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Quantitative cytology of the egg and central cell of *Plumbago zeylanica* and its impact on cytoplasmic inheritance patterns

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Summary. The egg and central cells of *Plumbago zeylanica* have an average volume of 543,000 μm^3 and 2,560,000 μm^3 respectively, with surface areas of 38,600 μm^2 and 154,000 μm^2. The egg contains an average of 39,900 mitochondria and 730 plastids. The majority of the plastids are perinuclear (>60%) with less than 40% in lateral areas or near the filiform apparatus. After fertilization, the number of maternal organelles exceeds paternal organelles by a ratio of 1:1,000 for mitochondria and 1:54 for plastids. The central cell contains an average of 178,700 mitochondria and 1,840 plastids. After fertilization, these organelles far exceed the number of sperm organelles transmitted, by a ratio of approx. 1:4,000 for plastids and 1:820 for mitochondria. Biparental inheritance of plastids in the embryo is possible, but not favored; the only comparable data in *Oenothera* and *Impatiens* reveals that biparental inheritance is possible in up to 1:24 ratios. Plants lacking biparental plastid inheritance do not contain plastids in the sperm, and thus the presence of even few sperm plastids may result in expression. The number of paternal mitochondria transmitted into the central cell is greater than that transmitted into the egg as the result of preferential fertilization with the mitochondrion-rich dimorphic sperm cell, although the ratio of paternal to maternal mitochondria is 1:1,000 in the egg and 1:820 in the central cell. The similarity in these ratios suggests that there is a critical dosage of mitochondria that is permissible within the zygotic and endospermatic lineages. This may represent either: (1) a maximum permissible value to prevent expression of paternal mitochondrial genome, (2) a minimum ratio required in order to permit recombination of maternal and paternal mitochondrial genomes, or (3) a cytoplasmic genome balance number.

Key words: Cytoplasmic inheritance – Egg – Endosperm – Mitochondrion – Plastid

Introduction

Cytoplasmic inheritance in angiosperms typically results in the maternal inheritance of plastids and mitochondria (Grun 1976; Gillham 1978). Biparental cytoplasmic inheritance of plastids occurs in only a third of the angiosperms studied to date (Kirk and Tilney-Bassett 1978). Patterns of mitochondrial inheritance in higher plants are known even more poorly. Although the non-transmission of paternal organellar genomes in animals and lower plants has been attributed to the occurrence of selective endonucleases that eliminate transmitted paternal mtDNA within the zygote (Gillham 1978), such mechanisms do not appear to occur in angiosperms. Therefore, other mechanisms must exist that result in the prevalence of uniparental cytoplasmic inheritance.

In flowering plants and in heterogenomic cytoplasmic cells where genome-specific endonucleases are unknown, it is postulated that the relaxed control of the cytoplasmic genome may result in multiple genomes coexisting in single cells with each organelle often containing duplicate genes (Birky 1983). The genome of individual organelles is postulated to be essentially free from the selective pressures exerted on cells or whole organisms and therefore the prevalence of a given genome is expected to be proportional to its organellar frequency (Birky 1978, 1983; Birky et al. 1983). Or-

Abbreviations: mtDNA = mitochondrial DNA; S_{ua} = sperm cell unassociated with the vegetative nucleus; S_{vn} = sperm cell physically associated with the vegetative nucleus

ganellar inheritance in this system is thus ultimately dependent on the probabilistic effects of sorting out of organelles within daughter cells during mitosis: the lesser the frequency of a given organellar genome, the greater the likelihood of its extinction in a given cell lineage.

The present study was undertaken to determine the relative numbers of plastids and mitochondria in the egg and central cell of *Plumbago zeylanica* in relation to data available on the sperm cells of this plant. Despite some limitations in using the number of organelles to predict cytoplasmic genomic frequency, such organelles are the exclusive routine vehicle of transmission. The genomic content of an organelle should be closely related in the gametes of a single species of flowering plant and therefore such a comparison provides data on the quantity of organelles transmitted and their relative numerical impact. Viable sperm organelles, both mitochondria and plastids, are transmitted during gametic fusion in *Plumbago*, as demonstrated by ultrastructural studies (Russell 1983, 1985). By comparing the quantitative cytology of the egg and central cells with the sperm, the impact of transmitted male cytoplasmic genome may be evaluated probabilistically, and the possibility of biparental inheritance predicted.

Materials and methods

Specimen preparation

Plants of *Plumbago zeylanica* L. were grown in soil from seed provided by Palmengarten, Frankfurt a. M., FRG, and maintained under 16 h days in growth chambers. Under these conditions, the plants are fertile with approx. 95% seed set (Russell 1983). Ovules were collected for electron microscopy from newly opened flowers and fixed by immersion in 3% glutaraldehyde in 0.067 M phosphate buffer at pH 6.8 at room temperature for the first hour and then transferred to 4C for an additional 5 h. Ovules were rinsed briefly in buffer, fixed for 2 h in cold 2% buffered osmium tetroxide, dehydrated in a graded ethanol series, and embedded in low viscosity resin (Spurr 1969).

Serial ultrathin sections were cut at 70 nm using a diamond knife, collected and mounted on Formvar-coated 1×2 mm slot grids, carbon coated and numbered consecutively. Images of the egg and central cell were photographed in a Zeiss 10a transmission electron microscope (Carl Zeiss, Inc., Oberkochen, FRG), photographically enlarged and printed at the same magnification. Light microscopic sections were photographed unstained with interference contrast optics using a Leitz Dialux 20 photomicroscope. Micrographs were photographically enlarged and printed at the same magnification.

Organellar fractions and volumes

Determination of average organellar volume was calculated differently for mitochondria and plastids. Mitochondria were small, ellipsoidal to spheroidal organelles typically less than 0.4 μm and could be represented as prolate spheroids (ellipses rotated on their major axis) whose major axes are randomly oriented with respect to the plane of sectioning in the tissue. The average volume of these organelles could therefore be determined by unfolding methods given by Cruz Orive (1978) and Weibel (1980) using measurements of the major and minor axis, and transforming a size-shape distribution array with a correction factor array as presented in Cruz Orive (1978). Volume and surface area distributions were then calculated based on average dimensions.

The volume of plastids was determined by direct measurement of organelle areas in section using 95 serial electronmicrographs magnified to X 8,460 and tracing the outline of each plastid using an Apple Graphics Tablet, Apple II+ microcomputer (Apple, Inc., Cupertino, California, USA) and a program written by the author. Total area and circumference were multiplied by thickness in order to obtain volume and surface area, respectively. Thickness was estimated by measuring spherical objects (e.g. small vacuoles or a nucleolus) and dividing by the number of sections in which the object was present. Section thickness was confirmed by measuring the width of median folds in the plastic and dividing by two (Weibel 1979) and by interference color of the sections.

Stereology and organelle counts

The cytoplasmic volume fractions of mitochondria and plastids were measured using point counting methods with a 7 mm regular grid (Weibel 1979) on median sections of the female gametophyte magnified to X 4,500. The correction factor for section thickness used for mitochondria was 0.810 ($g=0.156$) and for plastids was 0.934 ($g=0.0468$).

Female gametophyte, central cell, and egg volumes and surface areas were determined using photomicrographs of median sections of the egg and central cell, and tracing their perimeters on the digitizing tablet. Since these objects are axially symmetrical, their volume was estimated as that of stacked conical discs with axes located perpendicular to the plane of the discs. The diameter of each conical disc was equal to the width of the structure at a particular point. Although the operator manually determines the orientation axis for forming conical disc measurements, this serves only as a reference line, as the exact center of each conical disc is determined by the width of the disc at a particular point on the axis. Therefore, the object is simply divided into stacked discs that may deviate to some degree from the reference axis without causing great error. The digitized object was divided into 1,000 pseudosections with respect to the reference axis and their volumes and surface areas were added in order to determine total volume and surface area.

The volume of the nucleus was determined from median sections as that of an oblate spheroid (ellipsoid rotated about the minor axis). In this case, the major and minor axes are equal to the length and width of the object in median section, respectively. The object was sectioned parallel to the minor axis and therefore did not require a correction factor. Cytoplasmic volume was determined by subtracting the volume of the vacuole from that of the female gametophyte and egg.

Total organelle counts were calculated as volume fractions multiplied by cytoplasmic volume to determine total cellular volume occupied by a given organelle class and divided by average organelle volume to determine organelle numbers. Sampling errors for stereological data determined from equations given by Weibel (1979, 1980) are listed for all parameters dependent on stereological sampling.

Results

The female gametophyte of *P. zeylanica* is a large, essentially ellipsoidal object 50–70 μm in width and

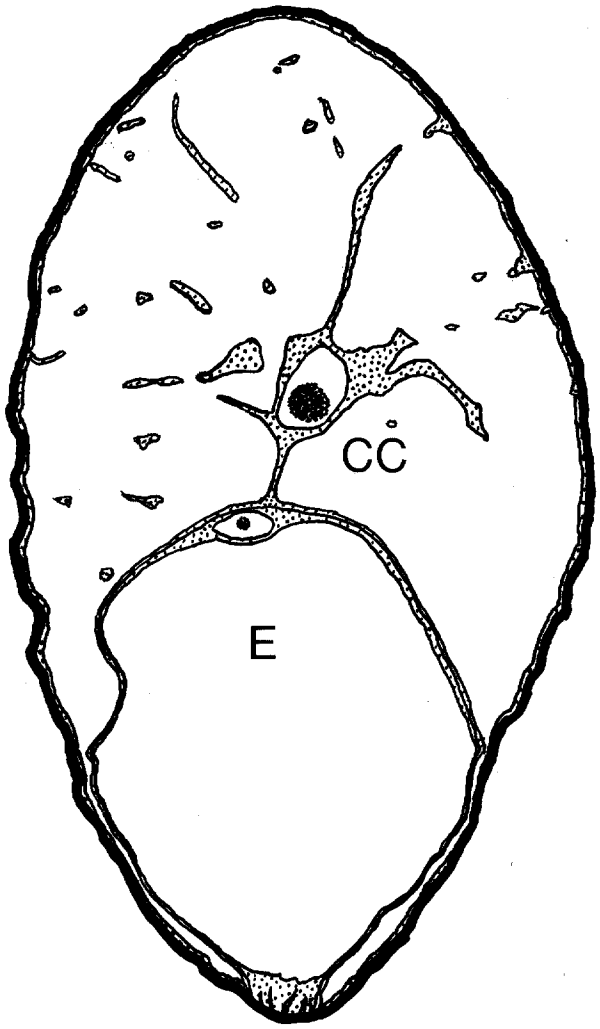

Fig. 1. Organization of the egg (E) and central cell (CC) in the female gametophyte of *Plumbago zeylanica*. ×250

100–140 μm in length (Fig. 1) with an average volume of $3.10 \times 10^6 \pm 3.87 \times 10^5$ μm³ and an average surface area of $1.16 \times 10^5 \pm 6.53 \times 10^3$ μm² (Table 1).

The largest cellular component is a highly vacuolated central cell, which occupies an average of 76.4% of the volume of the female gametophyte. Located near the center of the female gametophyte, the central cell nucleus is the product of fusion of four polar nuclei (Fig. 1) and accordingly has an average nuclear volume (2,920 ± 678 μm³, Table 1) that exceeds that of the egg (768 ± 132 μm³, Table 1) by 4.2 ± 0.2 times. The central cell nucleus is slightly in excess of 4 times the volume of the egg nucleus as the result of remnant cytoplasm that remains within the nucleus as a result of the previous fusion of the polar nuclei (Russell 1982). According to Dahlgren (1937), polar nuclei representing each of the four megaspores are present in this fusion nucleus, and development is therefore tetrasporic (Maheshwari 1950). Three additional cells occurring in the female gametophyte are located peripherally along the lateral and chalazal walls of the female gametophyte (Dahlgren 1937) and are named, based on position, as lateral or antipodal cells (Russell 1982). Up to two lateral cells and one antipodal cell may form, with these accessory cells occupying from 2–10% of the volume of the female gametophyte. Their exact volume depends on whether they are small and lenticular as typically occur in the female gametophyte, or whether one or more has become hypertrophied to nearly the size and volume of the egg (Dahlgren 1937; Russell 1981). Such cells have no apparent bearing on the fertilization process and may variably degenerate prior to fertilization or soon afterward (Russell 1982). The volume of these cells has been incorporated into central cell calculations in this study.

Table 1. Comparison of means of selected cellular parameters of the egg and central cell of *Plumbago zeylanica* with standard error of the mean (n = 6, except as noted)

Parameter	Egg	Central cell
Cell volume (in μm³)	543,000 ± 55,600	2,560,000 ± 331,000
Cell surface area (in μm²)	38,600 ± 3,800	154,000 ± 75,900
Nuclear volume (in μm³)	768 ± 132	2,920 ± 678
Nuclear surface (in μm²)	709 ± 615	1,100 ± 451
Cytoplasmic volume (in μm³)	42,300 ± 1,900	138,700 ± 16,000
Vacuole volume (in μm³)	500,000 ± 53,600	2,420,000 ± 314,000
Mitochondrial volume (in μm³)	7,170 ± 564	20,000 ± 1,980
Volume per mitochondrion (in μm³ [a])	0.177 ± 0.014	0.112 ± 0.011
Surface area per mitochondrion (in μm² [a])	1.829 ± 0.083	1.221 ± 0.072
No. of mitochondria	39,900 ± 3,140	178,700 ± 17,700
Plastid volume (in μm³)	609 ± 92	858 ± 346
Volume per plastid (in μm³ [b])	0.833 ± 0.129	0.599 ± 0.043
Surface area per plastid (in μm² [b])	5.778 ± 0.813	3.638 ± 0.226
No. of plastids	730 ± 110	1,840 ± 740

[a] n = 131 for central cell mitochondria; n = 160 for egg mitochondria
[b] n = 64 for central cell plastids; n = 62 for egg plastids

The egg is a large vacuolated cell occupying an average volume of $5.43 \times 10^5 \pm 5.56 \times 10^4$ μm³ with an average surface area of $3.86 \times 10^4 \pm 3.8 \times 10^3$ μm² (Table 1). The vacuole occupies 92.8% of the volume of the egg. Egg cytoplasm can be divided into three cellular regions based on cytological differences: (i) perinuclear cytoplasm, typified by a predominance of plastids; (ii) lateral cytoplasm; and (iii) micropylar cytoplasm. These regions are identical to the ultrastructural regions defined by Cass and Karas (1974) and are retained in the cellular calculations made in the present work.

The perinuclear region is dominated by a prominent nucleus (average volume, 768 ± 132 μm³) located chalazally in the egg (Table 1). Plastids are found most frequently in this region, accounting for 63% of the plastids in the egg. Only 6.8% of the mitochondria are present in the perinuclear cytoplasm, just slightly greater than the 6.3% of the cytoplasm that this region occupies. Lateral cytoplasm thinly surrounds the prominent vacuole located at the center of the egg and that occupies 92.1% of the egg volume (Table 1). Transvacuolar strands occur infrequently, but are essentially comparable in organellar constituency to lateral cytoplasm. Lateral cytoplasm occupies a total of 86.2% of the cytoplasmic volume of the egg and comprises 86.0% of the mitochondria and 18.6% of the plastids. Micropylar cytoplasm is defined by the presence of a filiform apparatus (Cass and Karas 1974) composed of linear to irregular cell wall ingrowths occupying the micropylar end of the egg, presumably representing a region of cytoplasmic specialization for the transport of solutes (Cass and Karas 1974). This area also serves as the entry point of the pollen tube during fertilization (Russell 1982). Micropylar egg cytoplasm occupies a small fraction of the cytoplasmic volume (7.4%), with 7.8% of the mitochondria and 18.1% of the plastids.

Central cell cytoplasm is located at the periphery of the embryo sac and egg, and was similarly divided into three distinct regions: (i) perinuclear and chalazal-egg associated cytoplasm; (ii) lateral cytoplasm occurring near cell walls; and (iii) transvacuolar cytoplasm bounded on both sides by the prominent central cell vacuole (Fig. 1). The mean volume of the central cell was $2{,}560{,}000 \pm 331{,}000$ μm³ dominated by a vacuole which occupies 94.5% of the cellular volume. The perinuclear region is dominated by a prominent nucleus (average volume, $2{,}920 \pm 680$ μm³) located centrally in the cell (Table 1). Despite the relatively small volume of this region, which occupies less than 2.6% of the central cell cytoplasm, the perinuclear region contains 50.6% of the plastids. Less than 3.5% of the mitochondria are located in the perinuclear cytoplasm. Lateral cytoplasm is distributed around the periphery of the embryo sac and surrounds interfaces with the egg and accessory cells (Fig. 1). Although thin, this cytoplasm accounts for most of the $154{,}000 \pm 75{,}900$ μm² external surface area of the central cell and over 98% of the $115{,}000 \pm 72{,}100$ μm² nutritive surface area of the inner nucellus. Lateral cytoplasm occupies 93.7% of the cytoplasmic volume of the central cell and contains 92.8% of the mitochondria and 47.5% of the plastids. Transvacuolar cytoplasm is defined as that cytoplasm that traverses the vacuole and then belongs neither to the perinuclear or lateral cytoplasm. Transvacuolar cytoplasm occupies a small fraction of the cytoplasmic volume (1.9%), with 1.9% of the mitochondria and 0.9% of the plastids.

The mean volume fractions of three organelle classes are presented in Table 1 as the sums of the volume fractions for each of the regions processed for this study. The respective volume determination for each female gametophyte was used to calculate absolute cytoplasmic volumes for each organelle (Table 1) in order to compare the variability of each of the gametophytes.

The mean number of organelles in the egg and central cell was determined by tabulating volume frequencies in each region, summing the volume frequencies and dividing by the average volume of each organelle type as measured in the egg (mean volume: 0.1797 μm³) and central cell (mean volume: 0.1121 μm³). As a result of these calculations, the egg was determined to contain an average total of $39{,}900 \pm 3{,}200$ mitochondria and 730 ± 110 plastids (Table 1). Egg mitochondria each have an average surface area of 1.829 μm², for a total of 73,000 μm² of mitochondrial surface in the egg. The central cell contained an average of $178{,}700 \pm 17{,}700$ mitochondria and $1{,}840 \pm 740$ plastids. Central cell mitochondria each have an average surface area of 1.221 μm² and a total of 218,000 μm² for the central cell. Both plastid and mitochondrial frequencies were lower in the central cell than those of the egg. Overall, mitochondria were distributed roughly equally throughout the egg and central cell, whereas plastids are located preferentially in the perinuclear cytoplasm of both cells and less frequently in other regions of either cell (Table 1).

Discussion

The dimorphic egg and central cell are fertilized by dimorphic sperm cells (Russel and Cass 1981; Russell 1985). Of the two sperm cells, the larger sperm cell possesses a long cellular projection that associates this cell specifically with the pollen vegetative nucleus, and is termed the S_{vn}, and the smaller cell, unassociated with the vegetative nucleus is termed the S_{ua}. The S_{vn} is known to contain largely mitochondria (average: 256) and infrequent plastids (average: 0.45, see Table 2), whereas the S_{ua}

Table 2. Cellular parameters of the dimorphic sperm cells of *Plumbago zeylanica* ± standard error of the mean, as derived from serial sections (n = 11; after Russell 1984)

Parameter	S_{vn}	S_{ua}
Cell volume (in μm^3)	69.5± 7.5	48.9±4.4
Cell surface (in μm^2)	147.9±12.0	84.7±5.3
Nuclear volume (in μm^3)	19.9± 1.4	12.1±0.5
Nuclear surface (in μm^2)	32.4± 2.1	20.4±0.6
Cytoplasmic volume (in μm^3)	45.2± 6.0	33.4±2.7
No. of mitochondria	256.2±18.0	39.8±3.3
No. of plastids	0.4± 0.2	24.3±3.9

contains numerous plastids (average: 24.3) and fewer mitochondria (average: 39.8, see Table 2). In following the fate of paternal cytoplasmic organelles ultrastructurally, 93% of the embryo sacs studied in detail revealed that the S_{ua} fused with the egg and that the S_{vn} fused with the central cell (Russell 1985). Additional segments traceable to paternal cytoplasm (Russell 1983) are commonly found unfused between the egg and central cell and represent severed cytoplasm torn from the S_{vn} at pollen tube discharge. These bodies reseal through hydrophobic interactions with the aqueous medium to form isolated organelle-containing cytoplasmic bodies (Russell 1983, 1985). Such bodies, contain up to 15% of the mitochondria of the S_{vn}, and are the major source of paternal organelle reduction at fertilization, for the rest of the cytoplasm appears to be transmitted as viewed in the electron microscope (Russell 1983).

Since sperm cell morphotypes each contain different characteristic organelle numbers, paternal organelles are transmitted in a differential manner: one sperm cell contains few mitochondria, and the other sperm often lacks plastids. The S_{vn} contains an average of 6.4 times more mitochondria than the other sperm cell before pollen tube discharge, and after pollen tube discharge and severance of the projection, still contains 5.5 times more mitochondria than the S_{ua}. Paternal plastid content in the S_{ua}, however, exceeds that of the S_{vn} by nearly 54 times (Table 2).

The plastid-rich S_{ua} sperm cell, in fusing with the egg therefore inserts an average of 24.3 plastids into an egg that contains an average of 730 plastids: a ratio of 1 : 54 paternal to maternal plastids (Russell 1984, present study). The plastid-poor S_{vn} however, in fusing with the central cell usually does not even transmit a single plastid, but on the average, 0.45 plastids are inserted into a central cell that contains an average of 1,840 plastids: a ratio of less than 1 : 4,000 paternal to maternal plastids (Russell 1984, present study).

Quantification of plastid transmission has been reported in only two angiosperms previously and both are known to transmit plastids biparentally. In *Impatiens,* the sperm cells contained an average of 20 plastids, compared to 150–480 plastids in the egg (Richter-Landmann 1959), representing from 7.5 to 24 maternal plastids for each paternal plastid. In *Oenothera erythrosepala,* the sperm cells contained 8–13 plastids, compared to 25–32 plastids in the egg (Meyer and Stubbe 1974), representing an average of 2.8 maternal plastids for each paternal plastid. Data for central cell plastids were not reported. Both authors note that in fertilized zygotes, the number of plastids increased consistently with the number present in the sperm cell. In *Oenothera,* the maturing zygote displayed a subsequent increase in both maternal and paternal plastids after fertilization up to the first division (Meyer and Stubbe 1974). In *Plumbago,* the ratio of 1 : 54 paternal to maternal plastids presumably would strongly favor maternal inheritance, but would not eliminate the possibility of biparental inheritance.

Mitochondrial transmission, however, overwhelmingly favors maternal mitochondria in both the egg and central cell. The only other such study in an angiosperm was conducted with *Impatiens glandulifera* using paraffin microtomy of basic fixation images and revealed that the sperm cells contained an average of 50 mitochondria and spherosomes, compared to 1,000–2,500 mitochondria and spherosomes in the egg (Richter-Landmann 1959). This ratio of 200 to 500 maternal mitochondria for each paternal mitochondrion reflects a inborn bias for maternal inheritance. No data were presented for endosperm. In relative frequencies, the endosperm of *Plumbago* receives 820 maternal mitochondria for each transmitted paternal mitochondrion and the embryo receives 1,000 maternal mitochondria for each paternal mitochondrion transmitted. This ratio reflects a similar inborn bias for maternal inheritance. The differential of mitochondria in the dimorphic sperm (1 : 5.5) nearly matches the 1 : 4.5 differential of egg to central cell mitochondria observed here; the 1 : 1,000 and 1 : 820 transmission rates are therefore essentially similar in both egg and central cell.

Although the absolute number of mitochondria or plastids may not directly indicate the number of genomic units present in the cytoplasm of the cell because of multiple copies of the genome (Gillham 1978), the natural dynamism of organelle structure (Gunning and Steer 1975), and the fact that a small fraction of organelles apparently lacks a genome in some plants (Bendich and Gauriloff 1984), it is likely however, to be somewhat proportional to the real number of genomes present. Overall volume of a given organelle type is predicted (Bendich and Gauriloff 1984) to be more closely correlated with total organellar DNA content than is individual organelle size. This assumption negates an argument by Pearson (1981), that the in-

dividually smaller organelles of the male cytoplasm (as evidenced in Russell 1983, 1985) are incapable of playing an equal role in the transmission of organellar DNA. This factor may in actuality be neutral except as it alters overall chondriome volume. If individual organelles are regarded as being essentially proportionate to the individual transmission and recombination units present (Birky 1978; 1983), the relatively low frequency of male organelles observed in sperm cells (Table 2) compared to female reproductive cells (Table 1) is predictive of exceedingly low levels of biparental inheritance of mitochondria.

That the ratio of mitochondrial transmission in the egg and central cell are so close suggests that a critical ratio of maternal to paternal mitochondria exists in each plant and that male and female mitochondrial content may adapt to this ratio. Cytoplasmic sperm dimorphism in *Plumbago* may represent a form of compensation for organellar differences between the egg and central cell. Whether this critical ratio represents a *minimum* number of paternal organelles required to gain expression or a *maximum* number of paternal organelles that can be transmitted without gaining expression through survival or recombination remains to be determined through genetic and theoretical studies of angiosperms. An alternative possibility exists of a cytoplasmic genome balance number similar to proposed nuclear genomic balance numbers proposed in other plants (Johnson et al. 1980). To date, cytoplasmic heterospermy has been detected in *Plumbago zeylanica* (Russell 1984), *Brassica campestris, B. oleracea* (McConchie et al. 1987) and *Spinacia oleracea* (Wilms 1986), and has been proven to be absent only in the grasses, to date (Mogensen and Rusche 1985, for review see Russell 1986). If preferential fertilization occurs in these plants and follows patterns similar to those in *Plumbago,* mitochondrial differentials in the perm may assure that neither the egg nor central cell receives a disproportionate number of paternal mitochondria.

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