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# Occurrence of Mitochondria in the Nuclei of Tobacco Sperm Cells

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**Tobacco sperm cells contain intact mitochondria within their nuclei with a frequency of 0.35 ± 0.13 per cell. These inclusions appear to originate from mitochondria found among chromatids in the highly elongated metaphase plate of the dividing generative cell. These organelles are apparently captured during the reconstitution of the nuclear envelope. Only sperm cells were observed to contain these nuclear mitochondria; generative cells, vegetative pollen cells, transmitting tissue cells, unfertilized egg cells, and central cells lacked them. Nuclear mitochondria were also seen in the nuclei of the egg and central cell after fusion with sperm nuclei, suggesting that nuclear mitochondria are transmitted into the zygote and primary endosperm cells during double fertilization. Organellar inclusions in the sperm nucleus provide a potential mechanism for transmitting organellar DNA into the next generation and could potentially facilitate the transfer of genetic material between the nucleus and other organelles.**

## INTRODUCTION

DNA-containing organelles of plant cells—the nucleus, mitochondrion, and plastid—are usually considered to be spatially separated cellular components. Exceptions have largely been limited to relationships between the outer membranes of these organelles. In several chrysophycean and cryptophycean algae, doubled membranes of the outer envelope of the nucleus were observed to enclose chloroplasts (Gibbs, 1962). Chloroplasts containing mitochondria within surface invaginations have been reported in *Hyptis* and maize (Montes and Bradbeer, 1976) and completely enclose mitochondria in *Panicum* (Brown et al., 1983). Chloroplasts and mitochondria were seen joined by their outer membranes in the fern *Pteris* (Crotty and Ledbetter, 1973) and may be located in a single compartment composed of their outer membranes in *Euglena* (Calvayrac et al., 1981). Linked outer membranes of the nuclear envelope and mitochondria have occasionally been observed in cells of wheat and *Pteris* (Crotty and Ledbetter, 1973), whereas in *Cosmos* microsporocytes, mitochondria appear to aggregate around the nucleus and adhere to the nuclear envelope (Dickinson, 1986). These reports most commonly explain the closeness of these interorganellar relationships as evidence of physiological cooperation.

A more unusual situation occurs in tobacco, where normal-appearing mitochondria are entirely enclosed within the nuclei of sperm cells. To verify their commonness and determine their fate after fertilization, specimens containing sperm nuclei were serially thin sectioned at 100 nm. Thereafter, all sections were collected, stained, and photographed using a transmission electron microscope. Use of this serial technique eliminates the possibility that these are merely cytoplasmic invaginations into the nucleus. Here, we report on the occurrence, frequency, and formation of nuclear mitochondria and discuss these in the context of their possible significance.

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## RESULTS

The sperm cells of tobacco occur as a linear assemblage in the pollen tube consisting of two sperm cells and a vegetative nucleus, as in the majority of flowering plants. One sperm cell is consistently associated with the vegetative nucleus, whereas the other sperm cell is unassociated with the vegetative nucleus and bound together with the first sperm cell by a common pollen tube plasma membrane (Figure 1A). Sperm cells are similar in size and are statistically identical in surface area and volume of the cytoplasm, nucleus, and selected organelles early in development (Yu et al., 1992) and differ only in cell surface area later in development (Yu and Russell, 1994a).

Mitochondria with normal ultrastructural features (Figures 1B and 1C) are found within occasional sperm nuclei; these appear to occur from sperm inception to fertilization. The ultrastructural features of these organelles include the occurrence of intact double membranes, electron-dense inclusions, and internal membranes that presumably represent cristae (Figure 1C). Such mitochondria are found singly or occasionally in small groups (Figure 1D). They are usually found nearer to the periphery than to the center of the nucleus (Figures 1B and 1D). These mitochondria are completely enclosed within the nuclear envelope. A group of these mitochondria is shown

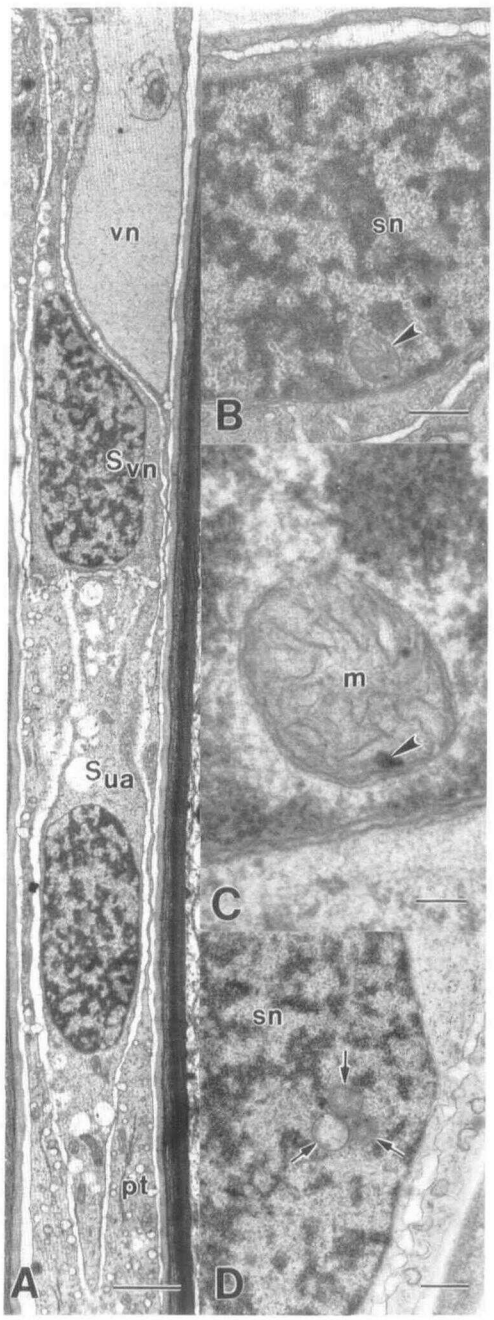


**Figure 1.** Transmission Electron Micrographs of Sperm Cells within Pollen Tubes and Sperm Nuclei Containing Mitochondria in Tobacco.

**(A)** Typical sperm cells and vegetative nucleus in the pollen tube. Bar = 2 μm.

**(B)** Low-magnification view of mitochondrion (arrowhead) in the sperm nucleus. Bar = 0.5 μm.

**(C)** Higher magnification of same nuclear mitochondrion shown in **(B)**. Arrowhead indicates osmiophilic body apparently unique to mitochondria. Bar = 0.1 μm.

**(D)** Low-magnification view of an aggregation of three mitochondria (arrows). Bar = 0.5 μm.

m, mitochondrion; pt, pollen tube; sn, sperm nucleus; $S_{vn}$, sperm associated with the vegetative nucleus; $S_{ua}$, sperm not associated with the vegetative nucleus; vn, vegetative nucleus.

at higher magnification in serial sections in Figures 2A to 2C. These remain as separate organelles although they are aggregated. Because sperm mitochondria are rarely more than 0.3 μm thick, each is visible in at most four 100-nm-thick sections. The appearance of cristae and the crispness of outer membranes are influenced by section thickness, section position, and sectioning orientation (Figures 2A and 2B). Other types of nuclear inclusions were sometimes observed although not all could be clearly identified (Figure 2C).

The occurrence of nuclear mitochondria was limited to sperm cells. No other cells examined in this study contained similar nuclear structures prior to fertilization, including generative cells, vegetative cells, transmitting tissue cells, egg cells, and central cells (Table 1). Nuclear mitochondria appear to occur with a similar frequency from sperm cell formation (~8.5 hr after pollination) to fertilization (normally 36 to 50 hr after pollination) ($P > 0.05$, anova). At 9 hr after pollination, each sperm nucleus contains an average of 0.44 ± 0.13 mitochondria ($n = 32$) with up to three mitochondria observed in a single nucleus. At 26 hr after pollination, the average number of nuclear mitochondria is 0.29 ± 0.13 mitochondria ($n = 14$), and in unfused sperm cells within the embryo sac at 45 hr after pollination, their occurrence is 0.13 ± 0.13 mitochondria ($n = 8$).

Mitochondria are still present in sperm nuclei after cellular fusion of the gametes. Figure 3A illustrates the presence of a nuclear mitochondrion in a sperm nucleus within the egg prior to fusion with the egg nucleus. The cytoplasm of the sperm cell is evident at the periphery of the egg cell, immediately adjacent to the degenerated synergid (Figure 3A, arrowheads). The sperm cell had apparently just completed cellular fusion with the egg cell when this ovule was fixed. Although the nuclear mitochondrion in this cell seems to retain its size, shape, and internal organization, the cristae appear wider (Figure 3B) than in nuclear mitochondria of younger sperm cells (cf. Figure 1C).

Nuclear mitochondria are also found in the fusion products of the sperm and the egg nuclei (Figure 3C), and the second sperm and the polar nuclei (Figure 4A), indicating that nuclear mitochondria are transmitted during double fertilization. Nuclear mitochondria in the fusion nuclei of the zygote and primary endosperm continue to display double membranes and electron-dense deposits, but the number of cristae appears reduced compared to cytoplasmic mitochondria (Figures 3D, 4B, and 4C). The size of the nuclear mitochondria in fusion nuclei corresponds closely with sperm mitochondria (~0.25 μm long in median section) and is less than that in

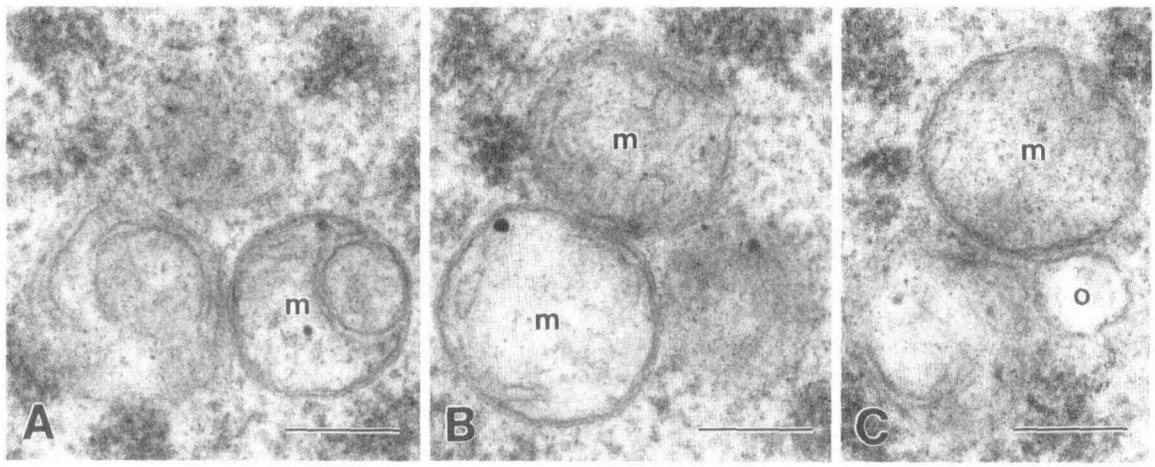


**Figure 2.** Higher Magnification of Consecutive Serial Sections of Three Aggregated Nuclear Mitochondria Shown in Figure 1D.

**(A)** Median section of one mitochondrion (m) and oblique sections of two adjacent mitochondria (top and left). Glancing sections of circular internal membranes are evident in two mitochondria.

**(B)** Higher magnification of the section shown in Figure 1D. A different view of the cristae is evident in this section compared to **(A)**.

**(C)** Adjacent section showing two mitochondria and an unidentified organelle (o) located adjacent to the lower right mitochondrion in **(A)** and **(B)**.

Bars = 0.2 μm.

female cytoplasm (~0.4 μm long in median section) (Figures 3C and 4A). Nuclear mitochondria occur with a frequency of 0.33 ± 0.13 in the zygote nucleus and 0.13 ± 0.09 in the primary endosperm nucleus, closely corresponding to the frequency of nuclear mitochondria in the sperm cells (Table 1). These data support a paternal origin of nuclear mitochondria. These inclusions were absent in unfertilized egg and polar nuclei.

Nuclear mitochondria are first observed in the male lineage only after the division of the generative cell. We therefore examined dividing generative cells to determine how mitochondria and cytoplasmic inclusions might be incorporated into sperm nuclei. Because generative cells divide within the narrow confines of the growing pollen tube, their mitotic spindle is highly constrained, and the metaphase plate may be almost vertical. This unusually long spindle does not apparently successfully exclude cytoplasmic organelles from the mitotic spindle and nuclear region. Figure 5A illustrates two mitochondria present near chromatids during late anaphase. Slightly later, during early telophase (Figure 5B), mitochondria (unlabeled arrow) are also observed among decondensing chromatids and at the edge of the reforming nucleus (labeled mitochondrion). Mitochondria present within this region may conceivably be captured during the reformation of the nuclear envelope. Another potential mechanism for uptake is the incorporation of mitochondria through cytoplasmic invaginations into the interior of the nucleus; however, this seems less likely because

**Table 1.** Occurrence of Nuclear Mitochondria in Reproductive and Gynoecial Cells of Tobacco Based on Analyses of Ultrathin Serial Sections

| Cell Type | No. of Nuclei Examined | No. of Nuclear Mitochondria | Frequency (Mean ± SE) |
|---|---|---|---|
| Newly formed generative cell | 9 | 0 | 0 |
| Generative cell in mature pollen | 10 | 0 | 0 |
| Generative cell 9 hr after pollination | 7 | 0 | 0 |
| Newly formed sperm nucleus | 32 | 14 | 0.44 ± 0.13 |
| Sperm nucleus 26 hr after pollination | 14 | 4 | 0.29 ± 0.13 |
| Sperm nucleus in embryo sac | 8 | 1 | 0.13 ± 0.13 |
| Vegetative nucleus | 4 | 0 | 0 |
| Transmitting tissue nucleus | 5 | 0 | 0 |
| Egg nucleus | 7 | 0 | 0 |
| Polar nucleus | 12 | 0 | 0 |
| Zygote nucleus | 15 | 5 | 0.33 ± 0.13 |
| Primary endosperm nucleus | 15 | 2 | 0.13 ± 0.09 |

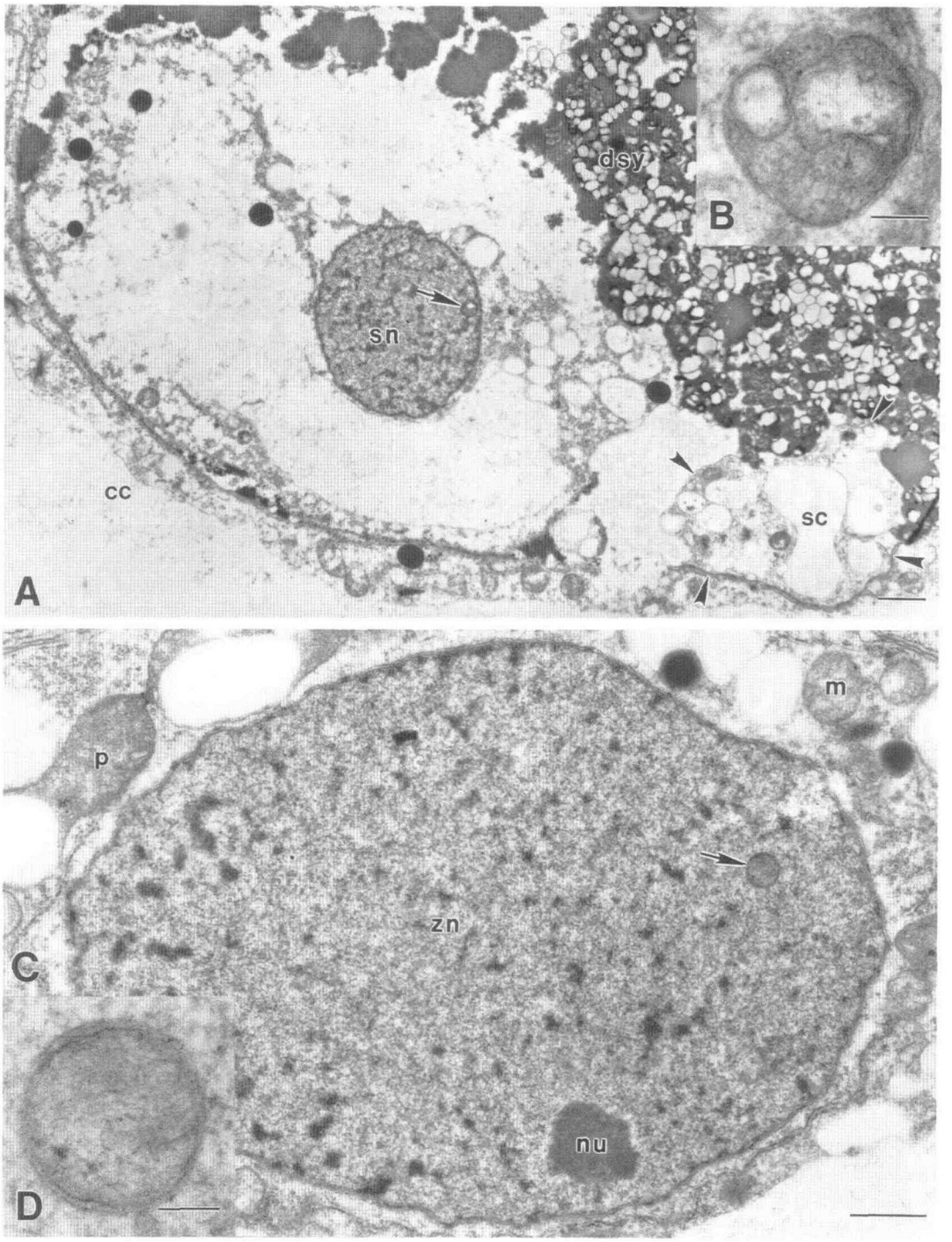


**Figure 3.** Nuclear Mitochondria during Fertilization Events in the Egg/Zygote.

**(A)** Immediately after gametic fusion, the sperm nucleus apparently enters the egg cell/zygote via the degenerated synergid. A mitochondrion (arrow) is present within the sperm nucleus. Arrowheads indicate a region containing sperm cytoplasm at the presumed site of cellular fusion. Bar = 2 μm.

**(B)** Inset of the same nuclear mitochondrion shown in **(A)**. Bar = 0.1 μm.

**(C)** After nuclear fusion, a mitochondrion (arrow) of presumed sperm origin is evident within the nucleus of the zygote. This nuclear mitochondrion is significantly smaller than maternal mitochondria found in the cytoplasm of the zygote. Bar = 1 μm.

**(D)** Inset of the same nuclear mitochondrion shown in **(C)**. Bar = 0.1 μm.

cc, central cell; dsy, degenerated synergid; m, mitochondrion; nu, nucleolus; p, plastid; sc, sperm cytoplasm; sn, sperm nucleus; zn, zygote nucleus.

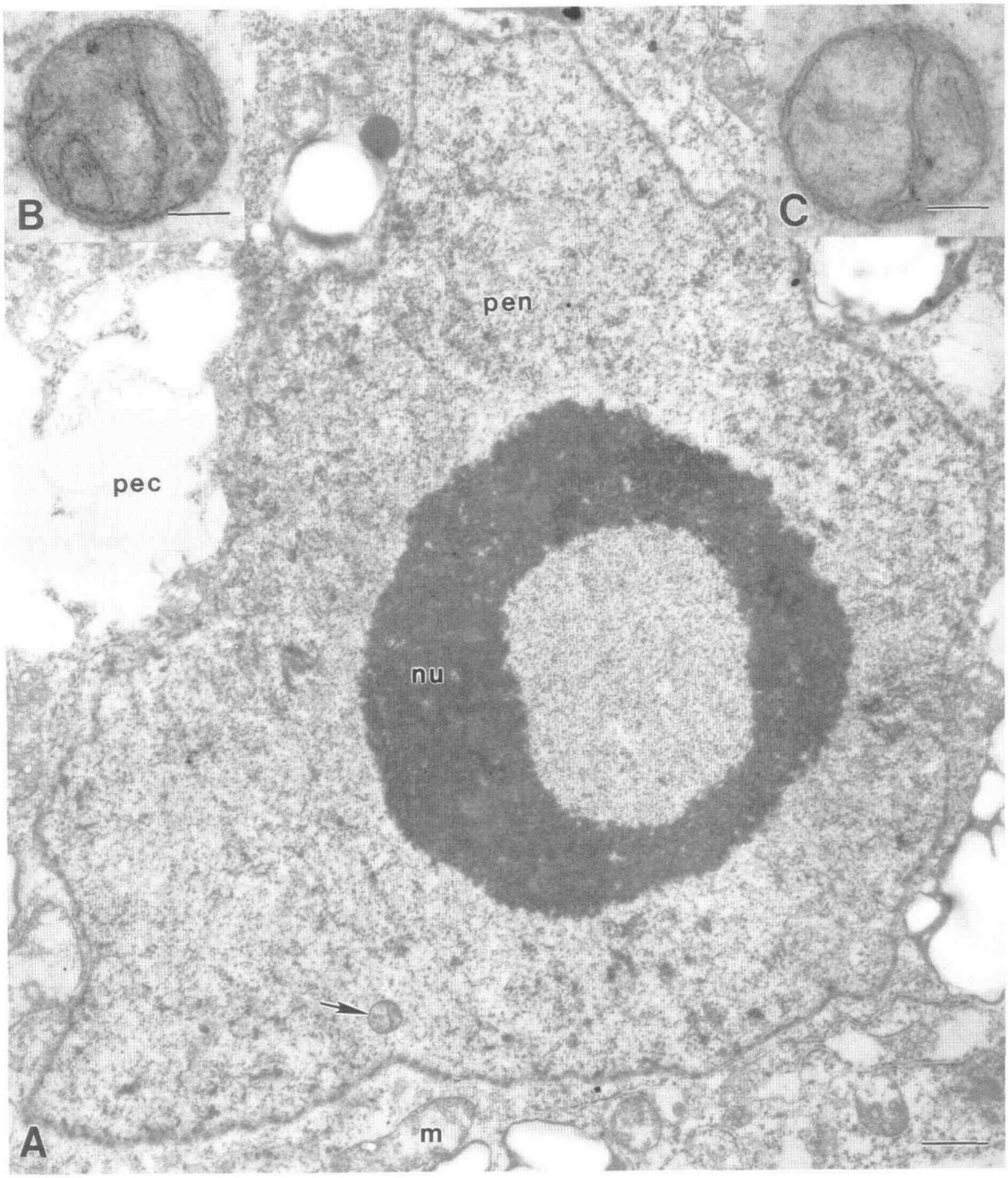


**Figure 4.** Nuclear Mitochondria of Putative Sperm Origin during Fertilization Events in the Primary Endosperm Cell.

**(A)** After the fusion of the two polar nuclei with a sperm nucleus, a mitochondrion (arrow) of presumed sperm origin is found within the primary endosperm nucleus. The nuclear mitochondrion is significantly smaller than maternal mitochondria found in the primary endosperm. Bar = 1 μm.
**(B)** Inset of a serial section of the same nuclear mitochondrion shown in **(A)**. Bar = 0.1 μm.
**(C)** Inset of nuclear mitochondrion shown in **(A)**. Bar = 0.1 μm.
m, mitochondrion; nu, nucleolus; pec, primary endosperm cell; pen, primary endosperm nucleus.

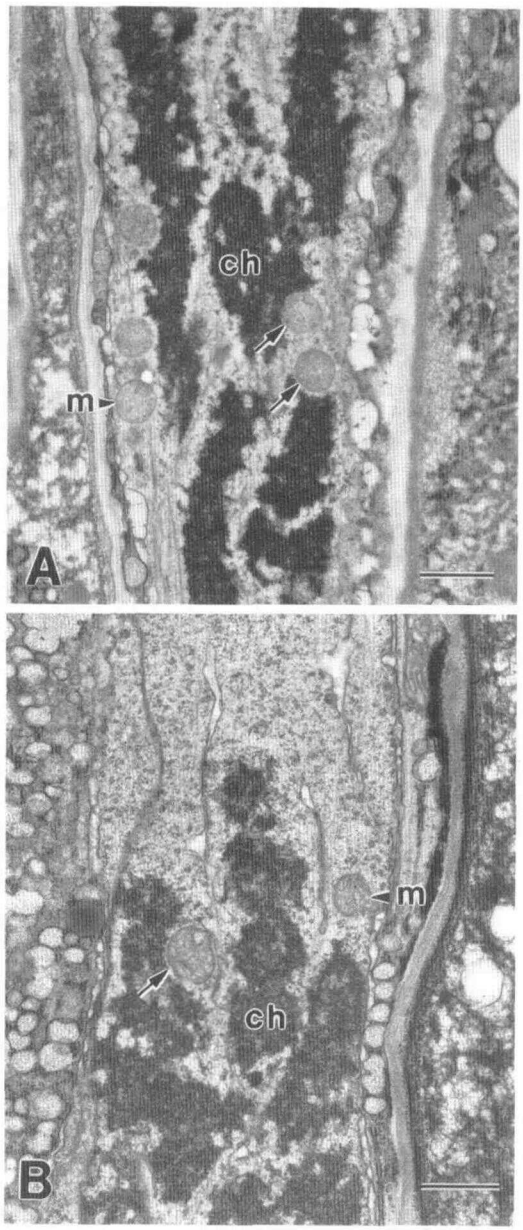


**Figure 5.** Late Stages of Generative Cell Division.

Mitochondrial incursion into the nuclear region apparently results in entrapment within sperm nuclei during later reconstitution of the nuclear envelope.

**(A)** Late anaphase. Spindle fibers are evident among the chromatids and extending to the poles. Mitochondria (arrows) are infrequently present among the chromatids. Bar = 1 μm.

**(B)** Early telophase. The phragmoplast has begun to form at the equatorial plane. Occasionally, one mitochondrion (arrow) or more are present among the decondensing chromatids. During the reconstitution of the nuclear envelope, this mitochondrion would presumably be captured within the nucleus. Bar = 1 μm.

ch, chromatid; m, mitochondrion.

nuclear mitochondria are not surrounded by cytoplasm, and suitable invaginations were not observed.

## DISCUSSION

### Nonartifactual Nature of Mitochondria in the Sperm Nucleus and Its Successors

Because serial sectioning and reconstruction were used throughout this study, it is evident that nuclear mitochondria are located entirely within the nucleoplasm. They are found throughout the lifespan of the sperm, but not in other cell types, and appear to be ultrastructurally similar to mitochondria in the cytoplasm. The size, organization, position, and frequency of nuclear mitochondria seem unchanged during development, suggesting that nuclear mitochondria remain functional and are not eliminated from the sperm. Nuclear mitochondria are present regardless of the method used to culture pollen tubes and regardless of the fixation technique used. These findings attest to the nonartifactual nature of nuclear mitochondria.

According to a prior study, the cytoplasm of the sperm cells is transmitted during fertilization (Yu et al., 1994); apparently, nuclear mitochondria are also transmitted. Nuclear fusion in tobacco conforms to the premitotic pattern and involves the direct fusion of intact nuclear envelopes prior to division, thus transmitting the contents of both the male and female nuclei to the progeny. Because the frequencies of nuclear mitochondria in fusion nuclei of the zygote and primary endosperm are so similar to those of sperm cells, apparently all of the sperm nuclear mitochondria are transmitted during double fertilization. Nuclear mitochondria do not seem to occur after the division of the zygote and primary endosperm nuclei.

### Mechanism of Nuclear Mitochondrion Incorporation

That the frequency of nuclear mitochondria does not change significantly during the lifespan of the sperm cells suggests that no mechanisms are active for either the accumulation or elimination of nuclear mitochondria during sperm maturation and that the included mitochondria remain conventional in organization, although unconventional in position, within the nuclear matrix.

Although it is remotely possible that mitochondria are incorporated through the invagination of cytoplasmic processes into the nucleus, mitochondria were never surrounded by cytoplasm in the nucleus. In addition, there was no evidence of cytoplasmic invaginations, which would have been expected if this mechanism was active throughout development.

Given these observations, the most reasonable assumption is that nuclear mitochondria are taken up during the division cycle that forms the sperm cells. During this division, the mitotic spindle is subjected to extreme spatial constraints inside pollen tubes grown in vivo; the mitotic apparatus may extend

over 35 μm in length at the close of anaphase and may occupy a width of less than 4 μm, creating an essentially vertical mitotic spindle (Yu and Russell, 1993). Most organelles accumulate at the poles of the dividing generative cell at prometaphase. Mitochondria that are not excluded from the field of the mitotic spindle may become located among the chromosomes during division and fail to migrate away from the reforming nucleus during the reconstitution of the nuclear envelope. During anaphase, chromatids then migrate to the poles of the generative cell. At telophase, fragments of nuclear envelope first reappear on the outer surface of the decondensing chromatids, and then these fragments join to enclose the nuclear matrix (Yu and Russell, 1993). Any mitochondria present in this region would likely be enclosed within the nucleus.

If this is true, the occurrence of nuclear mitochondria lends support to the model of nuclear envelope restoration proposed by Stracke and Martin (1991). Using electron spectroscopy, they provided evidence that preassembled envelope cisternae attach to chromosomes at random surface sites and enclose the chromosomal mass by coalescence of external fragments. The alternative mechanism, based on cell-free systems, suggests that the cell nucleus is restored by nuclear envelope fragments that cover individual chromosomes entirely and coalesce to form the new nucleus (for review, see Benavente, 1991). Were the latter model true, mitochondria would be excluded during nuclear envelope formation; however, nuclear mitochondria are present at a consistent low frequency. The absence of stray nuclear envelope fragments within the newly formed nucleus also supports the former model. Cytoplasmic structures are normally absent from the chromatin-rich region in cells with conventionally disposed mitotic spindles. In the generative cell, however, the mitotic spindle is not conventionally disposed, and its mitotic field is apparently not strong enough to exclude mitochondria and occasional organelles from being incorporated into the nucleus. Since similar spatial constraints occur during generative cell division in pollen tubes of other flowering plants, it seems unlikely that nuclear mitochondria would be restricted to tobacco.

### Presumed Significance

Two possibilities seem evident for the underlying significance of nuclear mitochondria in the sperm cells of tobacco. First, the transmission of nuclear mitochondria during karyogamy may provide an alternative and novel pathway for the leakage of paternal mitochondrial DNA into the progeny. In tobacco, ~25 mitochondria are present in each sperm cell prior to fusion (Huang et al., 1993). If these paternal mitochondria are inherited into the embryo—and cytological evidence indicates that this is true (Yu et al., 1994)—then there is no special consequence for the redundant transmission of mitochondria via the nucleus. Results from genetic analysis, however, indicate that tobacco displays purely maternal mitochondrial inheritance (Medgyesy et al., 1986). If this is the case, male cytoplasmic mitochondria may be selectively eliminated prior to the first zygotic division. Nuclear mitochondria reentering the zygotic cytoplasm after this division might escape this elimination and be inherited, albeit infrequently, in the embryo. In plants without the transmission of male cytoplasm, the occurrence and transmission of organelles through their inclusion in the nucleus may be a plausible mechanism for infrequent biparental inheritance. The occurrence of unidentified organelles in the nucleus suggests that a similar mechanism may very infrequently function for plastids and chloroplast DNA in light of their lower frequency (Medgyesy et al., 1986; Yu and Russell, 1994b).

Second, the occurrence of nuclear mitochondria provides an opportunity for recombination to occur between cytoplasmic DNA and nuclear DNA in a manner that could be heritable and would occur in the founding cell of the next generation. Comparisons of plastid, mitochondrial, and nuclear genomes have indicated significant movement of genes between compartments in higher plant cells (Schuster and Brennicke, 1988, 1994). Fragments of the mitochondrial genome have been found in nuclear chromosomes in species as diverse as yeasts and humans. This has been explained by a number of mechanisms, including (1) physical contact and fusion between organelles; (2) direct addition or exchange of organellar or nuclear DNA; (3) transfer of DNA or RNA intermediates via a plasmid, virus, or other mobile element; (4) transformational-like lysis of organelles and passage of their nucleic acids from the cytoplasm to the nucleus; or (5) by a combination of the above-mentioned mechanisms (reviewed in Gellissen and Michaelis, 1987; Schuster and Brennicke, 1988). Additional mechanisms that are not currently recognized may also prove to be important in intercompartmental gene transfer. The occurrence of mitochondria in the nucleus provides an additional model for how the compartmental barriers of the cell might have broken down allowing DNA recombination between the nucleus and mitochondrion. Although the breakdown of mitochondrial membranes may still be required for the recombination of cytoplasmic DNA with that of the nucleus, the infrequent failure of the mitotic spindle to exclude cytoplasmic organelles from the nuclear region may occur often enough to provide for the presence of mitochondrial DNA within the nucleus. During evolutionary time, this may allow the migration of genes in a population between otherwise discrete cellular compartments.

## METHODS

Tobacco (*Nicotiana tabacum*) plants were grown under natural light in the greenhouse of the Department of Biology, Peking University, Beijing, China or in growth chambers at the University of Oklahoma under controlled growth conditions (19 to 25°C, 80 to 90% humidity, 15-hr day length, light intensity 600 $\mu E\ m^{-2}\ sec^{-1}$ at 10 cm). Selected stages of reproductive development from immature pollen to fertilization were examined. Pollinations were conducted using mixed pollen from several plants on stigmas of emasculated flowers. After 7 or 25

hr of growth, the style was excised just ahead of the growing pollen tubes and cultured on a medium containing 10% sucrose and 0.01% boric acid (see Yu et al., 1989, 1992). Cut styles were cultured 1 to 2 hr at 28°C, and segments 1 mm in length were harvested from those with emerging pollen tubes. Fertilization stages were prepared using individual ovules dissected after 45 hr of in vivo pollen tube growth.

Specimens were fixed using 3% glutaraldehyde in 0.1 M cacodylate buffer containing 0.1 M sucrose, pH 7.1, for 4 hr at room temperature, rinsed with the same buffer for 1 hr (four changes), and fixed with 1% osmium tetroxide in buffer, pH 7.1, for 12 hr at 4°C. After rinsing in buffer, material was dehydrated in a graded ethanol series followed by propylene oxide and embedded in Spurr's low-viscosity resin. All observed generative and sperm cells during pollen tube growth in stylar culture were chosen from tissues at least 200 μm from the cut end of the style.

Serial sections were cut at ~100 nm using a diamond knife and collected on Formvar-coated, carbon-stabilized slot grids (1 × 2 mm). Sections were stained with uranyl acetate and lead citrate and observed using a transmission electron microscope operated at 60 kV.

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