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Quantitative, three-dimensional analysis of alfalfa egg cells in two genotypes: Implications for biparental plastid inheritance

Tong Zhu[1], **H. Lloyd Mogensen**[1], **Steven E. Smith**[2]

[1] Department of Biological Sciences, Box 5640, Northern Arizona, Flagstaff, AZ 86011, USA
[2] Department of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA

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Abstract. In alfalfa (*Medicago sativa* L.), plastids are inherited biparentally. Patterns of plastid transmission vary according to the genotypes involved, but there is a strong bias in favor of male plastid transmission. Previous cytological studies on the male gametophyte of this species have not provided an adequate explanation for the differences in plastid transmission frequencies among genotypes. In the present study, we compared egg cells from genotypes classified as strong or weak plastid transmitters to determine whether plastid transmission strength is correlated with egg cell structure before fertilization. We found that plastids in the mature egg cells of the strong female (genotype 6-4) are significantly larger than in mature eggs of the weak female (genotype CUF-B), and that significantly more plastids are positioned in the apical portion of the mature egg cell of genotype 6-4 than in CUF-B. Immature eggs in the two genotypes show the same pattern as mature eggs with regard to plastid number and polarization. Since only the apical portion of the egg cell/zygote gives rise to the functional embryo, these results indicate that the potential input of female plastids, in terms of plastid size and number, may be an important factor in determining the inheritance patterns of these organelles in alfalfa.

Key words: Cytoplasmic inheritance – Female gametophyte (ultrastructural reconstruction) – *Medicago* (egg cell) – Plastid inheritance

Introduction

Genotypes of alfalfa (*Medicago sativa* L.), a species exhibiting a strong bias with regard to paternal plastid inheritance (Smith et al. 1986; Lee et al. 1988; Schumann and Hancock 1989; Masoud et al. 1990), have been characterized according to the strength of their plastid transmission (Smith 1989). We have previously analyzed strong, intermediate, and weak male plastid transmitters and found that there is no consistent correlation among genotypes between the mean number of plastids per generative cell (sperm mother cell) and the strength of male plastid transmission (Zhu et al. 1990, 1991a). Likewise, the mean number of plastid nucleoids (DNA aggregates) per generative cell correlates poorly with the strength of paternal plastid transmission among genotypes (Shi et al. 1991). Analysis of sperm cells has shown that neither the number of plastids nor plastid nucleoids are significantly different in the two sperms of a pair compared with their progenitor, the generative cell. Moreover, plastid number is essentially the same in each sperm cell of a pair, and no significant changes in plastid number were observed up to 17 h after pollen tube growth in vitro (Zhu et al. 1992). Thus, cytological studies on the male gametophyte of alfalfa have not provided an adequate explanation for observed variation in the patterns of plastid inheritance among genotypes of this species.

In the present study, we analyzed unfertilized eggs of a strong and a weak female genotype in order to assess whether the strength of potential plastid transmission is correlated with egg cell composition prior to fertilization. Using quantitative, three-dimensional electron microscopy, we have concentrated primarily on the number, size, and distribution of plastids within egg cells of mature, receptive flowers. Preliminary results of this study have been published previously (Zhu et al. 1991b).

Materials and methods

Alfalfa plants for this study were grown and maintained under the same conditions as for previous studies (Smith et al. 1986; Smith 1989; Zhu et al. 1990). Genotypes 6–4 and CUF–B were used because of their significantly different patterns of plastid transmission. Genotype CUF–B is considered a weak female because when it is crossed with genotype 301 as the male parent, over 90% of the progeny contain only male plastids (Smith 1989). Genotype 6–4 is classified as a strong female because when it is crossed with the same male parent, 41% of the progeny contain only paternal plastids (Smith 1989).

Correspondence to: H. Lloyd Mogensen; FAX: 1(602)523 2626

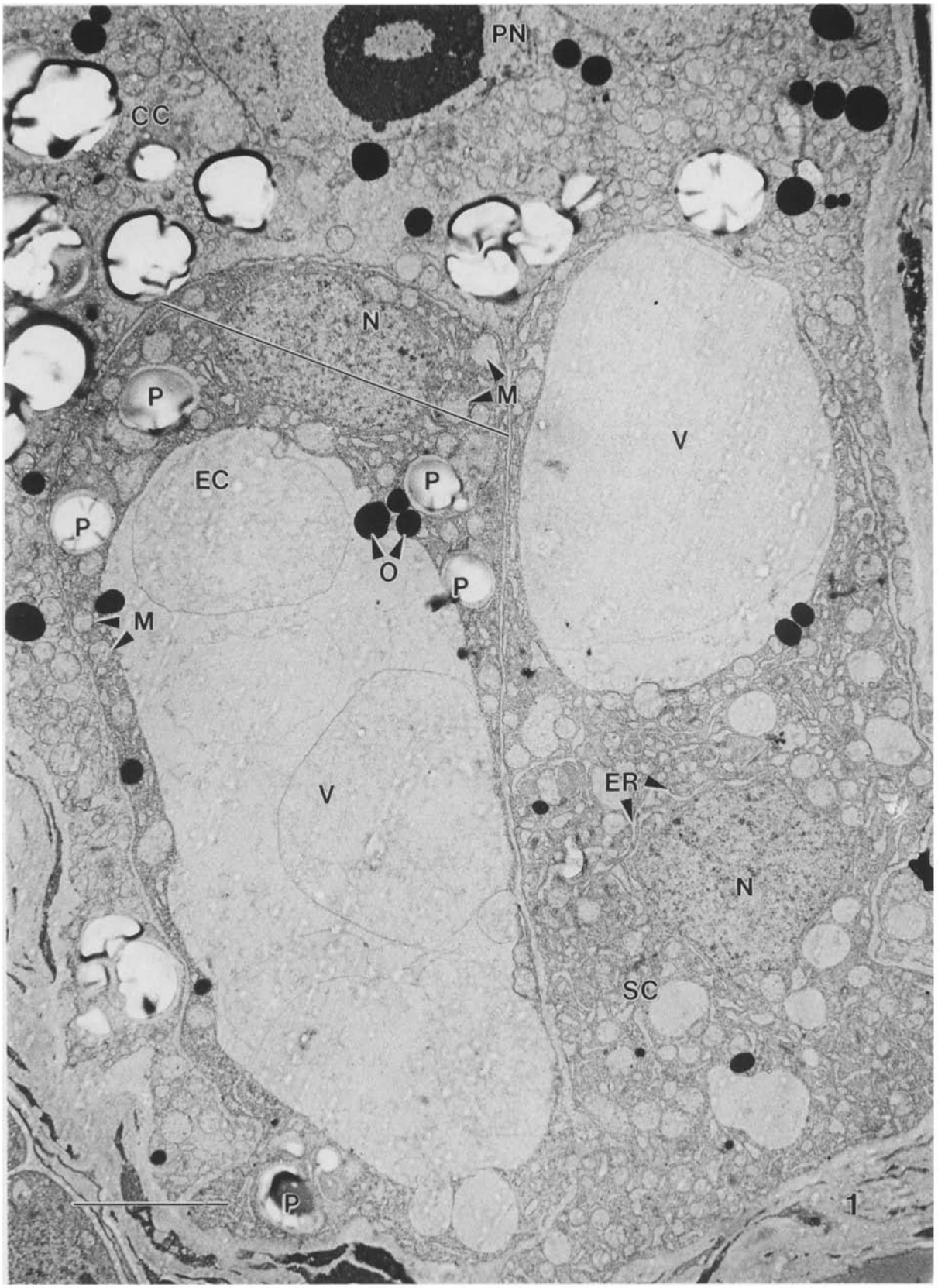


Fig. 1. Near median longisection of the mature egg cell (*EC*) of alfalfa genotype CUF–B showing the nucleus (*N*), amyloplasts (*P*), osmiophilic bodies (*O*), mitochondria (*M*) and the central vacuole (*V*). Note the fewer number and more micropylar position of the plastids in the egg cell of this genotype compared to that of Fig. 2. *CC*, central cell; *ER*, endoplasmic reticulum; *PN*, polar nuclei; *SC*, synergid cell. The *solid line* marks the predicted plane of division of the cell following fertilization. × 5200; bar = 5 μm

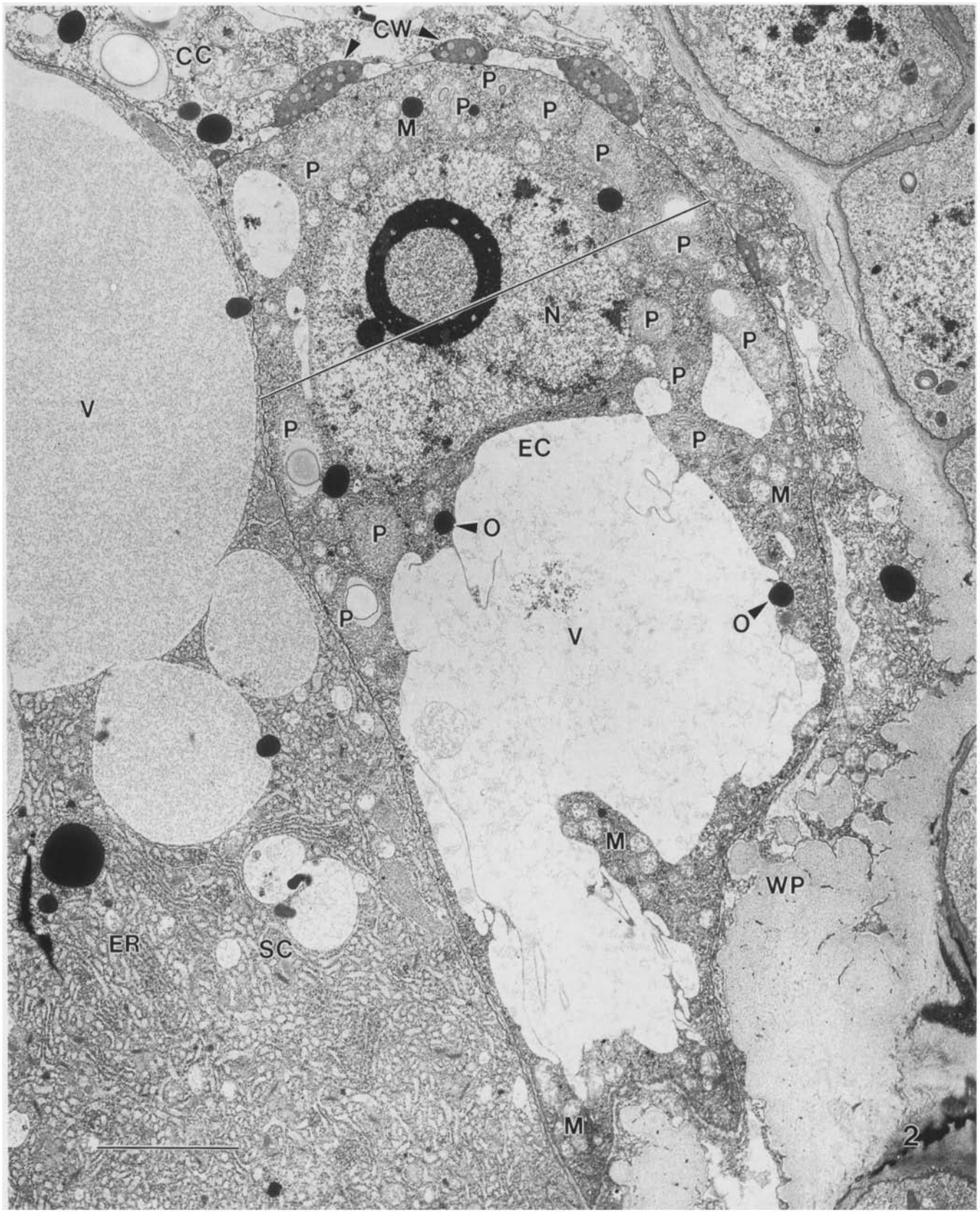


Fig. 2. Near median longisection of the mature egg cell (*EC*) of alfalfa genotype 6–4 showing the nucleus (*N*), partially dissolved chalazal cell-wall material (*CW*), plastids (*P*), mitochondria (*M*) and the large central vacuole (*V*). Note the greater number and more chalazal location of the egg-cell plastids of this genotype compared to that of Fig. 1. *CC*, central cell; *ER*, endoplasmic reticulum; *O*, osmiophilic bodies; *SC*, synergid cell; *WP*, proliferation of central-cell wall. The *solid line* marks the predicted plane of division of the cell following fertilization. ×5200; bar = 5 μm

Ovules were dissected under moist conditions from mature, unpollinated flowers, and fixed in Dalton's OsO_4-chromate for 3.5 h on ice (Juniper et al. 1970). Fixed ovules were then rinsed in distilled water for 1 h (three changes), dehydrated through a graded ethanol-acetone series and embedded in Spurr's resin (Spurr 1969). Embedded ovules were first serially sectioned at 3–4 μm for observations using phase-contrast microscopy. Sections containing egg cells were then re-embedded (Mogensen 1971) and serial ultrathin sections prepared. The ultrathin sections were collected on single-slot (1×2 mm^2), formvar-coated, carbon-stabilized grids, stained with uranyl acetate and lead citrate, and then viewed and recorded on film with a transmission electron microscope (1200 EXII; Jeol, Tokyo, Japan) at 50 kV.

Data entry and computer reconstruction and quantitation were performed as described previously (Young et al. 1987; Zhu et al. 1990, 1991a), except that for this study the computer images of Figs. 3–6 were created using the Wavefront software program and a Silicon Graphics Personal Iris 4D–25 workstation. Twenty egg cells, ten from each of the two genotypes 6–4 and CUF–B, were reconstructed. The Wilcoxon two-sample test was used to rank the quantitative data and compare the sums of the ranks for each class (Zar 1984). A significance level of $P < 0.05$ was selected for comparing values.

Results

Although all ovules for this study were collected from mature flowers with receptive stigmas, we found that not all egg cells were at the same stage of maturity. Therefore, based upon various criteria as indicated below, we placed the egg cells into two classes: immature and mature. Results for each category are presented separately.

Mature egg cells

Mature egg cells have several features in common between the weak (CUF–B) and strong (6–4) genotypes, including a chalazally positioned nucleus, mostly perinuclear cytoplasm, and a complement of plastids, mitochondria, endoplasmic reticulum, ribosomes, vacuoles, and osmiophilic bodies (Figs. 1, 2). Dictyosomes are also occasionally present. A large vacuole (Figs. 1, 2) is a more common feature of genotype 6–4 (Fig. 2) than of CUF–B (Fig. 1), which often has several small vacuoles, rather than a single large one.

There are no significant defferences between the mature egg cells of genotypes CUF–B and 6–4 with regard to their volume and surface area (Table 1). Nuclear size (volume and surface area) is nearly identical in the mature eggs of the two genotypes (Table 1).

The mean number of plastids per egg cell is 49% greater in genotype 6–4 than in CUF–B (41.3 vs. 27.8 plastids/cell); however, this difference is not statistically significant due to the high variance among CUF–B eggs (Table 1). The volume and surface area of individual plastids are significantly greater in genotype 6–4 than in CUF–B, as are the volume and surface area of all the plastids combined (Table 1).

Plastid distribution within the mature egg cells of the two genotypes contrasts greatly. If a separation is made along what is known from an earlier embryological study (Cooper 1935) to be the approximate division plane of the zygote, the egg can be divided into an apical portion (chalazal end of the egg) and a basal portion (the rest of the egg; Figs. 1, 2). The plastid number is significantly greater in the apical end of the egg of genotype 6–4 than in that of CUF–B (Table 1). Within the egg cells of each genotype, there is a reverse order in terms of which portion of the cell has the most plastids (Figs. 3–6; Table 1). In genotype 6–4, the plastid number is greater in the apical portion compared with the basal portion; whereas in CUF–B, the basal portion of the egg cell has a greater plastid number compared with the apical portion (Figs. 3–6; Table 1).

Mitochondria in egg cells were not compared quantitatively within or between genotypes; however, no ap-

Table 1. Comparison of quantitative data at different developmental stages of the alfalfa egg cell in two genotypes. Values are means ± SE

	CUF–B			6–4			Diff. between genotypes	
	Immature ($n=5$)	Mature ($n=5$)	Diff.[d]	Immature ($n=6$)	Mature ($n=4$)	Diff.[d]	Immature	Mature
Protoplast								
Volume[a]	4839.8 ± 441.5	10124.1 ± 1470.7	**	8312.7 ± 977.3	15727.6 ± 2571.2	**	**	n/s
Surface Area[b]	1631.3 ± 168.4	2800.0 ± 405.7	**	2527.0 ± 315.8	3469.6 ± 324.6	n/s	**	n/s
Nucleus								
Volume	311.4 ± 54.4	1063.0 ± 225.2	**	591.4 ± 104.0	991.2 ± 158.0	n/s	**	n/s
Surface Area	188.4 ± 22.9	461.1 ± 77.6	**	323.4 ± 34.4	470.8 ± 47.0	**	**	n/s
Plastids								
Volume	260.3 ± 30.2	435.3 ± 99.0	n/s	280.7 ± 41.6	1185.1 ± 225.8	**	n/s	**
Surface Area	617.8 ± 67.5	802.0 ± 191.0	n/s	778.7 ± 119.6	2009.3 ± 308.3	**	n/s	**
Total Number	25.4 ± 4.0	27.8 ± 8.0	n/s	55.5 ± 8.8	41.3 ± 2.4	n/s	**	n/s
Apical Number[c]	8.8 ± 2.3	9.0 ± 2.5	n/s	29.5 ± 6.5	25.8 ± 4.8	n/s	**	**
Basal Number	16.6 ± 2.2	17.2 ± 4.4	n/s	25.5 ± 7.6	15.5 ± 3.6	n/s	n/s	n/s
Vol./Pl.	10.7 ± 1.0	17.3 ± 3.3	n/s	5.6 ± 1.0	28.2 ± 4.1	**	**	**
Surf. Area/Pl.	25.3 ± 2.0	30.2 ± 3.3	n/s	14.7 ± 1.7	48.0 ± 5.0	**	**	**

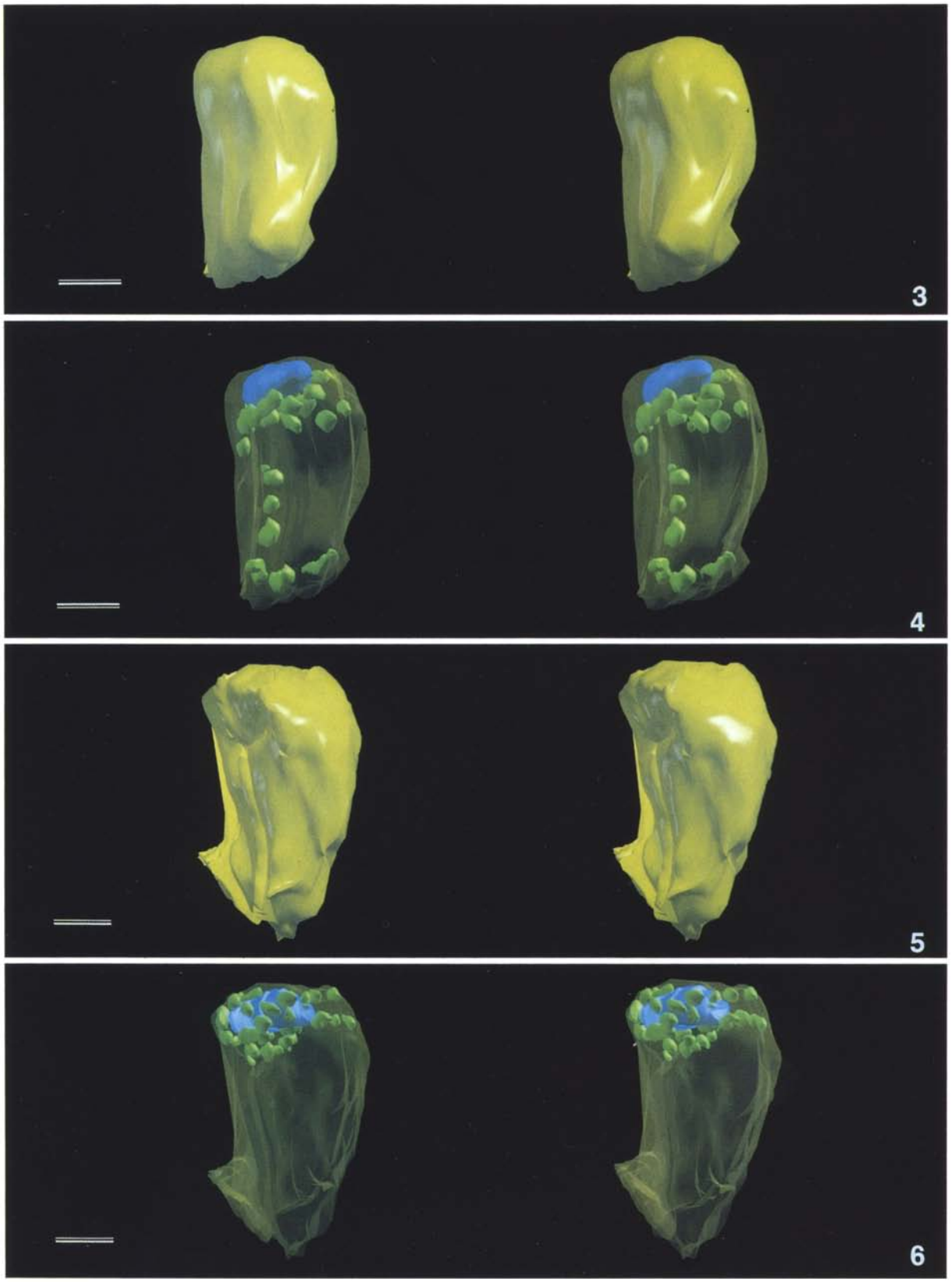
[a] Volume is expressed as μm^3

[b] Surface area is expressed as μm^2

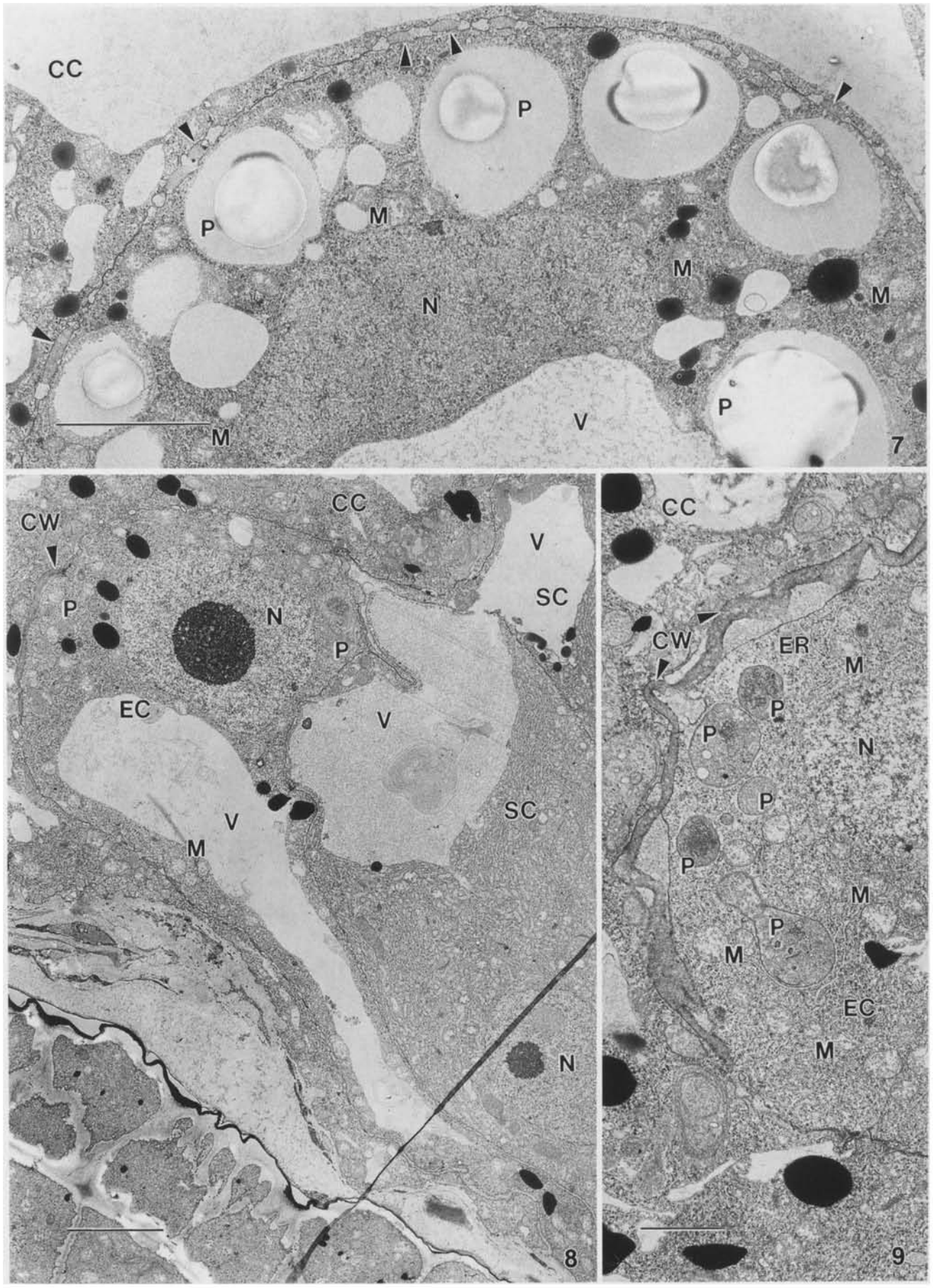
[c] Plastid number in apical and basal part of cells separated by presumed nuclear division plane

[d] Difference between immature and mature egg cells within genotype

** significantly different; n/s = not significantly different ($P < 0.05$)



Figs. 3–6. Stereoscopic pairs of computer-assisted reconstructions from serial ultrathin sections. Key to color coding: *blue* = egg nucleus; *green* = plastids. × 1000; bar = 10 μm. **Fig. 3.** Mature egg cell of genotype CUF–B showing the external surface. **Fig. 4.** Same egg cell as in Fig. 3, but with a transparent surface showing the distribution of plastids within the egg cell. Note that most plastids are positioned below the presumed plane of nuclear division (see Fig. 1). **Fig. 5.** Mature egg cell of genotype 6–4 showing the external surface. **Fig. 6.** Same egg cell as in Fig. 5, but with a transparent surface showing plastid distributed around the nucleus. There is no obvious difference in plastid number above and below the presumed division plane (see Fig. 2)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parent differences in abundance or distribution were observed. In one representative egg cell of genotype CUF–B, more than 500 mitochondria were counted.

Immature egg cells

Comparisons between immature and mature eggs. The immature egg cells of both genotypes resemble mature egg cells by having a chalazally positioned nucleus, mostly perinuclear cytoplasm, and a complement of plastids, mitochondria, endoplasmic reticulum, ribosomes, vacuoles, osmiophilic bodies (Figs. 7–9) and occasional dictyosomes. Immature egg cells are primarily distinguishable from mature eggs by the presence of proplastids (compare Figs. 7 and 9), and by the generally smaller volume and surface area of the cell, nucleus and central/micropylar vacuole (Figs. 1, 2, 7–9; Table 1). Immature egg cells also have a densely staining cell-wall component around the chalazal end (Figs. 8, 9), which becomes discontinuous at egg maturity (Figs. 1, 2, 7).

The volume and surface area of individual plastids do not differ significantly between mature and immature eggs in genotype CUF–B. However, in 6–4, the volume and surface area per plastid are significantly greater in mature eggs than in immature eggs (Table 1). Likewise, the volume and surface area of all plastids combined are not significantly different between young and mature eggs in CUF–B; but they are significantly greater in mature than in immature eggs of 6–4 (Table 1).

The number of plastids per egg cell, and the number of plastids within the apical and basal portions of the egg cells are essentially the same in immature and mature eggs within each genotype (Table 1).

Comparisons between genotypes. Contrasting features of immature eggs between the two genotypes include smaller cells and nuclei (volume and surface area), as well as fewer and smaller plastids in genotype CUF–B compared with 6–4 (Table 1). Plastid distribution in the immature eggs parallels that of mature egg cells; i.e., there is a significantly larger plastid number in the apical portion of the immature egg of genotype 6–4, than in that of CUF–B. In genotype CUF–B, there are twice as many plastids in the basal portion of the immature egg than in the apical portion, whereas in 6–4 the number of plastids is nearly equal in the two cell portions (Table 1).

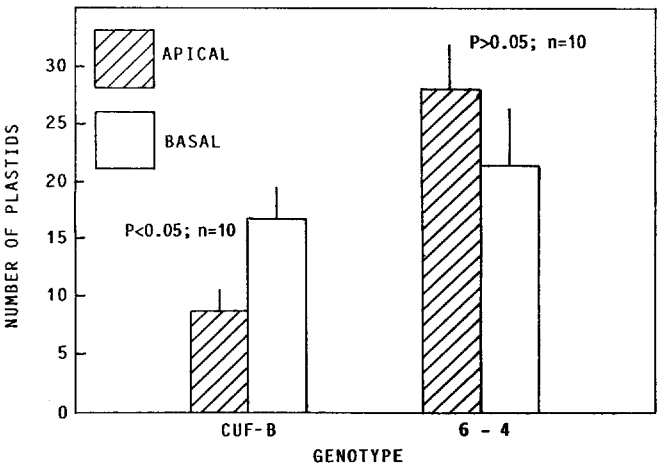

Fig. 10. Comparison of plastid number and distribution within two portions (apical and basal, see Figs. 1 and 2) of the alfalfa egg cell in two genotypes. Values represent data pooled from immature and mature egg cells (Table 1). Note that in genotype CUF–B (weak female) there are significantly more plastids in the basal end of the egg cell than in the apical end; whereas in genotype 6–4 (strong female) the number of plastids is not significantly different in the two portions of the egg cell. It is also apparent that the plastid number per egg cell is greater in genotype 6–4 than in CUF–B

Since there are no significant differences between immature and mature eggs with regard to plastid number and distribution (Table 1), these data have been pooled and are illustrated in Fig. 10.

Discussion

Whether the young eggs, as described in this study, have the potential to be fertilized is not known at this time. Conceivably, they could develop further after pollination and be receptive by the time pollen tubes arrive approximately 24 h after collections were made in this study (Cooper 1935; our data, not shown). Alternatively, the presence of young, unreceptive eggs in mature flowers could be a factor leading to the high rate of ovule abortion in alfalfa (Cooper et al. 1937; Sayers and Murphy 1966; Dane and Melton 1973). Postpollination and postfertilization studies are needed to answer these questions.

The mature egg cells of the weak (CUF–B) and strong (6–4) genotypes of this study have many structural features in common, and they are typical of angiosperm eggs in general (Willemse and van Went 1984). Among egg-cell characteristics that differ between the two genotypes, it would appear that the number, distribution, and size of the plastids are the most likely features to have an influence on the patterns of plastid inheritance.

The mean number of plastids per mature egg cell is 33% higher in genotype 6–4 than in CUF–B, but due to the great variability in plastid number within genotype CUF–B (Table 1), this number may not be critical. Rather, the number of plastids positioned within the apical portion of the egg cells may explain the difference in plastid inheritance between the genotypes. Cooper (1935) has shown that the alfalfa zygote, which has the same cytological organization as the egg, divides to form a two-celled embryo consisting of a smaller apical cell and

Fig. 7. Portion of the mature egg cell of alfalfa genotype 6–4 showing details of amyloplasts (*P*), mitochondria (*M*), and a portion of the central vacuole (*V*). Note that dense cell wall material between the egg and central cell (*CC*) is not continuous (*arrowheads*). × 5800; bar = 5 μm

Fig. 8. Median longisection of the young egg cell (*EC*) of genotype 6–4 showing the nucleus (*N*), dense cell-wall material (*CW*), proplastids (*P*), mitochondria (*M*) and central vacuole (*V*). *CC* = central cell, *SC* = synergid cell. × 3600; bar = 5 μm

Fig. 9. Portion of the young egg cell (*EC*) of genotype 6–4 showing the dense cell-wall material (*CW*) in the chalazal end of the cell, and proplastids (*P*), nucleus (*N*), mitochondria (*M*), and endoplasmic reticulum (*ER*), *CC* = central cell. Note that some proplastids contain crystal-like structures. × 8000; bar = 2 μm

a larger basal cell. Subsequent embryogenesis results in the functional embryo being derived from the apical cell only, while the basal cell contributes to the suspensor, which eventually degenerates. Postulating the future division plane of the cell (Figs. 1, 2) allows us to predict the number of plastids that would most likely be included in either the apical or basal cell of the two-celled embryo in each genotype. Using this assumption, our data show that the mean number of plastids in the apical end of eggs of genotype 6–4 is significantly greater than in CUF–B eggs, regardless of developmental stage (Table 1). Moreover, plastid size (volume and surface area) is significantly larger in mature eggs of genotype 6–4 than in those of CUF–B. Since plastid number and distribution show the same basic differences between genotypes in immature and mature eggs (Table 1), these distinctions between genotypes are apparently established early and are maintained during egg development. We found no evidence that the smaller number of plastids within the eggs of genotype CUF–B compared with 6–4 is the result of plastid degradation, as has been suggested to occur in the eggs of *Daucus muricatus* (Hause 1991).

The results of this study further support our earlier suggestion (Zhu et al. 1991b) that the greater potential for plastid input of genotype 6–4, in terms of both number and size, may be an important factor leading to the more dominant female influence on plastid inheritance in this genotype compared with that of CUF–B (Smith 1989). Other factors may play a role in determining plastid-inheritance patterns among alfalfa genotypes, such as: (1) differing multiplication rates between male and female plastids and between plastids of certain genotypes during zygote and embryo development (Chiu et al. 1988; Schumann and Hancock 1989; Hause 1991), (2) preferential plastid (e.g. male, female genotype) degeneration during embryogenesis (Hause 1991), and (3) the degree of mixing between male and female plastids within the zygote and embryo cells (Tilney-Bassett and Almouslem 1989). Ongoing studies in this laboratory are addressing these possibilities.

Although not a focus of this study, it is of interest to note that the number of mitochondria within alfalfa egg cells is enormously greater than that in alfalfa sperm cells, which have fewer than an average of ten mitochondria per cell (Zhu et al. 1992). In the present study, over 500 mitochondria were counted in one egg cell of genotype CUF–B, and the observation of an apparently equal abundance of mitochondria in all eggs of this study indicates that the likelihood of any significant paternal mitochondrial inheritance is minimal at best. This supports the findings of Schumann and Hancock (1989) that mitochondrial inheritance is strictly maternal in alfalfa.

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References

Chiu, W-L., Stubbe, W., Sears, B.B. (1988) Plastid inheritance in *Oenothera:* organelle genome modifies the extent of biparental plastid transmission. Curr. Genet. **13,** 181–189

Cooper, D.C. (1935) Macrosporogenesis and embryology of *Medicago*. J. Agric. Res. **51,** 471–477

Cooper, D.C., Brink, R.A., Albrecht, H.R. (1937) Embryo mortality in relation to seed formation in alfalfa (*Medicago sativa*). Am. J. Bot. **24,** 203–213

Dane, F., Melton, B. (1973) Effects of temperature and method of pollination on the frequency of aborted seed in alfalfa. Crop Sci. **13,** 753–754

Hause, G. (1991) Ultrastructural investigations of mature embryo sacs of *Daucus carota, D. aureus* and *D. muricatus*. Possible cytological explanations of paternal plastid inheritance. Sex. Plant Reprod. **4,** 288–292

Juniper, B.E., Cox, G.C., Gilchrist, A.T., Williams, P.R. (1970) Techniques for plant electron microscopy. Blackwell, Oxford London

Lee, D.J., Blake, T.K., Smith, S.E. (1988) Biparental inheritance of chloroplast DNA and the existence of heteroplasmic cells in alfalfa. Theor. Appl. Genet. **76,** 545–549

Masoud, S.A., Johnson, L.B., Sorensen, E.L. (1990) High transmission of paternal plastid DNA in alfalfa plants demonstrated by restriction fragment polymorphic analysis. Theor. Appl. Genet. **79,** 49–55

Mogensen, H.L. (1971) A modified method for re-embedding thick epoxy sections for ultratomy. J. Ariz. Acad. Sci. **6,** 249–250

Sayers, E.R., Murphy, R.P. (1966) Seed set in alfalfa as related to pollen tube growth, fertilization frequency, and postfertilization ovule abortion. Crop Sci. **6,** 365–369

Schumann, C.M., Hancock, J.F. (1989) Paternal inheritance of plastids in *Medicago sativa*. Theor. Appl. Genet. **78,** 863–866

Shi, L., Zhu, T., Mogensen, H.L., Smith, S.E. (1991) Paternal plastid inheritance in alfalfa: plastid nucleoid number within generative cells correlates poorly with plastid number and male plastid transmission strength. Curr. Genet. **19,** 399–401

Smith, S.E. (1989) Influence of paternal genotype on plastid inheritance in *Medicago sativa*. J. Hered. **80,** 214–217

Smith, S.E., Bingham, E.T., Fulton, R.W. (1986) Transmission of chlorophyll deficiencies in *Medicago sativa,* evidence for biparental inheritance of plastids. J. Hered. **77,** 35–38

Spurr, A.R. (1969) A new low viscosity resin embedding medium for electron microscopy. J. Ultrastruct. Res. **26,** 31–43

Tilney-Bassett, R.E.A., Almouslem, A.B. (1989) Variation in plastid inheritance between pelargonium cultivars and their hybrids. Heredity **63,** 145–153

Willemse, M.T.M., van Went, J.L. (1984) The female gametophyte. In: Embryology of angiosperms, pp. 159–197, Johri, B.M., ed. Springer-Verlag, Berlin Heidelberg New York

Young, S.J., Royer, S.M., Goves, P.M., Kinnamon, J.C. (1987) Three-dimensional reconstructions from serial micrographs using the IBM PC. J. Electron Microsc. Tech. **6,** 207–218

Zar, J.H. (1984) Biostatistical Analysis. 2nd ed. Prentice-Hall, Inc. Englewood Cliff, New Jersey

Zhu, T., Mogensen, H.L., Smith, S.E. (1990) Generative cell composition and its relation to male plastid inheritance patterns in *Medicago sativa*. Protoplasma **158,** 66–72

Zhu, T., Mogensen, H.L., Smith, S.E. (1991a) Quantitative cytology of the alfalfa generative cell and its relation to male plastid inheritance patterns in three genotypes. Theor. Appl. Genet. **81,** 21–26

Zhu, T., Mogensen, H.L., Smith, S.E. (1991b) Maternal plastids in alfalfa egg cells: A qualitative and quantitative study. In: Proc. Electron Microsc. Soc. Amer, pp. 210–211, Bailey, G.W., Hall, E.L. eds. San Francisco Press, San Francisco

Zhu, T., Mogensen, H.L., Smith, S.E. (1992) Heritable paternal cytoplasmic organelles in alfalfa sperm cells: ultrastructural reconstruction and quantitative cytology. Eur. J. Cell. Biol. **59,** 211–218