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Video microscopic observations of living, isolated embryo sacs of *Nicotiana* and their component cells

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Summary. Living embryo sacs and megagametophytic cells of *Nicotiana alata* and *Nicotiana tabacum* were obtained using enzymatic maceration and microdissection. The yields of isolated embryo sacs, egg apparatus and central cells were up to 35%, 40% and 35%, respectively. Vectorial movement of organelles and undulations of tubular structures, presumably endoplasmic reticulum, were observed in eggs, synergids and central cells using video-enhanced microscopy. Despite evident viability using the fluorochromatic reaction, the egg displays much less organelle movement and therefore appears to be quiescent. The large vacuole of the central cell is traversed by mobile strands of cytoplasm through which organelles migrate. A polygonal network is located at the periphery of the central cell, which may contribute to anchorage of the cell with the embryo-sac wall. The observation of organelle movement provides direct evidence of the condition of the cell and may be a useful approach for assessing cell vigor.

Key words: Female germ unit – *Nicotiana* – Megagametophyte – Organelle movement – Video microscopy

Introduction

The isolation of female gametes provides an opportunity to develop valuable techniques for manipulating haploid cells and embryogenetic cell lineages in vitro (Dumas et al. 1984; Zhou and Yang 1986; Theunis et al. 1991). To meet these objectives, it is of importance to follow an isolation procedure that retains full viability of the cells. Assessing the initial quality of the isolated materials is at least as important as prolonging the viability of the cells in culture, which is a problem of its own. Although such enzymatic indicators as the fluorochromatic reaction (FCR, Heslop-Harrison et al. 1984) and metabolic indicators like luciferase (Roeckel et al. 1990) may be indicators of cell viability, physiological parameters such as the intracellular movement of organelles may be a more sensitive indicator of cell vigor and probable success in culture.

The isolation of living embryo sacs (ES) and their constituent cells allows detailed in vivo observations of the developmental events that occur during gametophyte maturation and fertilization. The movement of organelles and gametes has been intensively investigated in pollen tubes (Heslop-Harrison and Heslop-Harrison 1987, 1990; Pierson et al. 1990); however, these aspects are scantily documented in their female counterparts, with the notable exception of the cinematographic works of Erdelská (1974, 1983) and brief descriptions of cytoplasmic movement in protoplasts isolated from the ES of *Torenia fournieri* (Mól 1986) and *Zea mays* (Kranz et al. 1991).

Past studies have reported on the isolation and observation of fixed ES in *Nicotiana* (Enaleeva and Dushaeva 1975; Hu et al. 1985; Sidorova 1985; Tyrnov et al. 1975; Zhou and Yang 1985, 1986) and presented some information on the organization of isolated ES.

We present a protocol for isolating living cells of the female gametophyte from two species of *Nicotiana* and report our observations on organelle movement in these cells as visualized by video-enhanced microscopy (VEM). This protocol provides viable ES cells and tissues as tested by FCR and observed using VEM for movement of organelles. These isolated products may be used to examine the integral structure of the cytoskeleton in the ES (Huang et al. 1990; Webb and Gunning 1990), the deployment of its cytoplasm (Russell et al. 1989) and fertilization events (Zhou 1987; Huang and Russell 1991).

Materials and methods

Flowers of *Nicotiana alata* L. and *N. tabacum* Link & Otto were collected on the day of anthesis from greenhouse-grown plants. Ovarian segments were dissected and incubated in 0.5 ml enzyme

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solution [0.5% cellulase (w/v) and 0.5% pectinase (w/v) (Sigma, St. Louis, Mo., USA)] in 0.65 *M* sorbitol in distilled water for 1 h at room temperature. After three rinses in 0.65 *M* sorbitol solution, ovules were dissected to remove remaining integuments and adherent nucellar cells. Isolated egg apparatus, central cells and intact ES were located using an inverted phase contrast microscope and collected using a micropipette. Recovery rates were calculated by taking the number of ES recovered, dividing by the number of ovules used and multiplying by 100.

Viability was indicated with a positive fluorochromatic reaction (FCR+) using 5 μg/ml fluorescein diacetate in the sorbitol solution (Heslop-Harrison et al. 1984). Differential interference contrast (DIC) and fluorescence microscopy were conducted using a Zeiss Axiophot microscope equipped with a Grundig FA76 video camera. Organelle movement was recorded by a Sony U-matic videocassette recorder (VO-5800PS). The images of organeller movements were photographed using Kodak T-Max 100 film at ISO 100.

Results

Embryo sac isolation

The isolated ES of *Nicotiana* contains two synergids, an egg, a central cell and three antipodals (Fig. 1), all of which display a positive reaction with FCR (Fig. 2), as seen in *N. alata*. The unfertilized central cell is characterized by a large vacuole and a strong aggregation of amyloplasts (Figs. 3, 4). The egg and synergid are more densely cytoplasmic and strongly fluorescent in the FCR (Figs. 5, 6). The egg contains a large vacuole and is essentially wall-less, whereas the synergid is more densely cytoplasmic and has a strong FCR+ reaction throughout the cytoplasm. In *N. alata* the egg is larger than the synergid, whereas in *N. tabacum* the opposite pattern occurs (Figs. 10–12). The applicability of this technique to developmental studies is shown in Figs. 7–9 in *N. alata* and has also been applied to *N. tabacum* (unpublished data). The linear arrangement of the adherent megaspores, shown in Fig. 7, is followed by expansion of the functional megaspore (Fig. 8) and the formation of the two-nucleate ES (Fig. 9). Because ovules develop at different rates within the flower, different developmental stages may be obtained from the same ovary. Figure 12 illustrates the condition of ES cells upon prolonged (5–6 h) digestion of the ES wall in *N. tabacum*. Each of the component cells of the ES assumes a spheroidal shape after sufficient incubation, presumably indicating that the shape-maintaining constituents of the cell wall have been dissolved.

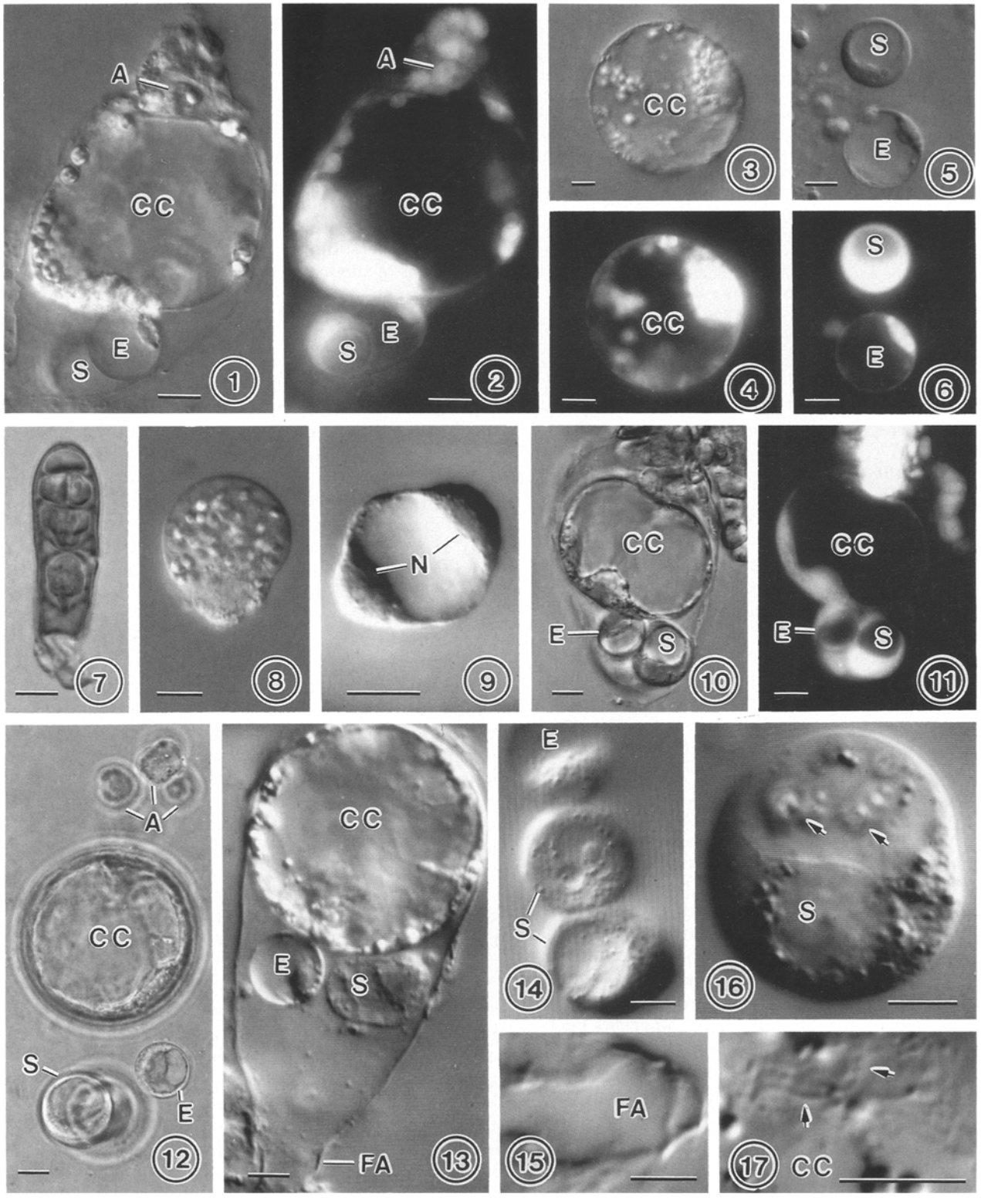
Observations of living ES and isolated component cells

Figure 13 illustrates the organization of the living ES of *N. alata* as viewed by differential interference contrast video-enhanced microscopy (DIC-VEM). Although the outer ES wall and filiform apparatus are evident, ES component cells are spherical, presumably indicating that these two cell walls are more resistant to enzyme digestion than internal cell walls (Fig. 13). Principally Brownian movement is observed at this magnification, but at higher magnification vectorial movement is also evident.

The synergids contain a vacuole and dense perinuclear cytoplasm (Fig. 14). In vivo, the synergid of *Nicotiana* is a highly polarized cell with a chalazal vacuole, centrally positioned nucleus and a micropylar grouping of cell-wall invaginations forming the filiform apparatus (Mogensen and Suthar 1979). In vitro, however, the synergid separates from the filiform apparatus (Fig. 15) and becomes spherical (Fig. 16). Vectorial movement of organelles, local oscillations of particles in the cytoplasm and undulations of tubular structures resembling ER are evident within the synergids (documented in time sequences shown in Figs. 18 and 19 and schematically in Fig. 25). Movement of particles within the synergid may be divided into three general types of movement (Fig. 25, Table 1): oscillatory movement (in which the particle exhibits both forward and retrograde motion, but displays some overall directionality); vectorial movement (in which the particle displays distant migration, typically with sudden acceleration and stopping); and Brownian movement (in which the particle exhibits random motion but no directionality). Such movements are not conspicuous within the associated egg cell (Fig. 24); however a few organelles display vigorous directional movement (Fig. 27, Table 1). The infrequent observation of organelle movement leads to an impression that the egg, although strongly viable (Figs. 6, 11), is physiologically quiescent.

The central cell is the largest cell in the ES, with a vacuole occupying over 80% of the volume of the cell (Figs. 12, 13). Given its large size in the ES, it is sometimes inadvertently released during the isolation procedure. Although most of the cytoplasm is peripheral, dynamic strands of cytoplasm traverse the central vacuole. Figures 20 and 26 illustrate the vectorial movement of cellular organelles within such cytoplasmic strands, providing firm evidence that the cell is still vital. The rate and behavior of particle movement within these strands appears to be closely related to organellar size in relation to the cytoplasmic strand (Table 1). The largest organelles (average: 11.3 μm) display the slowest rate of vectorial movement (Fig. 26A), frequently accumulating in clusters of similar organelles near the edge of the cell (Fig. 3). Small- (average: 0.66 μm) and medium- (average: 1.71 μm) sized organelles display a similar rate of vectorial movement within cytoplasmic strands, but the smaller organelles (Fig. 26B) display a more constant speed than the medium-sized organelles (Fig. 26C). Tubular structures present in the peripheral cytoplasm of the central cell (Fig. 17) also display significant intracellular movement. These tubules demonstrate undulations and rapid configurational changes (Fig. 21). Their structure and behavior resembles that of tubular cisternae of ER as reported in other VEM studies of onion epidermal cells and pollen tubes (Allen et al. 1988; Lichtscheidl and Url 1990; Pierson et al. 1990).

A polygonal network at the periphery of the central cell seems to form a reticulum on the surface of the cell (Fig. 22). This may be of a similar nature as that of the fibers (Fig. 23) found to link the ES wall with



Figs. 1–17. Isolated ES and ES cells of *Nicotiana alata* (**Figs. 1–9, 13–17**) and *N. tabacum* (**Figs. 10–12**) viewed using DIC (**Figs. 1, 3, 5, 7, 8, 10**), DIC-VEM (**Figs. 9, 13–17**), phase contrast (**Fig. 12**) and fluorescence microscopy of FCR (**Figs. 2, 4, 6, 11**). *A* Antipodal cell, *CC* central cell, *E* egg cell, *FA* filiform apparatus, *S* synergid cell. *Bars*: 10 µm. **Fig. 1.** Isolated ES. Notice the numerous amyloplasts near the surface of the central cell. **Fig. 2.** Localization of FCR in same ES shown in Fig. 1 (2 h after isolation). **Fig. 3.** Isolated central cell. Note numerous amyloplasts aggregating around the cytoplasm of the central cell. **Fig. 4.** Localization of FCR in same central cell as Fig. 3 (4 h after isolation). **Fig. 5.** Isolated egg and synergid. Note prominent vacuole occupying most of the egg cell. **Fig. 6.** Localization of FCR in same egg and synergid as Fig. 5 (4 h after isolation). **Fig. 7.** Isolated linear tetrad of megaspores. **Fig. 8.** Enlarging functional megaspore with numerous amyloplasts 4.5 h after isolation. **Fig. 9.** Binucleate ES 4 h after isolation. Note two nuclei (*N*) and a large vacuole. **Fig. 10.** Isolated ES. **Fig. 11.** Localization of FCR in same ES as Fig. 10 (7 h after isolation). **Fig. 12.** Isolated ES cells 7 h after isolation. This preparation was incubated 30 min in enzyme solution followed by 6.5 h

Table 1. Observations of organelle movement in megagametophytic cells of *N. alata*

Megagametophyte cells	Type of movement	Observation notes	Maximum speed (μm/s)	Minimum speed (μm/s)	$\bar{v} \pm SD$ (μm/s)	Figure number
Synergid	Oscillation	Local oscillations and slow movement	4.8	0.6	1.9 ± 1.4	Fig. 25. I
	Vectorial	Distant migration with sudden acceleration and stopping	7.6	1.2	3.8 ± 2.0	Fig. 25. II
	Brownian	Irregular, fast motion, no net movement	9.7	2.7	6.7 ± 3.2	Fig. 25. III
Central cell	Vectorial	Small (0.7 μm) particles following cytoplasmic strands ± constant movement	5.1	3.2	4.2 ± 0.5	Fig. 26B
	Vectorial	Medium (1.7 μm) particles inconstantly moving along cytoplasmic strands	6.1	2.2	4.3 ± 1.3	Fig. 26C
	Vectorial	Large (11.3 μm) particles move slowly along large cytoplasmic strands	2.5	1.4	1.9 ± 0.8	Fig. 26A
Egg cell	Vectorial	Moving organelles infrequently observed	7.2	0.5	2.7 ± 2.6	Fig. 27

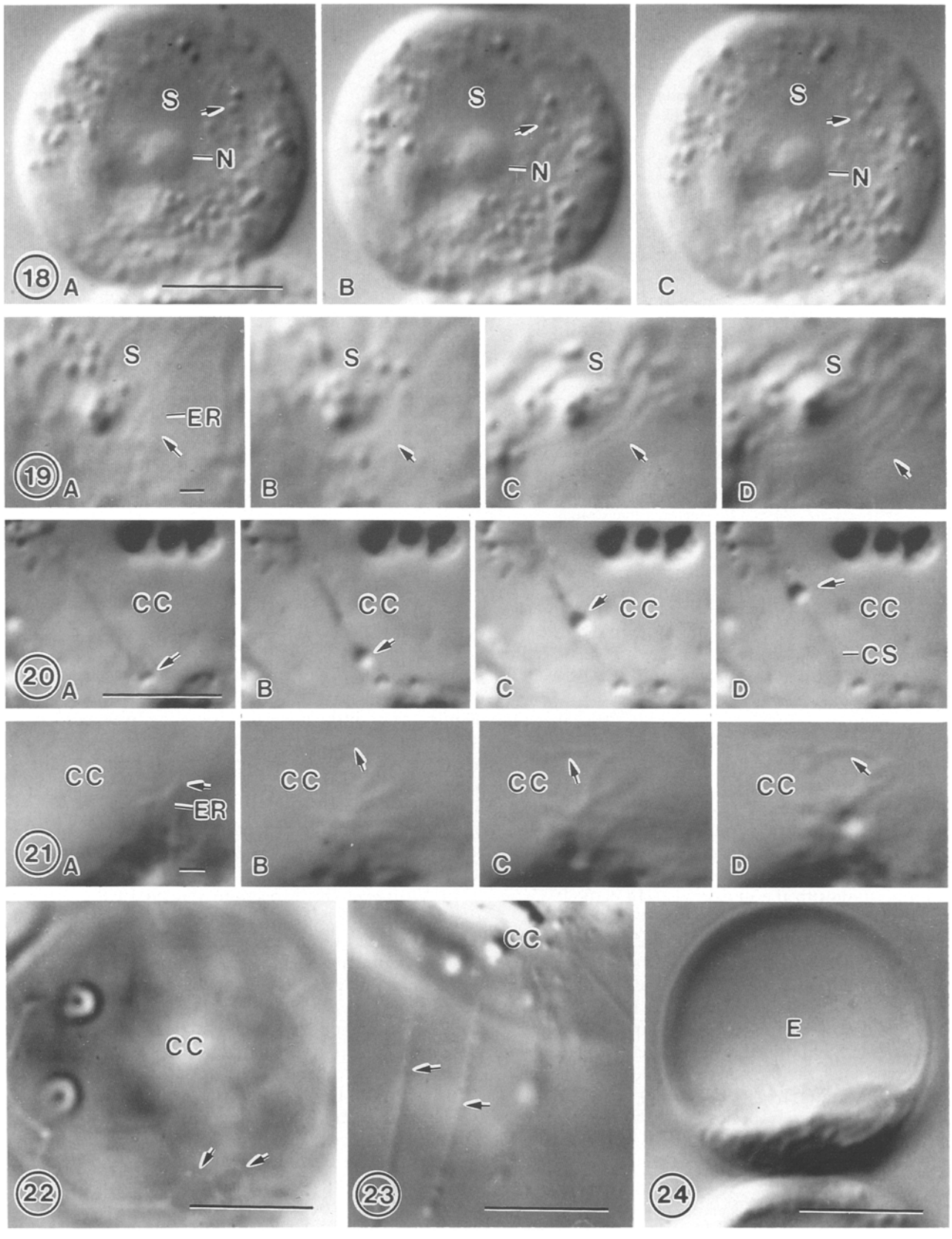
the surface of the central cell. Although the role of these fibers is not clear, we presume that they are involved in the anchorage of the central cell within the ES wall during ES formation and maturation.

Discussion

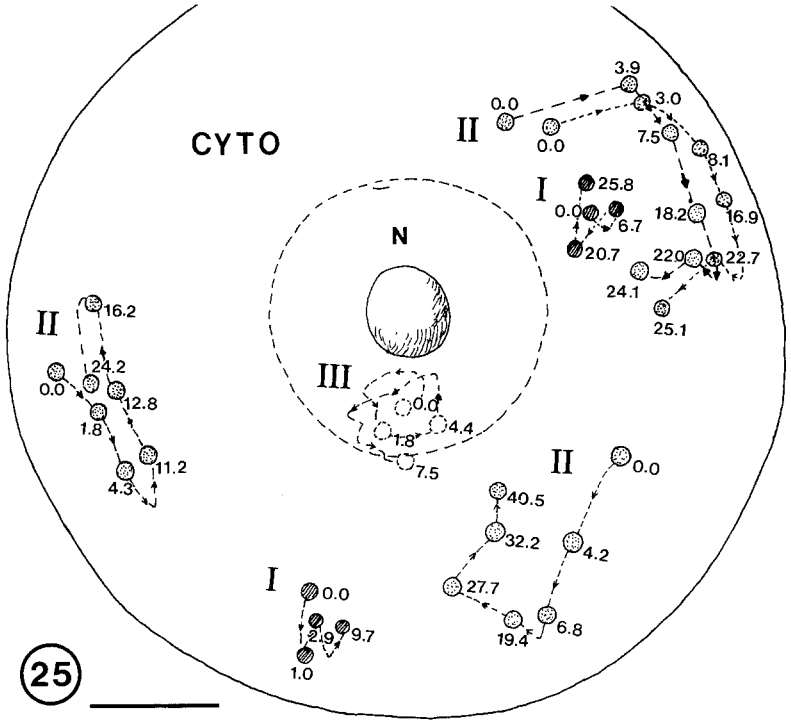
The isolation of *Nicotiana* ES was first reported in 1975 by a Russian group (Enaleeva and Dushaeva 1975; Tyrnov et al. 1975), but was published without a detailed protocol or any illustrations. This work was followed by a group of studies in the mid-1980s (Hu et al. 1985; Sidorova 1985; Zhou and Yang 1982, 1985). Hu et al. (1985) isolated the ES with driselase in 0.65 *M* mannitol and 0.25% potassium dextran sulfate using a gentle squash technique to release the ES from the ovule; viability was tested with FCR. Sidorova (1985) used 2% pectinase, 1% driselase and 0.5% xylosidase in 0.2 *M* mannitol to isolate the ES and then assessed quality by transmission electron microscopy, which revealed apparently intact cells. Zhou and Yang (1982, 1985) reported the isolation of ES in several species including *Nicotiana*, but did not show any living ES in that plant. In the current work, *Nicotiana* ES were subjected to a simplified version of the procedure described by Huang and Russell (1989) in which only two of the four enzymes mentioned were used (cellulase and pectinase) for 30 min; very little micromanipulation was needed to isolate the ES or component cells. The major differences in protocol between these reports are the enzymes used, temperature, incubation time and osmoticum. The viability of the ES is apparently enhanced by short enzyme incubations; the 30-min incubation period used in this study proved to be the minimum exposure required to release the ES.

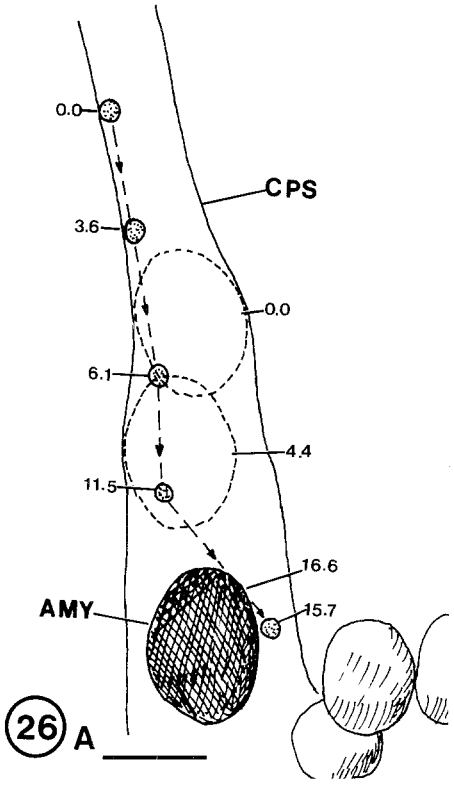
In *Nicotiana*, this procedure yielded FCR+ cells in approximately 35% of the ES, 40% of the egg apparatus cells and 35% of the central cells. Additional micromanipulation of the intact ES can be skillfully used to isolate the component cells. Cytoplasmic movement was observed in approximately 80–90% of the successfully isolated ES and component cells. In *Plumbago*, the ES retained FCR+ at room temperature for 54 h, the egg for 33 h and the central cell for 29 h (Huang and Russell 1989). Van Went and Kwee (1990) noted retention of FCR+ in *Petunia* for up to 80 h at 4° C. Mól (1986) reported extended viability of ES cells and noted that FCR+ could be sustained for up to 2 weeks in cultures supplemented with 10% coconut water, 2 mg/l 2,4-dichlorophenoxyacetic acid and 720–840 mOs/kg mannitol, but cell wall re-establishment was not observed. Kranz et al. (1991) observed some cytoplasmic streaming for more than 2 weeks in eggs maintained in breeder cell cultures. In *Nicotiana*, the movement of intracellular particles in the central cell was observed up to 8 h in the unsupplemented medium, although movement is most evident within the first 2–4 h of isolation. Cells retained FCR+ for at least twice as long or longer.

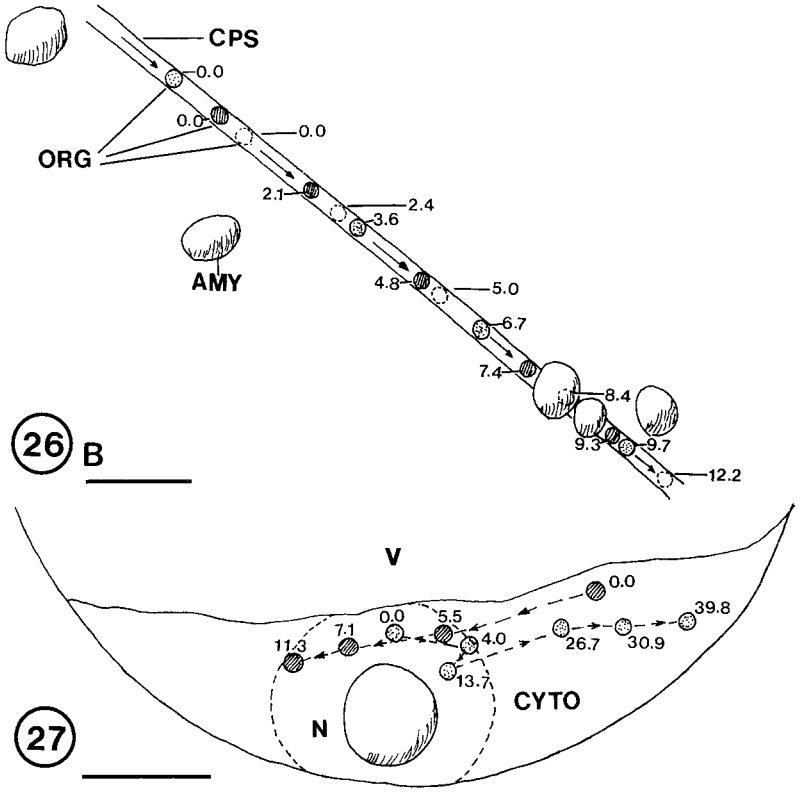
in buffer. (Presumably, continued separation of these cells is the result of residual enzymatic digestion.) Note two synergids, one of which is out of focus, egg, central cell and three antipodal cells. **Fig. 13.** Video image of ES 4 h after isolation. Note filiform apparatus, central cell, egg and synergid. **Fig. 14.** Egg apparatus cells including the two synergids and egg. **Fig. 15.** Filiform apparatus in ES. **Fig. 16.** Two synergids at different focal planes, one showing the nucleus with dense surrounding cytoplasm and the other showing the vacuolate region (*arrow*). **Fig. 17.** Endoplasmic reticulum-rich area (*arrow*) in the peripheral cytoplasm of the central cell

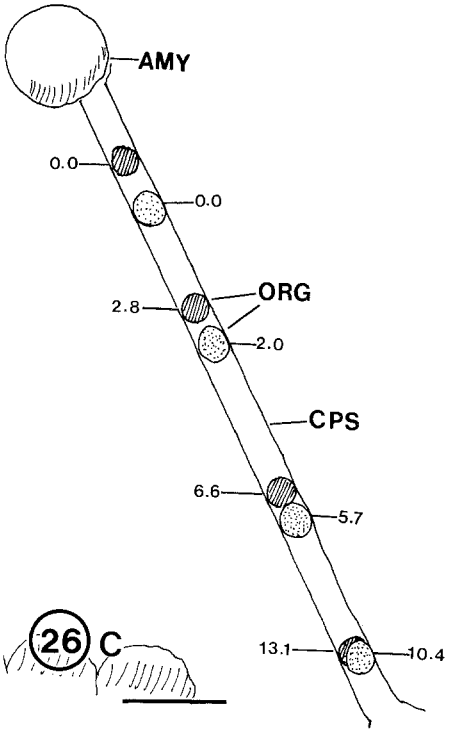


Figs. 18–24. DIC-VEM micrographs of timed sequences of organelle movement (**Figs. 18–21**) and static views of cytoplasmic structures (**Figs. 22–24**) in isolated ES cells of *N. alata*. *CC* Central cell, *CS* cytoplasmic strand, *E* egg cell, *N*, nucleus, *S* synergid cell. **Fig. 18 A–C.** Timed sequence showing organelle movement in the synergid. *Arrow* indicates an organelle moving slowly in the cytoplasm of the synergid near the nucleus; **A** 0 s **B** 9 s **C** 16 s. **Fig. 19 A–D.** Undulating movement of tubular structure (*arrow*) in synergid: **A, B** 24 s **C, D** 11 s. **Fig. 20 A–D.** Vectorial movement of organelle along cytoplasmic strand in the central cell: **A** 0 s, **B** 4 s, **C** 7 s, **D** 9 s. **Fig. 21 A–D.** Undulation of tubular structure (*arrow*) in central cell. **A** 0 s **B** 1 s **C** 7 s **D** 17 s. **Fig. 22.** Network-like structure (*arrows*) on the surface of the central cell. **Fig. 23.** Tubular fibrils (arrows) link the surface of central cell with the outer ES wall, presumably anchoring the central cell. **Fig. 24.** Video image of the highly vacuolate egg. Organelle movement was not observed in the impoverished cytoplasm of the egg. *Bars*: 10 μm (**Figs. 18, 20, 22–24**); 1 μm (**Figs. 19, 21**)









Figs. 25–27. Schematic representations of organelle movement in synergids, central cells and eggs traced from timed sequences recorded using DIC-VEM. Time sequence of organelle positions is given in seconds. **Fig. 25.** Selected tracks of organelle movement in the synergid. *I* Slow oscillation movement, *II* long-distance tracking movement, *III* Brownian movement, *N* nucleus, *CYTO* cytoplasm. **Fig. 26A–C.** Selected tracks of organelle movement in the central cell taken from timed sequences recorded using DIC-VEM. The unit used, is seconds. **A** Large organelle (amyloplast) moves slowly (average 1.92 μm/s) along the cytoplasmic strands (*CPS*). *AMY* Amyloplast. **B** Small organelles (presumably mitochondria) move constantly along the CPS (4.16 μm/s). *ORG* Organelle. **C** Medium organelles (presumably plastids) show inconstant movement along the CPS (average 4.25 μm/s). **Fig. 27.** Selected tracks of organelle movement in the egg cell. The egg cell is relatively inactive, with only a few organelles moving at the same time (average 2.66 μm/s). *V* Vacuole. *Bars*: 5 μm (**Figs. 25, 27**); 2 μm (**Fig. 26A–C**)

Isolated ES and gametophytic cells obtained by the procedure described in this paper display FCR+ and conspicuous intracellular movement, confirming that these are vigorous, strongly viable cells, as would be required for physiological experimentation or cell/tissue culture. Further, these cells and ES are structurally intact and readily identifiable, providing vigorous female cells for the observation of cellular structure and cytoskeletal organization. Physiologically, this technique provides a concentrated fraction of ES cells that may be used for in vitro study or biochemical characterization.

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