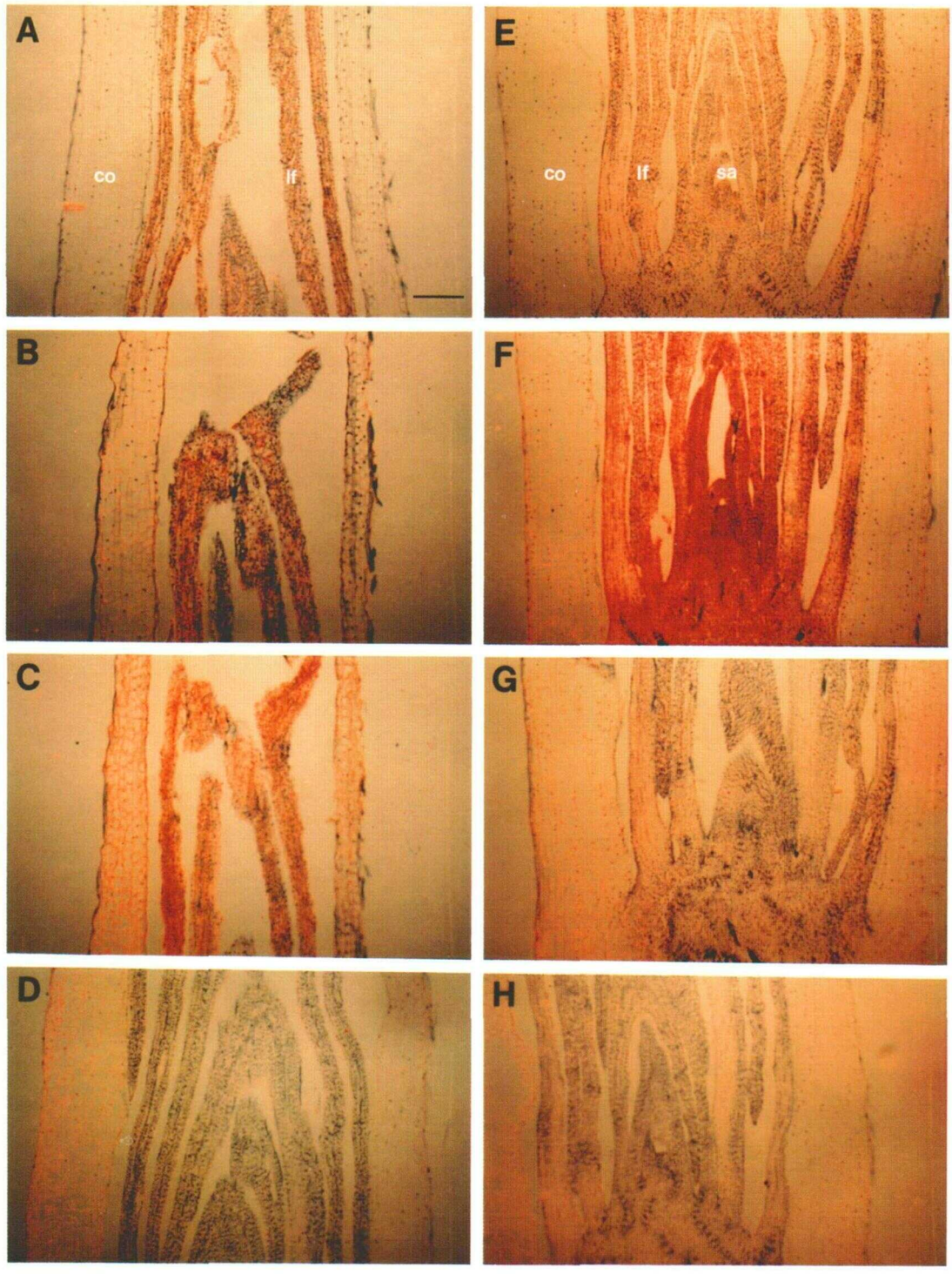

Figure 3. Transcript Localization in Longitudinal Sections of Seedling Shoots.

Sections were stained with toluidine blue after in situ hybridization with antisense RNA probes and visualized by bright-field and concomitant dark-field illumination using a red filter. Sections **(A)** to **(D)** pass through the upper portion of the shoot; sections **(E)** to **(H)** pass through the lower portion of the shoot near the shoot apex. co, coleoptile; lf, leaf; sa, shoot apex.

(A) and **(E)** Mitochondrial *atp6* RNA probe.

(B) and **(F)** Mitochondrial RNA*b* RNA probe.

(C) and **(G)** Mitochondrial 26S rRNA (*rrn26*) probe.

(D) and **(H)** Cytoplasmic 17S rRNA probe.

Bar in **(A)** = 0.2 mm for **(A)** to **(H)**.

it is not subject to artifacts resulting from, for example, variability in transfer of RNA from gels or effects of RNA abundance on hybridization efficiency.

The coleoptiles and leaves of 3-day-old shoots were separated from one another, and total RNA was isolated from each tissue. The two RNA samples were diluted such that their concentrations were made equal, a twofold dilution series of each sample was prepared, and equal volumes of each dilution were applied directly to hybridization membranes. Three such membranes were prepared on which the two RNAs were each fixed in dots in amounts that varied over a 32-fold range. These were then separately probed with cloned DNA fragments containing the *atp6*, RNA*b*, and 26S mitochondrial rRNA (*rrn26*) sequences. As shown in Figure 4A, differences in the amount of hybridization with coleoptile and leaf RNA were observed among the different probes. Both the *atp6* and RNA*b* probes hybridized to a greater extent with leaf than with coleoptile RNA, whereas the *rrn26* gene probe hybridized more with coleoptile than with leaf RNA. These results are consistent with the in situ hybridization experiments, which also indicate that in the lower shoot, from which the bulk of the material used for RNA isolation was obtained, the *atp6* and RNA*b* transcripts are more abundant in leaves than in coleoptiles, whereas the 26S mitochondrial rRNA transcripts are relatively more abundant in coleoptiles.

The blots were then stripped and rehybridized with the cytoplasmic 17S rRNA gene probe. As shown in Figure 4B, with this probe, no differences in the hybridization signals from the leaf and coleoptile RNA preparations were observed on any of the membranes. This result is consistent with the in situ hybridization experiments suggesting that the levels of the cytoplasmic rRNA in coleoptile and leaf tissue are similar. The equivalent hybridization of coleoptile and leaf RNA to the rRNA probe on all three membranes further indicated that the hybridization differences observed with the mitochondrial gene probes did not result from loading differences between the leaf and coleoptile RNAs.

Analysis of the autoradiographs by both visual inspection and scanning densitometry indicated that for the *atp6* and RNA*b* probes, generation of a hybridization signal equal to that obtained by a given amount of leaf RNA required roughly twice that amount of coleoptile RNA. For example, the *atp6* signals obtained from 1 and 0.5 μg of coleoptile RNA were found to correspond to those obtained from 0.5 and 0.25 μg of leaf RNA, respectively. From this result, we conclude that both *atp6* and RNA*b* transcripts are twice as abundant in leaf as in coleoptile RNA. By contrast, the 26S mitochondrial rRNA probe hybridization signals indicated that this transcript was approximately two times more abundant in coleoptile than in leaf RNA. Therefore, relative to the levels of 26S mitochondrial rRNA transcripts, there is a fourfold overall enrichment of *atp6* and RNA*b* transcripts in leaf versus coleoptile tissue. It should be pointed out that in coleoptiles, the *atp6* and RNA*b* transcripts are highly localized in the vascular tissue, and it is likely that the majority of the hybridizing transcripts in total coleoptile RNA are derived from these cells. In contrast, the 26S mitochondrial rRNA transcripts are abundant both in the coleoptile parenchyma

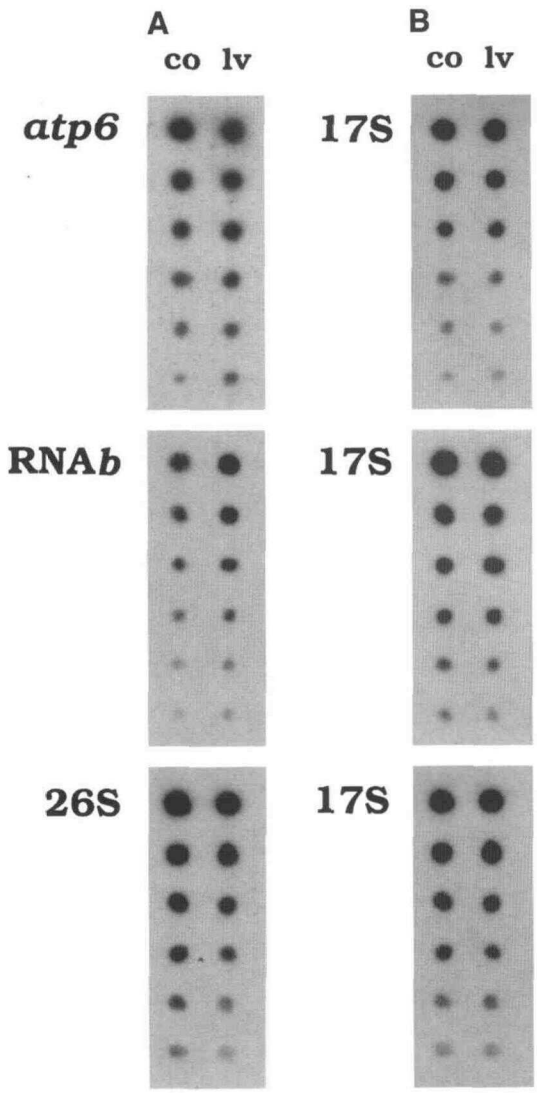

Figure 4. RNA Dot Blot Analysis of Coleoptile and Leaf Transcript Levels.

Serial dilutions of RNA preparations from coleoptiles (co) and leaves (lv) were spotted onto membranes and hybridized with labeled DNA probes for different mitochondrial transcripts or a cytoplasmic rRNA gene. The top dots on each filter contain 4 μg of leaf or coleoptile RNA. Each of the dots below corresponds to successive twofold dilutions of these RNA preparations. The time of exposure of the blots to x-ray film varied, depending on the abundance of the RNA species being probed; the relationship between the intensity of the dots in the autoradiographs and the absolute levels of the RNA examined therefore varies from probe to probe. The hybridization signals thus allow for comparison of the abundance of a particular RNA species in coleoptile versus leaf tissue but not for comparison of the abundance of different transcripts.

(A) Dot blots were hybridized with probes for the *atp6* gene, RNA*b*, or the mitochondrial 26S rRNA (*rrn26*) gene.

(B) After exposure to x-ray film, the probes were stripped from the membranes, and the same membranes were hybridized with a DNA probe for the maize cytoplasmic 17S rRNA.

and vascular tissue. It is therefore likely that the differences in abundance among the mitochondrial transcripts estimated by blot hybridization would have been greater if it were possible to compare leaf with only the coleoptile parenchyma tissue.

Cell-Specific Distribution of Other mtRNAs in Shoot Tissue

The observation that the three different types of mtRNAs showed different distribution patterns in different cell types of the seedling shoot and mesocotyl prompted us to examine the transcripts of a variety of other maize mitochondrial genes. Figure 5 shows the hybridization patterns of three additional maize mitochondrial mRNAs, encoding cytochrome *c* oxidase subunits I and II (*coxI* and *coxII*, respectively), the α subunit of the ATPase (*atpA*), and the 18S mitochondrial rRNA, in shoot cross-sections. The distribution of each of the mRNAs resembles that of the *atp6* and RNA*b* transcripts more than that of the *rrn26* transcripts because the transcripts are all more abundant in leaves than in the parenchyma of the coleoptile. Levels of these transcripts are also elevated in the vascular tissue and epidermis of the coleoptile. Two other mitochondrial mRNAs (*cob* and *rps12*) showed a similar pattern of distribution (data not shown). The distribution of the mitochondrial 18S rRNA (Figure 5B) shares features with the distributions of both the 26S rRNA and the mRNAs; it is most abundant in older leaves and least abundant in the younger leaves, with intermediate levels being present in the coleoptile.

Mitochondrial Transcript Localization in Vascular Tissues

Examination of in situ autoradiographs of shoot cross-sections at higher magnification indicated that the increased hybridization observed over the vasculature of the coleoptile is most intense in the procambial region, located between the xylem, which is identified by the large vessel elements in the region proximal to the leaves, and the opposing phloem (Figure 6A). Interestingly, as shown for the *rrn18* and *atp6* transcripts in Figures 6B and 6C, respectively, the abundance of mitochondrial transcripts is considerably higher in differentiating xylem cells than in the surrounding parenchyma, but these transcripts do not appear appreciably more abundant in differentiating phloem cells. A similar pattern of distribution could be observed for several other mitochondrial transcripts in both cross and longitudinal sections (data not shown). This suggests that mitochondrial transcript abundance correlates not only with cell division activity but with xylem differentiation as well.

Distribution of mtRNAs in Root Tissues

The correlation between cell division activity and mitochondrial transcript abundance was further investigated through the analysis of in situ autoradiographs of root sections. Cell division activity within the maize primary root is well characterized and varies widely among different sites within the apex. The boundary between the root cap and the remainder of the root apex is clearly delineated by a thick cell wall referred to as the cap junction (Clowes and Juniper, 1964). A narrow zone of nondividing cells, known as the quiescent center, is situated at the base of the stele, immediately above the cap junction (Figure 6E; Clowes, 1956). Cells of the root cap are nonmeristematic; meristematic activity is highest in the region of the cortex closest to the quiescent center and extends upward from this site for a distance of ~2 mm (Clowes, 1976).

In the root, the patterns of localization of the different mtRNAs, including the 26S rRNA, are more similar to one another than they are in the shoot. Figure 6F shows the distribution of *atpA* transcripts, which is largely characteristic of the other mitochondrial transcripts examined, in longitudinal sections of the lower root. A strong association between mitochondrial transcript abundance and meristematic activity was observed: levels of the transcripts are high in the dividing cells of the root apex but very low in the nondividing cells of the quiescent center and root cap. Like the shoot, the cytoplasm has similar 17S rRNA levels (Figure 6G) in both dividing and nondividing root cells. Examination of longitudinal sections at lower magnification indicated that mitochondrial transcript abundance decreased in the root cortex and became progressively more highly localized to the vascular cylinder as the distance from the root tip increased (data not shown). This observation is consistent with the pattern of meristematic activity, which is restricted to the root apex (Clowes, 1976) and the vascular cambium. As with the shoot, within the vascular cylinder, the procambium and developing xylem elements showed the highest levels of hybridization.

Previous studies (Clowes and Juniper, 1964; Juniper and Clowes, 1965) have indicated that the number of mitochondria per volume of cytoplasm drops modestly, approximately threefold, between the cap junction and a point 200 μm below it (about half the distance to the tip), then increases significantly in the area closer to the cap terminus. No differences in mitochondrial density between cells of the quiescent center and the surrounding dividing cells of the meristem were observed (Clowes and Juniper, 1964). The in situ hybridization signals from all the mitochondrial transcripts, however, showed large differences in intensity in the lower root; they were high in the meristem but nearly undetectable in the quiescent center and throughout most of the root cap. This strongly suggested that the levels of mitochondrial transcripts did not simply reflect the density of mitochondria in the different cells of the root.

An independent measure of the distribution of mitochondria in the root cap was obtained by using rhodamine-123, a fluorescent dye that allows specific visualization of functional mitochondria (Johnson et al., 1980) in individual living plant cells or whole plant organs by conventional fluorescence (Wu, 1987) or confocal (Gambier and Mulcahy, 1994) microscopy. Live root tips were exposed to rhodamine-123 after first cutting them longitudinally to allow penetration of the dye into cells in the central portion of the root. The distribution of rhodamine-

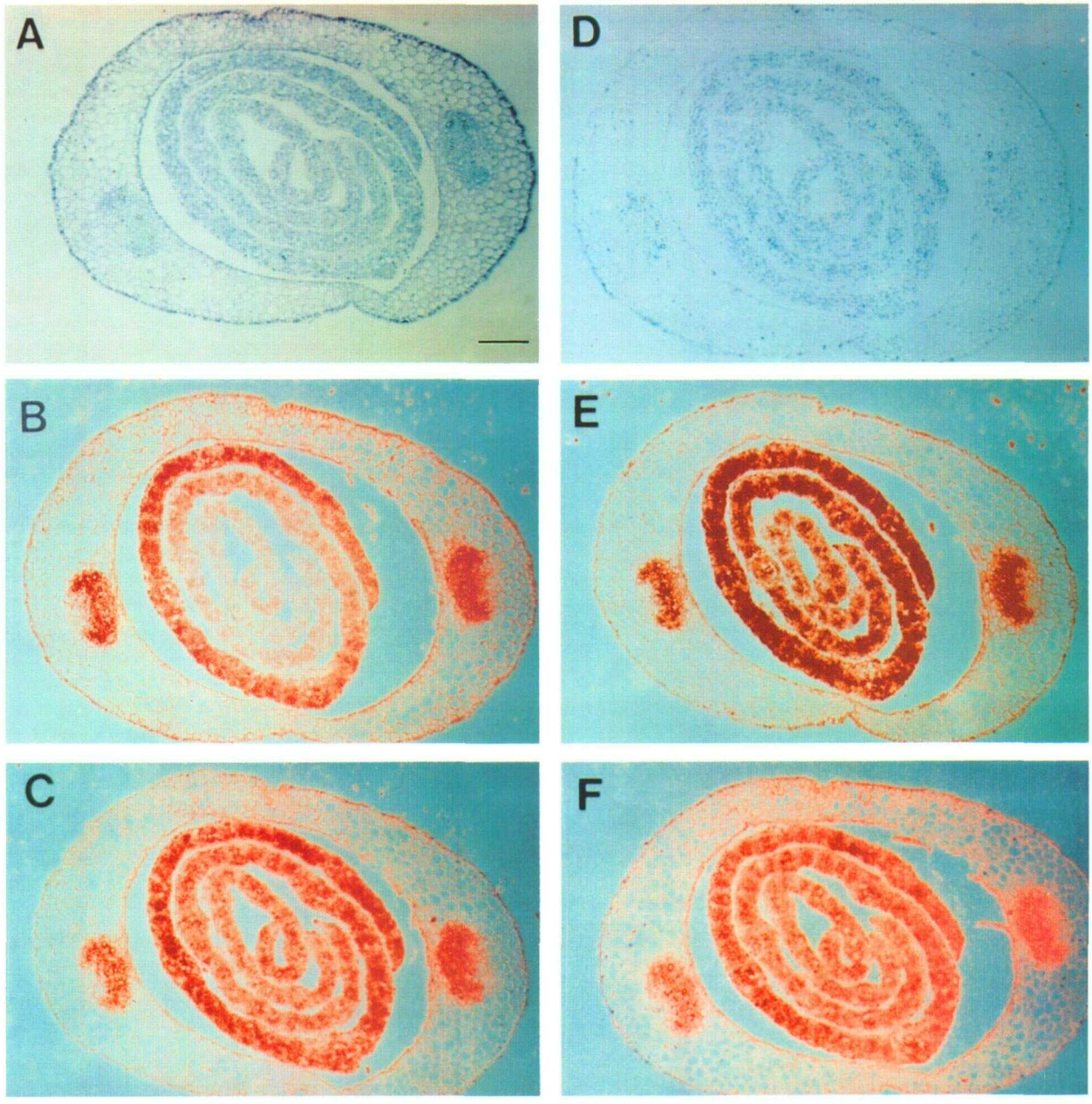

Figure 5. Localization of Different Mitochondrial Gene Transcripts in Shoot Cross-Sections.

Sections corresponding to position C1 of Figure 1 from the same seedling were stained with toluidine blue after in situ hybridization with RNA probes and visualized by bright-field illumination only in **(A)** or by bright-field with concomitant dark-field illumination with use of a red filter in **(B)** to **(F)**.

(A) Toluidine blue–stained section; no dark-field illumination.

(B) In situ hybridization of an antisense *rrn18* RNA.

(C) In situ hybridization of an antisense *coxI* RNA.

(D) In situ hybridization of a sense *atpA* RNA.

(E) In situ hybridization of an antisense *atpA* RNA.

(F) In situ hybridization of an antisense *coxII* RNA.

Bar in **(A)** = 0.2 mm for **(A)** to **(F)**.

123 fluorescence in the root cap and the meristem above it was then investigated by confocal laser microscopy. A confocal image of the signals from a single cell at the periphery of the root cap (Figure 6D) shows the distribution of punctate green fluorescence signals partially localized around the nucleus that is characteristic of the distribution of mitochondria observed in other plant cells (Wu, 1987). Only the green fluorescence characteristic of rhodamine-123 was observed in these and other sections; no fluorescence in vacuoles or at other wavelengths was detected. The pattern of rhodamine-123 fluorescence seen in the lower magnification image of a root tip longitudinal section (Figure 6H) is consistent with the distribution of mitochondria in the root cap and meristem predicted from earlier observations (Clowes and Juniper, 1964; Juniper and Clowes, 1965). The dramatic contrast between the distribution of mitochondria, as visualized by the pattern of rhodamine-123 fluorescence, and the in situ hybridization signals of Figure 6F provides a compelling demonstration of the absence of a strict correspondence between the levels of mitochondrial transcripts and the amount of mitochondria per se.

DISCUSSION

Specificity of Hybridization Signals

Several lines of evidence indicate that the signals observed resulted from hybridization of specific mtRNAs to the individual probes used. No significant signal was observed in negative control experiments, which included parallel hybridizations with sense strand probes (an example is shown in Figure 5D) and pretreatment of tissue sections with RNase before hybridization (data not shown). In many cases, distinct hybridization patterns were obtained from consecutive tissue sections when different probes were used, further suggesting that the signals did not result from nonspecific hybridization. The hybridization patterns were highly reproducible: in every case in which the same probe was used on consecutive sections, the same hybridization pattern was obtained, and all of the hybridizations were repeated using a different maize inbred S-type cytoplasm line, with essentially the same results. In addition, hybridization with mtDNA is not likely to contribute significantly to the observed signals. The sense and antisense strand probes would be expected to hybridize with the mtDNA equally well, but no appreciable hybridization was observed with any of the sense strand probes used. Moreover, as mentioned above, no clear signal was detected in RNase-treated sections, even after treatment with NaOH to ensure denaturation of mtDNA.

Cell-Specific Expression of Mitochondrial Transcripts

Our analysis indicates that mitochondrial transcripts are highly localized in both shoot and root tissues of maize seedlings. This localization is not simply a reflection of cytoplasmic density because neither the cytoplasmic rRNA probe nor toluidine staining shows a similar pattern of localization. In many tissues, the patterns of transcript localization are likely to correspond, at least to some degree, to the abundance of mitochondria in the different cells. The analysis of the distribution of mitochondria and mitochondrial transcripts in the root tip clearly shows, however, that the transcript localization patterns do not in all cases reflect the density of mitochondria and that the two patterns may in some cases correspond very little to one another.

The distribution of transcripts parallels to a large extent the cell division activity in various parts of the seedling. In the root, for example, mitochondrial transcript levels are high throughout the meristem but extremely low in the nondividing cells of the quiescent center and root cap. Their distribution corresponds closely to the pattern of mitotic activity as determined by ^{3}H-thymidine labeling of root tips (Clowes, 1956). In the shoot, Stein and Quastler (1963) found that cells near the tip of the coleoptile are the first to incorporate the label from ^{3}H-thymidine into nuclear DNA, followed, progressively, by the foliage leaves and shoot apex. By 60 hr after germination, mitosis begins to be restricted to the lower portion of the seedling, with the shoot apex being the last portion to resume division. At the stage we have analyzed, mitotic activity would be expected to be more prevalent in the leaves and in the shoot apex and thus would parallel the distribution of the *atp6* and RNA*b* transcripts we examined.

The correlation between mitochondrial transcript abundance and cell division is not surprising because mitochondrial biogenesis is likely to be most active in proliferating cells; mitochondrial gene expression may therefore be regulated as part of a more global activation of gene expression during cell proliferation. Because mitochondria are distributed throughout the root cap, mitochondrial transcript levels in this tissue more closely parallel cell division activity than the amount of mitochondria. In other tissues, however, such as developing microspores (Lee and Warmke, 1979; Smart et al., 1994), increased mitochondrial transcript levels are associated with an increase in mitochondrial number. It is likely that mitochondrial transcript levels are governed by a variety of factors, including cell division activity and the metabolic activities associated with the fate of newly formed cells.

In shoots, 26S rRNA transcripts appeared to be at least as prevalent in the parenchyma tissue of the coleoptile as in the leaves, whereas the other mtRNAs studied appeared to be more prevalent in leaves, at least in the older, outermost leaves. Similarly, differences were observed among the distributions of the *atp6*, RNA*b*, and *rrn26* transcripts in mesocotyl sections. RNA dot blot analysis confirmed that the levels of specific transcripts can differ between whole coleoptile and leaf RNA preparations and therefore provided strong support for the view that the in situ hybridization results reflect the in vivo distribution and levels of the analyzed transcripts. The lack of congruence among patterns of distribution of the different mtRNAs, especially between that of the 26S rRNA and the

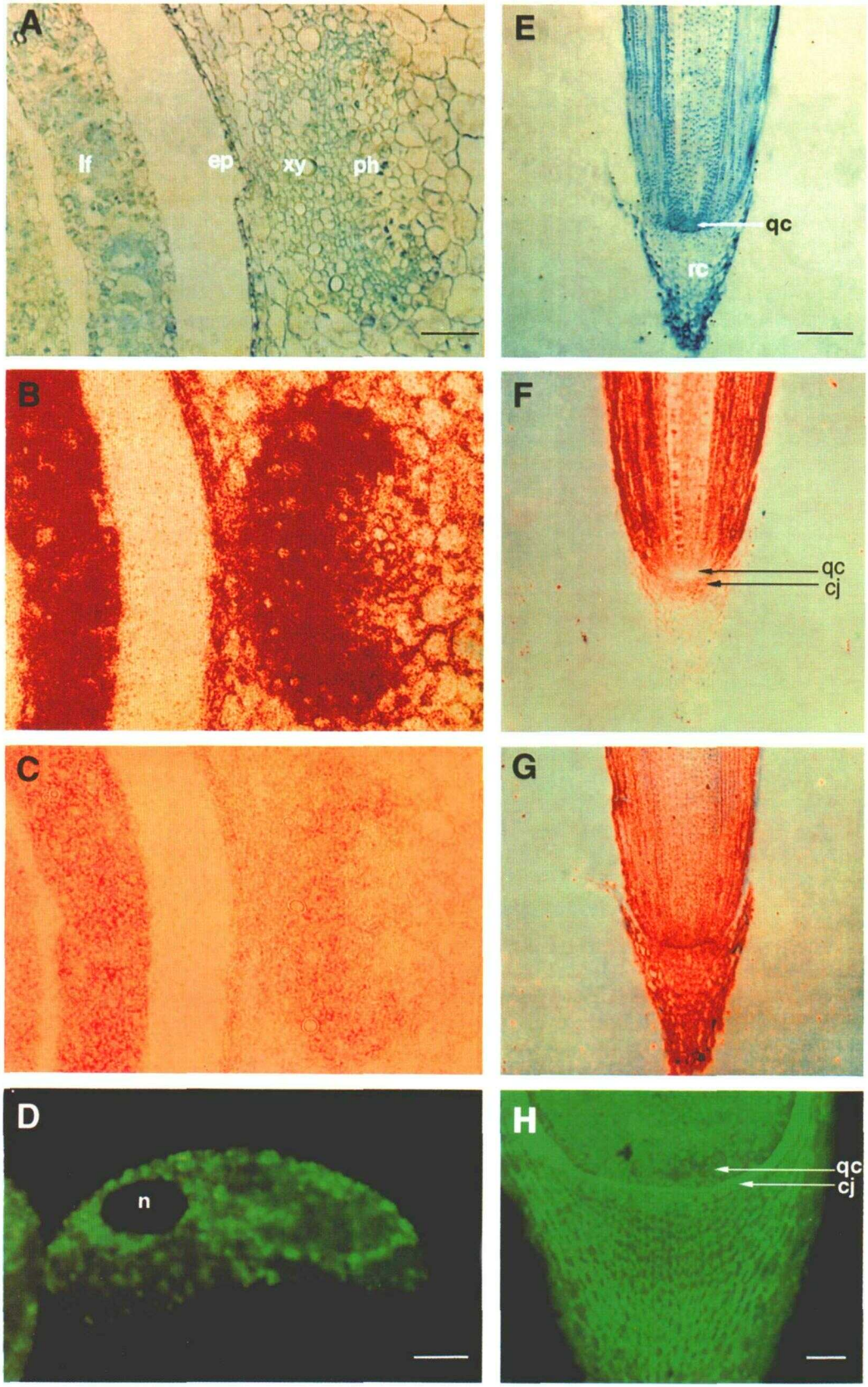

Figure 6. Localization of Transcripts in Cross-Sections through the Shoot Vascular Bundle and of Transcripts and Mitochondria in Root Longitudinal Sections.

other mtRNAs, was surprising because it suggested that controls governing the relative abundance of the different RNA species in the mitochondrion may be different in different cell types. Previous analyses of rates of RNA synthesis in maize tissue culture cells (Finnegan and Brown, 1990) and seedlings (Mulligan et al., 1991) indicated that mitochondrial transcript abundance is regulated at both the transcriptional and post-transcriptional levels. Thus, differences in the rates at which the different genes are transcribed as well as differences in transcript stability could contribute to the observed differences in the relative abundance of the various mitochondrial transcripts among different cell types.

Kuroiwa and colleagues (Kuroiwa et al., 1992; Fujie et al., 1993) have found that in both Pelargonium and Arabidopsis, the amount of mtDNA per mitochondrion is lowest in those meristematic cells more distal to the root apex, and that mtDNA synthesis, as monitored by ^{3}H-thymidine incorporation into mitochondrial nucleoids, was restricted to the meristematic region proximal to the apex. If the findings of Kuroiwa and colleagues can be extended to the maize seedling root, the distribution of mitochondrial transcript abundance that we have observed within the root meristem would more closely correspond to the distribution of mitochondria per se than the distribution of either mtDNA or mtDNA synthesis. Thus, it is highly possible that the developmental controls regulating mitochondrial transcript abundance and mtDNA levels are distinct.

Enhanced Expression of Mitochondrial Genes in Vascular Tissue

All of the mitochondrial transcripts examined were found to be more prevalent in vascular tissues of the root and coleoptile than in the surrounding parenchyma tissues. The hybridization signals appeared to be at least partially localized over meristematic procambium of the developing vascular tissues, and thus, the elevated levels of these transcripts are likely to be associated to some degree with the mitotic activity of these cells. The high levels of mitochondrial transcripts present in developing xylem elements are particularly interesting, especially because the levels of these transcripts in developing phloem cells do not appear to be higher than the levels in the surrounding parenchyma tissue. It is possible that the high level of mitochondrial transcriptional activity in the procambium persists during the differentiation of xylem elements, possibly to support a high level of mitochondrial energy production that may be required for xylem function or formation.

Mitochondrial Gene Expression during Vegetative Growth

Two recent investigations have reported cell-specific differences in mitochondrial gene expression in anther tissues. Smart et al. (1994) have shown that sunflower mitochondrial genes encoding subunits of the ATPase (*atpA* and *atp9*), cytochrome *b* (*cob*), and the large rRNA (*rrn26*) are all expressed at higher levels in developing microsporocytes, meiocytes, and tapetal cells than in surrounding anther tissue. Interestingly, transcripts of a nuclear-encoded mitochondrial protein, the β subunit of the ATP synthetase complex, were not subject to a similar cell-type enhancement of expression, and restoration of nuclear fertility was found to reduce the extent to which the expression of a CMS-associated gene, *orf522*, was enhanced in meiocytes. Conley and Hanson (1994) reported differences in the expression pattern of a number of different mitochondrial proteins in developing petunia anthers, but they did not examine the distribution of transcripts of these genes. Interestingly, the distribution of the ATPA and PCF (the product of the petunia CMS-associated fused gene) proteins observed by these authors differed from that of another mitochondrial gene product, the COXII protein, in that COXII was present only at low levels in sporogenous cells and vascular tissues, whereas levels of the other proteins were enhanced in these tissues. In maize seedlings, levels of *coxII* transcripts, like those of the other mitochondrial transcripts studied, are enhanced in vascular tissues.

Our findings show that expression of the mitochondrial genome is subject to a significant degree of spatial and temporal regulation during not only anther development but vegetative

Figure 6. (continued).

(A) and **(E)** were obtained after staining with toluidine blue and bright-field microscopy. **(B)**, **(C)**, **(F)**, and **(G)** were visualized by bright-field concomitant with dark-field illumination, with use of a red filter after in situ hybridization with antisense RNA probes followed by staining with toluidine blue. In **(D)** and **(H)**, mitochondria labeled with rhodamine-123 were visualized by confocal laser microscopy. cj, cap junction; ep, epidermis; lf, leaf; n, nucleus; ph, phloem; qc, quiescent center; rc, root cap; xy, xylem.

(A) Higher magnification of the vascular bundle portion of a shoot cross-section.

(B) Higher magnification of the vascular bundle portion of a shoot cross-section–in situ hybridization of an antisense *rrn18* transcript.

(C) Higher magnification of the vascular bundle portion of a shoot cross-section–in situ hybridization of an antisense *atp6* transcript.

(D) Confocal microscopy of rhodamine-123 fluorescence in a 5-μm optical section through an individual cell at the periphery of the root cap.

(E) Longitudinal section through the seedling primary root.

(F) Longitudinal section through the seedling primary root–in situ hybridization of an antisense *atpA* probe.

(G) Longitudinal section through the seedling primary root–in situ hybridization of an antisense cytoplasmic 17S rRNA probe.

(H) Confocal microscopy of rhodamine-123 fluorescence reconstructed from longitudinal optical sections taken through the root cap and meristem.

Bars in **(A)**, **(D)**, **(E)**, and **(H)** = 0.05 mm, 10 μm, 0.2 mm, and 0.05 mm, respectively.

plant growth as well. It should be emphasized that variations in transcript abundance represent only one aspect of the differences in mitochondrial gene expression. The transcripts isolated from plant mitochondria are often heterogeneous with respect to both the degree of editing and the occurrence of multiple 5′ and, in some cases, 3′ termini (Brown, 1992; Gray et al., 1992; Grienenberger, 1992). It is possible that part of this heterogeneity reflects qualitative, cell-type–specific transcript differences. Moreover, the differences between the levels of proteins synthesized by mitochondria isolated from different plant organs appear in general to be greater than transcript differences, and thus, developmental controls on the translation of specific mitochondrial transcripts may be more dramatic than the transcriptional differences observed here. The elucidation of the developmental controls on mitochondrial gene expression during both vegetative and reproductive growth should represent an interesting challenge for the future.

METHODS

Materials

WF9 maize seeds with S male sterile cytoplasm were from Pioneer Hi-Bred International (Johnston, IA). Cloned mitochondrial DNA (mtDNA) restriction fragments containing the maize *cob* and *coxI* genes were obtained from C.S. Levings III (North Carolina State University, Raleigh, NC), the wheat *rps12* gene clone from L. Bonen (University of Ottawa, Ottawa, Ontario), and the maize cytoplasmic 17S rRNA (clone 6L-1) from J. Messing (Waksman Institute, Rutgers University, New Brunswick, NJ). The sources of other mtDNA clones have been described previously (Finnegan and Brown, 1990; Singh and Brown, 1991). Specific restriction fragments, as indicated in Table 1, were subcloned into pBluescript vectors for in vitro transcription. The fragment orientation and vectors were chosen such that the amount of vector sequence included in the RNA transcript was minimized and the region in the multicloning sites that might hybridize with human rRNA (Witkiewicz et al., 1993) was not included. In addition, the fragments were oriented such that all the probes were transcribed from the T3 RNA polymerase promoter of the vectors; hence, all have the same extraneous sequence.

Tissue Preparation

Paraffin-embedded maize tissue sections were used for in situ hybridization with ^{35}S-labeled RNA probes (Cox and Goldberg, 1988). Briefly, maize seedlings were grown in the dark at 28°C for 50 to 60 hr, until the average length of the shoot was ~1.4 cm. Tissues were immediately fixed in FAA (3.7% formaldehyde, 50% ethanol, and 5% acetic acid) for 3 hr at room temperature, dehydrated through an ethanol series, and then embedded in paraffin. Tissue sections 10 μm thick were spread on a drop of water on microscopic slides subbed with poly-L-lysine (Sigma) and baked overnight on a slide warmer set at 48°C. After removing the wax with xylene, sections were rehydrated by an ethanol series and treated with 1 μg/μL proteinase K for 1 hr at 37°C and then with 0.25% (v/v) acetic anhydride in 100 mM triethanolamine, pH 8.0, for 5 min at room temperature. After a brief wash in 2 × SSC (1 × SSC is 0.15M NaCl, 0.015M sodium citrate), sections were dehydrated in an alcohol series, dried under vacuum for 1 hr, and stored under vacuum until hybridization.

Table 1. Genes Used as Probes for the in Situ Hybridization of Maize Seedlings

Gene	Plasmid	Species	Source	Fragment for Subcloning (kb)	Reference
atpA		*Zea mays*	pTA22 Braun and Levings (1985)	EcoRI-SacI (1.1)	Finnegan and Brown (1990)
atp6	KMA6.19	*Z. mays*	pT25H Dewey et al. (1985)	TaqI (0.95)	Finnegan and Brown (1990)
cob	pLP120	*Z. mays*	pZmEH680 Dawson et al. (1984)	HindIII-EcoRI (0.68)	This work
coxI	pLP128	*Z. mays*	pBN6601 Isaac et al. (1985)	EcoRI-BamHI (3.9)	This work
coxII	pLP130	*Z. mays*	pZmE1 Fox and Leaver (1981)	EcoRI-HindIII (2.0)	This work
RNA*b*	pKPB12	*Z. mays*	cDNA Zhang and Brown (1993)	EcoRI (0.6)	Zhang and Brown (1993)
rps12	pLP122	Wheat	pB303 Gualberto et al. (1988)	XhoI-EcoRI (0.2)	This work
rrn18	pLP124	*Z. diploperennis*	pZdmt6 Gwynn et al. (1987)	PstI-HindIII (2.5)	This work
rrn26	pKM26E12	*Z. mays*	pB406 Iams and Sinclair (1982)	SmaI-HindIII (2.0)	Finnegan and Brown (1990)
Cytoplasmic 17S rRNA	pMS17S1	*Z. mays*	p6L-1 from J. Messing	SacI (1.5)	This work

Labeling of RNA Probes

^{35}S-labeled RNA probes were synthesized by using a Stratagene in vitro transcription kit according to the manufacturer's instructions, except that 15 μM α-^{35}S-UTP (650 Ci/mmol) was substituted for the cold UTP and the reaction was performed at 40°C for 1 hr. After removal of unincorporated nucleotide with a Sephadex G-50 spun column, labeled RNA was precipitated with sodium acetate and hydrolyzed to an average size of 100 nucleotides with 120 mM sodium carbonate and 80 mM sodium bicarbonate at 60°C (Cox and Goldberg, 1988).

In Situ Hybridization

Tissue sections were hybridized overnight at 42°C in a moist chamber in 100 mL of hybridization solution per slide (50% formamide, 300 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 × Denhardt's solution [0.02% Ficoll, 0.12% PVP, and 0.02% BSA], 10% dextran sulfate, 70 mM DTT, 150 μg/mL yeast tRNA, and 200 ng/mL probe). After they were hybridized, sections were washed several times in 4 × SSPE (1 × SSPE is 0.15 M NaCl, 0.01 M NaH_2PO4, 1 mM EDTA, pH 7.4) plus 5 mM DTT. Single-stranded RNAs were removed from the sections with a 30-min incubation in 25 μg/mL RNase A in a high salt buffer (500 mM NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA) at 37°C. RNase was removed by three brief washes in 2 × SSPE and 5 mM DTT. Two low-stringency washes were performed for 20 min each in the same buffer at room temperature; these were followed by three high-stringency washes at 57°C (20 min each) in 0.1 × SSPE and 1 mM DTT. After dehydration through an ethanol series, microscopic slides with tissue sections were dipped into a 1:1 [v/v] dilution of Kodak NTB-2 liquid emulsion at 42°C, exposed for from 1 day to 2 weeks at 4°C, developed in Kodak D-19 developer at 15°C for 2.5 min, and fixed in Kodak fixer at 15°C for >5 min. The exposure time varied, depending on the abundance of the RNA species being examined. After the sections were washed in running tap water for 30 min, they were counterstained with 0.1% toluidine blue in 0.1% sodium borate for 30 sec, washed in tap water to remove the stain in the emulsion, dehydrated in ethanol, and mounted with Permount (Fisher Scientific, Fair Lawn, NJ). Photography was done with a Zeiss Photomicroscope II (Carl Zeiss, Oberkochen, Germany), using dark-field/bright-field double illumination techniques (Levine, 1980). In some cases, digital images of photographic prints were generated using a ScanJet II CX/T scanner (Hewlitt-Packard Co., Greeley, CO), and composites of individual prints were assembled using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA).

Quantitative RNA Dot Blot Hybridization

Shoots were removed from 120 seedlings at 50 hr after germination and sectioned longitudinally by hand with a scalpel. The coleoptiles were then separated from the leaves and shoot apices by using a fine forceps. Leaf tissue (0.3 g) and coleoptile tissue (0.5 g) were frozen in liquid nitrogen and ground into a powder with a mortar and pestle. Three milliliters of ice-cold isolation buffer (0.1 M Tris-HCl, pH 8.0, 50 mM EDTA, 1.3 M NaCl, 1% sarcosyl, 1% SDS, 0.5% Triton X-100, 1% PVP, 10 mM DTT, and 60 mM mercaptoethanol) was added, and the resulting suspension was extracted once with phenol, once with 1:1 [v/v] phenol-chloroform, and once with chloroform. Sodium acetate was added to a concentration of 0.3 M, and the nucleic acids were precipitated with 2.5 volumes of ethanol. The samples were dissolved in 100 μL of water and treated with RNase-free DNase, as described previously (Finnegan and Brown, 1990).

The resulting RNA preparations were then dissolved in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), and the concentrations of the coleoptile and leaf RNA samples were each adjusted 2 mg/mL based on their absorbance at 260 nm. A series of twofold dilutions of each RNA was then prepared, and 2 μL of each of the original and diluted RNAs was applied to GeneScreen Plus membranes (Du Pont–New England Nuclear) by the direct dot blot procedure of Shirley and Meagher (1990). This process resulted in a series of dots, for each sample, containing 4, 2, 1, 0.5, 0.25, and 0.125 μg of RNA, on each of three membranes. Direct rather than suction blotting of RNAs results in increased efficiency and linearity in binding of the RNA samples (Hightower and Meagher, 1985; Shirley and Meagher, 1990).

DNA fragments from the RNA*b*, *atp6*, 26S mitochondrial rRNA, and 17S cytoplasmic rRNA pBluescript clones used to generate the corresponding in situ hybridization probes were gel-purified and labeled by the random primer method using a Pharmacia T7 Quickprime kit, according to the manufacturer's instructions. Membranes were prehybridized at 60°C for 2 hr in a medium containing 5 × SSPE, 5 × Denhardt's solution, 1% SDS, 10% dextran sulfate, and 100 μg/mL denatured salmon sperm DNA. The membranes were then hybridized with the labeled DNA probes in the same solution, omitting the salmon DNA, for 16 hr at 60°C. After hybridization, the membranes were washed twice for 15 min at 60°C with 3 × SSC, 1% SDS, and twice for 15 min at 60°C with 0.3 × SSC, 0.1% SDS. Membranes were exposed to x-ray film, and the resulting autoradiographs were scanned with a densitometer. As with the in situ hybridization analysis, the exposure time varied depending on the abundance of the RNA species being examined. To estimate the relative amounts of each transcript in the leaf and coleoptile RNA preparations, the dots on the autoradiograph were scanned with a PDI densitometer, and the total hybridization signal was integrated over each dot using ImageMaster software (both from PDI Inc., Huntington Station, NY). The amount of dilution required to give dots with similar total hybridization signals was considered to define the relative levels of the specific transcript in the two RNA samples.

Confocal Imaging of Rhodamine-123 Fluorescence in Live Root Tissue

A manual longitudinal cut was made in roots of 3-day-old seedlings, and the tissue was immersed in 15 μg/mL rhodamine-123 for 5 hr in the dark. The tissue was rinsed for 30 min in distilled water, and fluorescence was visualized through a Leica confocal laser scanning microscope (Leica Lasertechnik GmbH, Heidelberg, Germany), using an argon 488-nm laser for dye excitation.

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