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Structure and function of the microtubular cytoskeleton during megasporogenesis and embryo sac development in *Gasteria verrucosa* (Mill.) H. Duval

M.T.M. Willemse and A.A.M. Van Lammeren
Department of Plant Cytology and Morphology, Agricultural University, Arboretumlaan 4, NL-6703 BD Wageningen, The Netherlands

Summary. Using immunocytochemical techniques, tubulin distribution in various stages of meiosis and embryo sac development was studied. In the archespore cell some microtubules appeared to be randomly oriented. During zygotene and pachytene, when the cell volume increases, a large number of microtubules in dispersed configurations and bundles were observed. During this stage the nucellar cells divide, and their parallel cortical microtubules play an important role in preparing the direction of cell enlargement. The protoderm cells show anticlinal-directed cortical microtubules. It can be concluded that the enlargement of the meiocyte during these early meiotic stages is influenced both by its own cytoskeleton and by growth of the nucellus. Thereafter, the microtubules function directly in meiosis and disappear for the greater part until the two-nucleate coenocyte is formed. In a four-nucleate coenocyte microtubules reappear around the nucleus; in a young synergid, randomly oriented microtubules are involved in cell shaping during the formation of the filiform apparatus; in the synergids of the mature embryo sac, many parallel arrays of microtubules are present. Microtubules are less abundant in other cells. It is concluded that the cytomorphogenesis of the developing coenocyte and embryo sac are due to cell growth of the nucellar cells together with vacuolation of the coenocyte.

Key words: Cytoskeleton – Embryo sac development – Megasporogenesis – Microtubules – *Gasteria.*

Introduction

In ovulated plants the development of the large coenocytic megagametophyte coincides with enlargement of the nucellus. At the archespore stage the nucellus is compact and small, but during meiosis and coenocyte formation the nucellus enlarges by cell division and stretching. The meiocyte enlarges before the first division and the functional megaspore grows by vacuolation and turns into a large coenocyte while some surrounding nucellus cells are crushed.

In an ultrastructural investigation, a random distribution of cytoplasmic microtubules in the megameiocyte of *Gasteria* was found during the leptotene, pachytene and diplotene stages, and in the tetrad (Willemse and Franssen-Verheijen 1978). Microtubules were also found around the nucleus of the megameiocyte in *Impatiens* during leptotene, zygotene, and diplotene, and in *Allium* during diplotene (De Boer-de Jeu 1978). At the coenocytic stage of embryo sac formation, mitoses need a directed spindle orientation (Rutishauser 1969) and according to Gerasimova-Navashina (1961), the ultimate position of the free nuclei is the result of nuclear repulsion and the drift to a common dynamic centre. Cytoplasmic microtubules have as yet not been observed in the interphase stages of coenocytic embryo sacs. However, during cellularization of the coenocytic embryo sac, microtubules are involved in cell wall formation (Cass et al. 1985, 1986).

In plant cells, microtubular configurations are related to cell division and cell stretching (Tiwari et al. 1984; Wick et al. 1984; Derksen et al. 1986; Bereiter-Hahn et al. 1987). During pollen development in *Gasteria*, three types of microtubular configurations were distinguished (Van Lammeren et al. 1985). Firstly, a random configuration of cytoplasmic microtubules which functions in the maintenance of cytoplasmic integrity; secondly, an asteroid arrangement which fixes the nuclear position, and thirdly, clustered microtubules functional in establishing and maintaining cell and spindle

Offprint requests to: M.T.M. Willemse

shapes. The cortical, parallel microtubules commonly present in directed stretching cells function in cytomorphogenesis, i.e., the development of cell shape in growing cells (Van Lammeren 1987), although the relation between microfibril deposition in the cell wall and parallel cortical microtubules is still unclear and under discussion (Emons 1982, 1986; Quader 1986). Considering the functions of the microtubular cytoskeleton during pollen development (Van Lammeren et al. 1985), the microtubular cytoskeleton of the developing megaspores and embryo sacs may also be related to cytomorphogenesis and cell enlargement which starts partly during meiosis, but especially after megaspore formation. In the meantime the surrounding nucellar tissue grows and influences this development.

The aim of this study is to show the existence of a relationship between microtubular configurations and cell differentiation during megasporogenesis and megagametogenesis in the developing nucellar tissue.

Materials and methods

Ten ovaries in different stages of development were selected from one inflorescence of *Gasteria verrucosa* (Mill.) H. Duval grown in a greenhouse at a temperature of 16–20° C. Placentae bearing the ovules were dissected. The developmental stages of megasporo- and megagametogenesis in the ovules were determined by phase-contrast microscopy on the sections used for immunocytochemistry.

A small lateral section of the ovules was cut to facilitate penetration of the fixative. For immunohistochemistry, the chemicals and procedure of Van Lammeren et al. (1985) were applied with two modifications: the ovules were fixed in a solution of 4% formaldehyde and fading of the fluorescence was reduced with Citifluor (Citifluor Ltd, London). Incubation with the polyclonal antibody raised in rabbit against tubulin gives some background labeling, and contamination with starch grains and some types of cell walls occurred. The fixation used induces a slight contraction of the cytoplasm.

Results

Megasporogenesis

During early nucellar development, the archespore cannot be distinguished from the nucellar cells until it increases in volume. The enlarged archespore is a cell with a large nucleus and much cytoplasm; microtubules are found in a random orientation in the cytoplasm (Fig. 1a, b). In the nucellar cells, a faint pattern of microtubules is present in the central cytoplasm and cortical microtubules are scarce.

At leptotene the volume of the meiocyte cytoplasm increases even more (Fig. 2a), and many microtubules and bundles of microtubules become visible (Fig. 2b). The microtubules are close to and oriented randomly around the nucleus; towards the periphery, the microtubules show a somewhat more dispersed pattern. At this moment, most of the still meristematic nucellar cells show a distinct

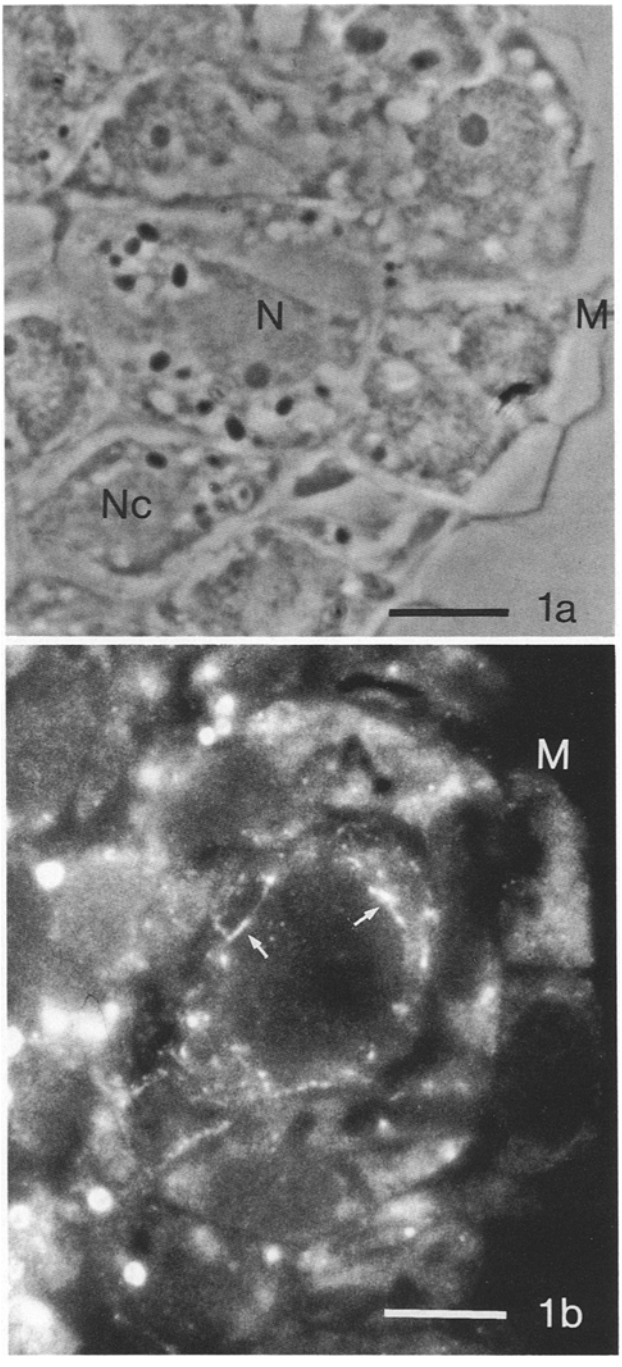

Fig. 1. a Survey of the archespore cell with a large nucleus (*N*). The micropylar direction in the nucellus (*Nc*) is indicated by *M* in most figures. **b** The archespore exhibits randomly positioned microtubules (*arrows*). The *bars* in all figures represent 20 μm

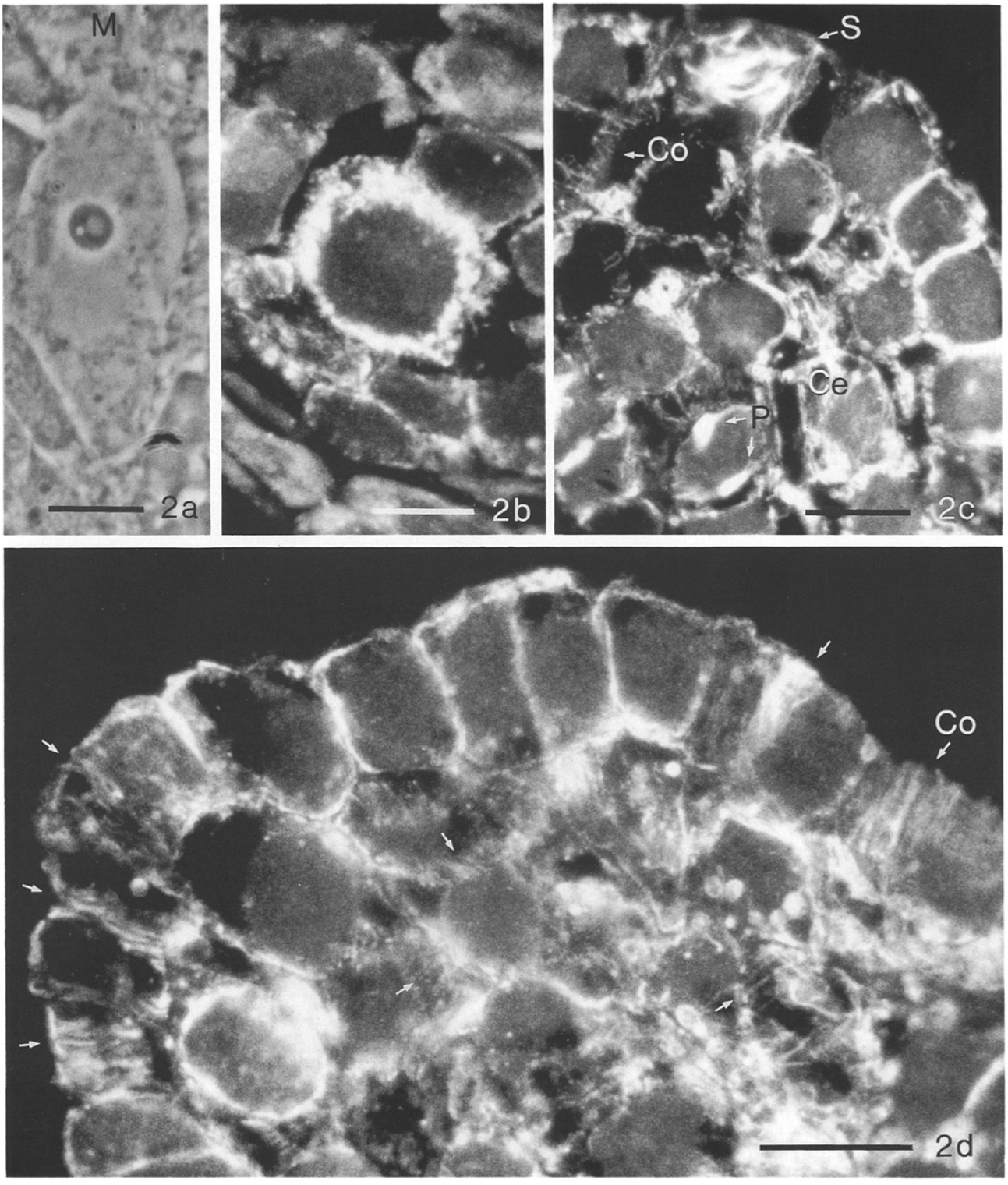

Fig. 2. **a** Survey of leptotene stage in a longitudinal section of the ovule. **b** Microtubules appear in bundles in a random orientation in the cytoplasm cross section. **c** Nucellar tissue showing a pre-prophase band (*P*), a mitotic spindle (*S*), randomly orientated microtubules in the central cytoplasm (*Ce*) and cortical microtubules (*Co*) adjacent to the cell membranes. **d** Microtubules in growing nucellus cells. Parallel cortical microtubules (*Co*) are to be found in an anticlinal direction especially in the protoderm. In the central nucellus cells the parallel cortical microtubules are arranged in various directions (*arrows*)

network of microtubules in their cortical and central cytoplasm (Fig. 2c). In the protoderm especially, strands of parallel, cortical microtubules were observed running in an anticlinal direction. In the central part of the nucellus, strands of parallel – arranged cortical microtubules can be observed; the direction of these microtubules varies from cell to cell (Fig. 2d). Cells divide frequently throughout the nucellar tissue (Fig. 8) as indicated by the presence of mitotic spindles and pre-prophase bands (Fig. 2c).

During pachytene, the meiocyte doubles in volume with respect to the young archespore (Fig. 3a). The microtubules are distributed in strands and bundles in the central cytoplasm, but cortical microtobules cannot be observed (Fig. 3b).

From pachytene onwards the cell volume of the meiocyte increases only slightly, and the cell becomes oval. The nucellus elongates and its cytoplasmic microtubules occur less frequently.

In the dyad a callose wall is present, especially between the two cells (Fig. 4a). At interkinesis microtubules radiate from the nuclei of both the micropylar and the chalazal cell (Fig. 4b, c). Cortical microtubules are frequently observed adjacent to the callose wall between the two cells (Fig. 4b). At this stage the nucellus is still elongating; the number of cell divisions, however, is decreasing (Fig. 8). In the growing integument, parallel cortical microtubules were observed perpendicular to the direction of stretching (Fig. 4b).

The tetrad, which is comprised of three degenerating and one functional megaspore, shows hardly any callose wall (Fig. 4d). As a result of the second meiotic division, the tetrad has enlarged. The cytoplasm of the functional megaspore contains cytoplasmic and cortical microtubules in a random orientation. In the degenerating megaspores the microtubules have disappeared (Fig. 4e), while in the nucellar cells, some cortical microtubules are present.

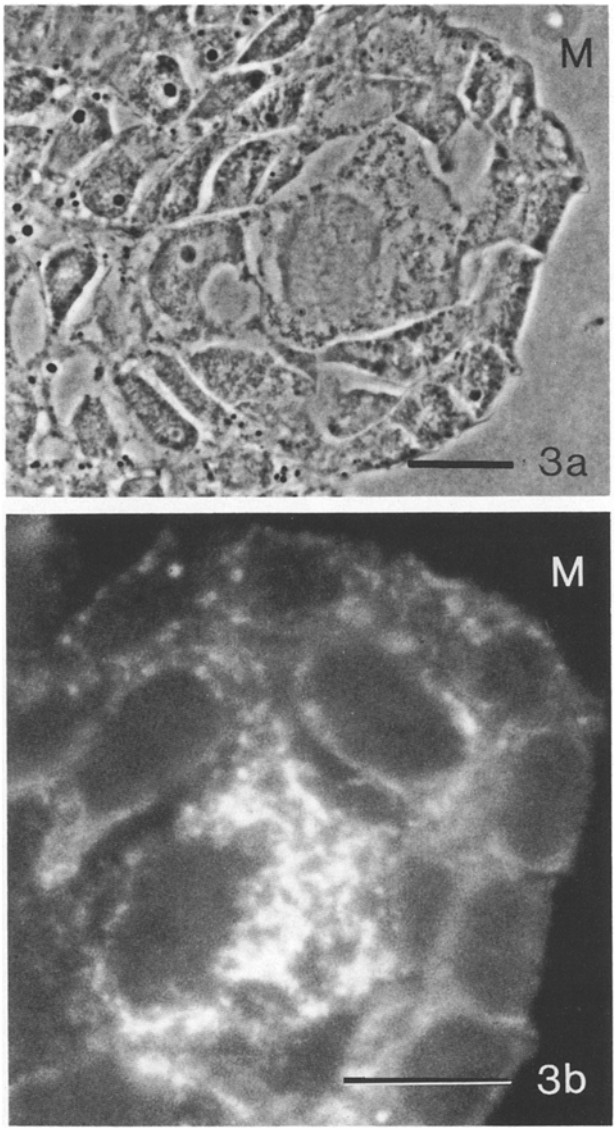

Fig. 3. a Survey of a pachytene cell. **b** Random-oriented microtubules and bundles of microtubules are scattered throughout the pachytene cell cytoplasm

Megagametogenesis

In the two-nucleate coenocytic stage vacuolation has started. The coenocyte elongates, and the degenerating megaspores become compressed (Fig. 5a). The coenocyte cytoplasm around the nucleus at the micropylar side shows few but the most microtubules (Fig. 5b). Except for the chalazal region, only a few cell divisions were observed in the nucellus (Fig. 8).

The four-nucleate coenocyte has a large vacuole and reached the ultimate dimensions of the embryo sac (Fig. 6a). In cross section only some microtubules were observed in the cytoplasm lining the cell membrane. Around the nucleus an intensive, diffuse fluorescence is present (Fig. 6b).

In a young embryo sac, the synergids (Fig. 6c) exhibit an intensive fluorescence. Microtubules run throughout the whole cytoplasm, especially in the region of the future filiform apparatus and in the chalazal part of the cell (Fig. 6d). In a full-grown embryo sac, the synergids developed a large filiform apparatus at the micropylar part of the cell (Fig. 7a). Lateral to that filiform apparatus fluorescence can still be observed, and at its chalazal

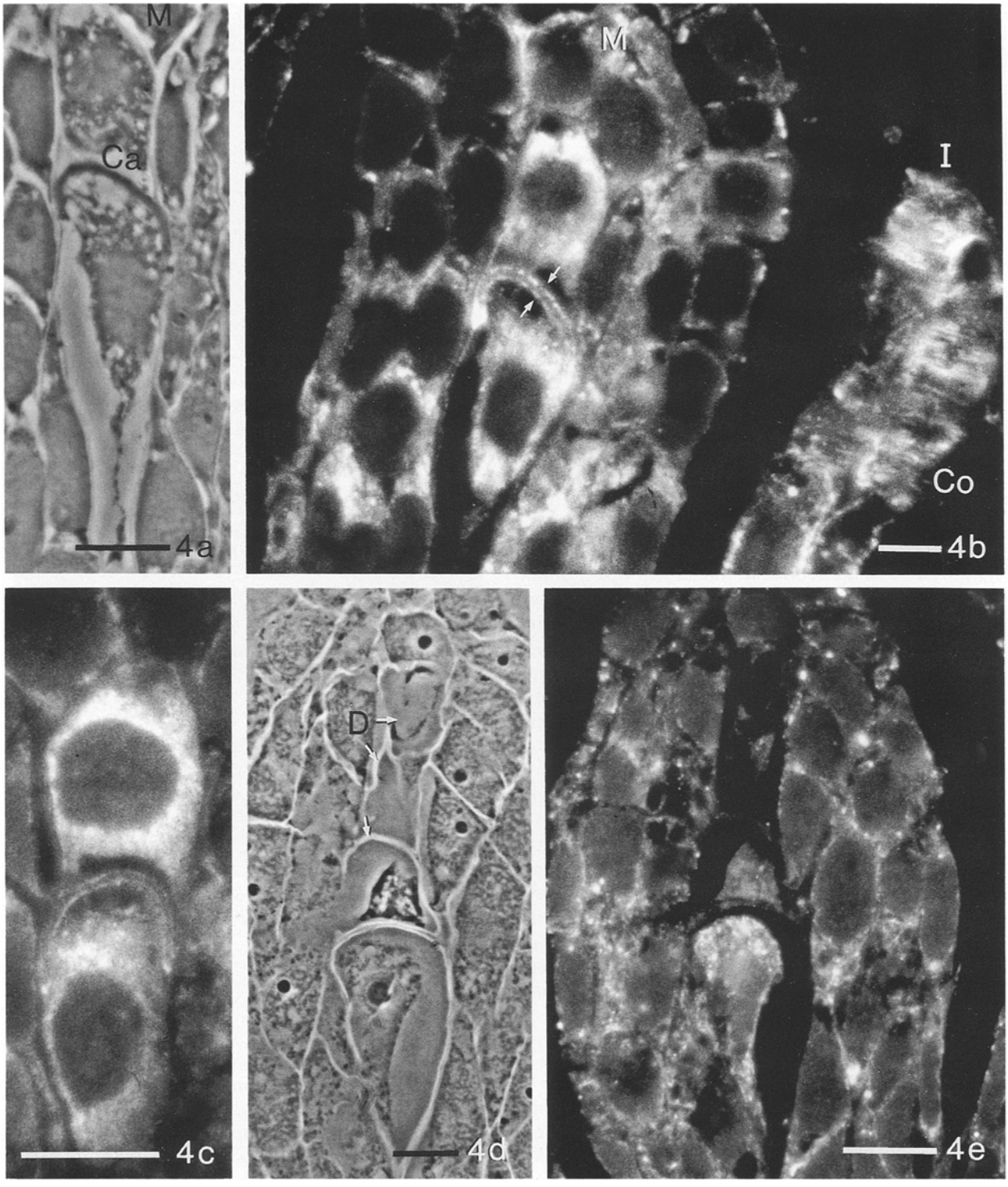

Fig. 4. a Survey of a dyad cell with callose wall (*Ca*). **b** Microtubular pattern during dyad stage. Note the microtubules against the callose wall (*arrows*) and the parallel cortical microtubules (*Co*) in the inner integument (*I*) cells. **c** Detail of the dyad with the radiating microtubules around the nuclei of both cells. **d** Survey of the tetrad with degenerating megaspores (*D*). **e** Microtubules in a random orientation in the cytoplasm of the functional megaspore

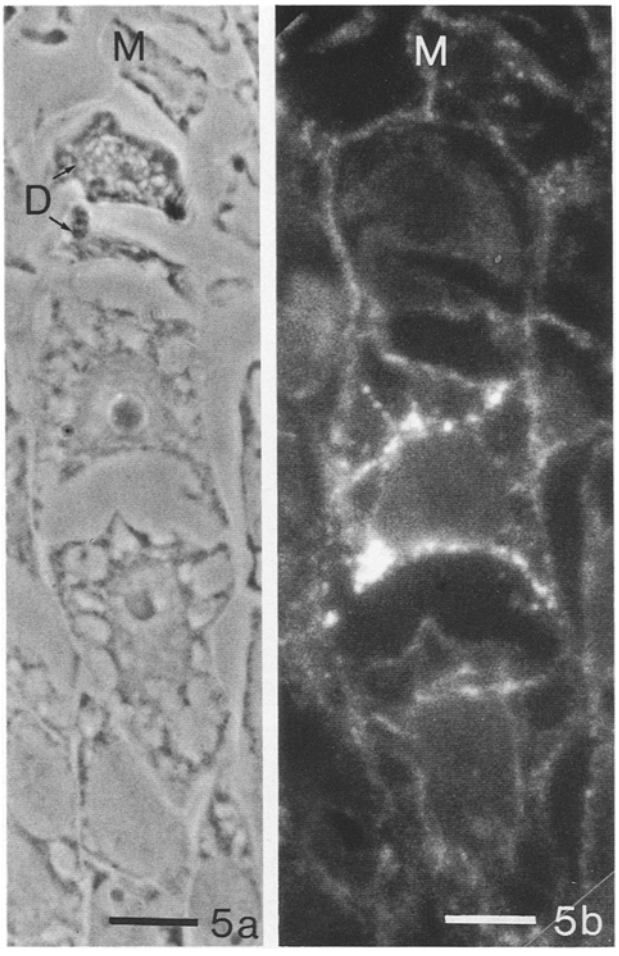

Fig. 5. a Survey of the two-nucleate coenocyte and degenerating megaspores (*D*). **b** Few microtubules can be observed in the cytoplasm of the coenocyte around the nucleus near the micropyle

side microtubules are arranged more or less parallel to the longitudinal axis of the synergid (Fig. 7b). The other parts of such an embryo sac, the egg cell (Fig. 7b) and the central cell (Fig. 7c, d) show only few microtubules, but the antipodes exhibit no reaction. In the chalazal part of the nucellus an hypostase develops, its cells show cortical microtubules along the thickened cell walls (Fig. 7d). The scheme in Fig. 8 summarizes the distribution of cell divisions, the position of the cortical microtubules in the ovule, and the cytoskeletal configurations during megasporo- and megagametogenesis.

Discussion and conclusion

Until meiosis the growth of the nucellus of *Gasteria* mainly occurs by cell multiplication. After meiosis an increase in the volume of the nucellar cells

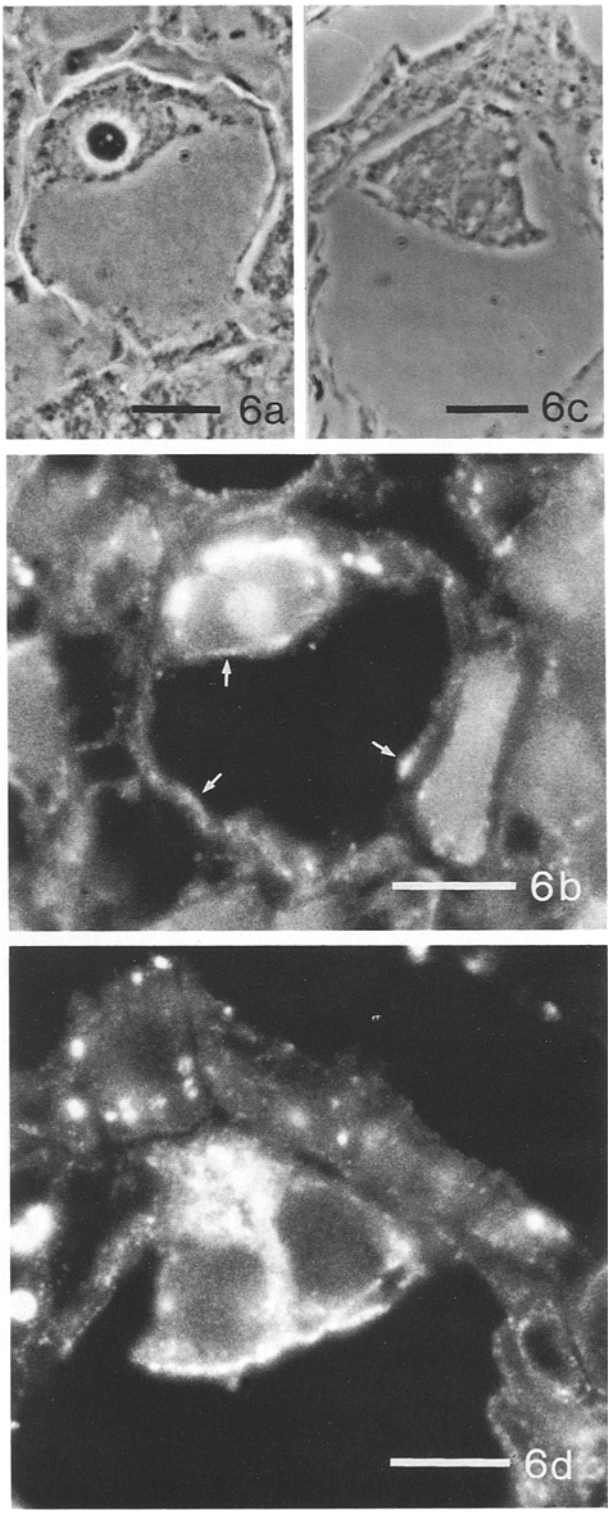

Fig. 6. a Survey of a cross section of the four-nucleate coenocytic stage showing one nucleus. **b** A positive diffuse fluorescence is observed in the cytoplasm around the nucleus and locally in cytoplasm against the cell border. It is partly caused by the presence of microtubules (*arrows*), partly by contamination with starch. **c** Survey of the young synergids. **d** Microtubules are present in the young synergids. Note the dense arrangement on the future place of the filiform apparatus

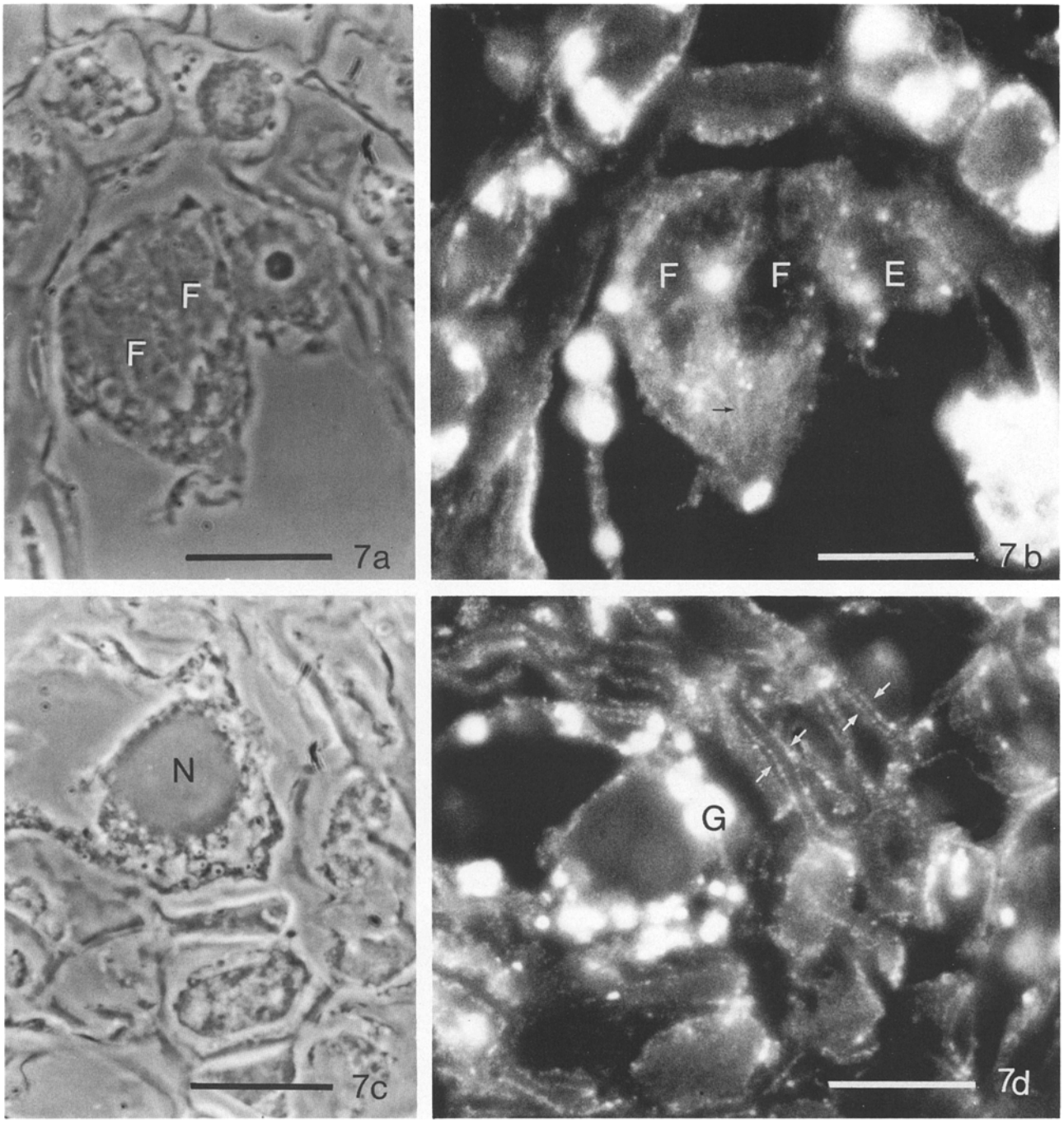

Fig. 7. **a** A mature egg apparatus with filiform apparatus (*F*) in the synergids. **b** Microtubules are in the cytoplasm next to the filiform apparatus. In the basal cytoplasm, small strands (*arrow*) run towards the chalazal side of the cell. The egg cell (*E*) shows few microtubules. **c** Central cell with nucleus (*N*). **d** Very little reaction is present in the central cell. The starch granules (*G*) show contamination. Note the microtubules bordering the thickened walls of the hypostase cells (*arrows*)

causes the elongated shape of the nucellus. At the beginning of meiosis the archespore cell and meiocyte increase in volume by synthesizing cytoplasm, and during pachytene-diakinesis an oval, large cell is realized (Willemse and Franssen-Verheijen 1978). Thereafter, during the two meiotic divisions the space available to the megaspores enlarges and megaspores become elongated when the whole nucellus elongates. The functional megaspore forms the coenocyte which in its turn mainly enlarges by vacuolation. After nuclear division the coenocyte first occupies the space of the tetrad, then

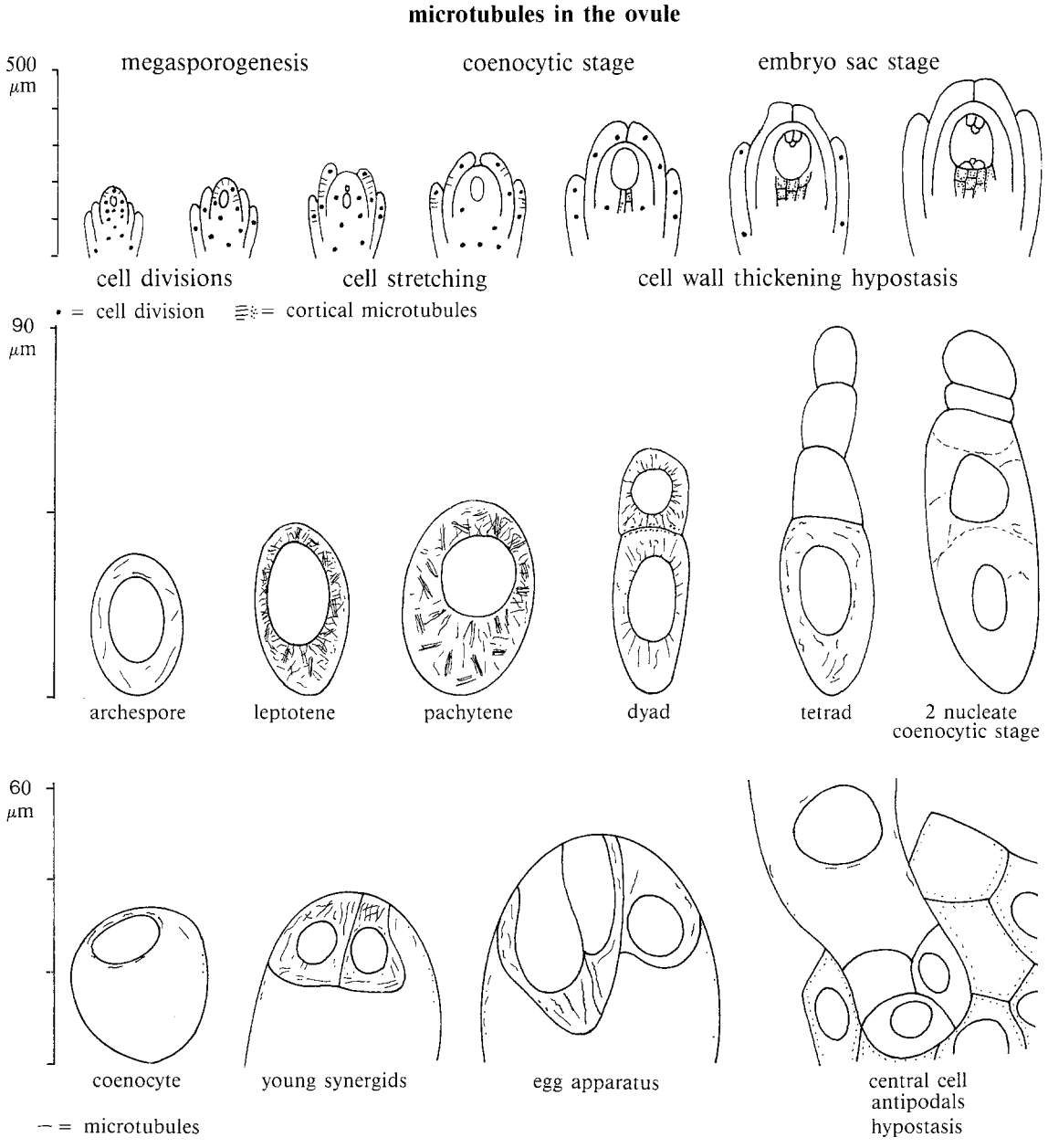

Fig. 8. Scheme of the nucellar development with cell divisions and the microtubular configuration during megasporo- and megagametogenesis

increases in volume by the extension of vacuoles and crushes the surrounding nucellar cells.

It is clear that the cortical parallel microtubules in the nucellus and integument induce the change in nucellar shape by preparing for the directed cell stretching in the sense described recently by Van Lammeren (1987). The uniformity of the microtubular arrangements in the protoderm cells which govern the shape of the nucellus is very remarkable. The underlying nucellar cells follow this growth pattern with another, more individual orientation of the cortical microtubules. Since cortical microtubules cannot be observed in the meiocytes during megasporogenesis until the dyad stage, the elongation of the megaspore and embryo sac is partly a passive phenomenon induced by the cytomorphogenesis of the nucellar cells.

At the onset of meiosis, a large number of microtubules appear during the increase in volume of the meiocyte. Three types of cytoplasmic microtubules were observed in the archespore cells and meiocytes during megasporogenesis: (a) randomly distributed microtubules running throughout the

cytoplasm; (b) bundles of microtubules found throughout the cytoplasm but predominantly lying against the nucleus; and (c) microtubules which appear to radiate from the nuclear membrane. The microtubular cytoskeletons as described in types a and b developed in the cytoplasm of the meiocytes. They function particularly in the maintenance of cytoplasmic integrity and in the positioning of organelles during development. Their increase in number and the appearance of bundles may strengthen these functions but may also cause cell enlargement. Type c is present in the dyad. The microtubular radiating from the nucleus may be related to the positioning of the nuclei in those cells prior to the second cell division. Comparable functions have been reported during the process of microsporogenesis (Van Lammeren et al. 1985). In the dyad, the cortical microtubules along the callose wall may function in the development of cell shape during the thickening of the cell walls and be independent of the composition of the cell wall.

After formation of the functional megaspore, the microtubular cytoskeleton becomes less extensive. The cortical microtubules are not arranged in a parallel fashion and do not function in directed cell elongation. However, the cytoplasmic microtubules can be related to the preparation of the positions of the nuclei, especially the micropylar one. The same seems to happen in the fully-developed coenocyte, which has a microtubular cytoskeleton surrounding the nuclei in particular.

As soon as the coenocytic embryo sac becomes cellular, the microtubular cytoskeletons of its individual cells reappear. Microtubules seem to be involved in the shaping of the synergid. Few microtubules are present in the other cells of the mature embryo sac.

Cortical microtubules oriented in a parallel fashion were observed in both the nucellar protoderm and the integument cells during stretching. The directed stretching protoderm cells are assumed to be especially responsible for the elongation of the nucellus by which the megaspore and embryo sac are enlarged. During this period the vacuolation of the coenocyte starts and increases by the uptake of water and nutrients, a process also leading to an increase in volume. Integument cells show the most obvious parallel orientation, and the growth of the integuments exceeds the elongation of the nucellus. In the hypostase cells, microtubules are involved in the organization and maintenance of cell shape during the thickening of the cell wall.

In conclusion, the shaping of the increasing meiocyte is induced partly by its microtubular cytoskeleton and partly by the growing nucellus. The cytoskeleton also functions in the positioning of the organelles and the maintenance of cytoplasmic integrity. After meiosis, the developing megagametophyte enlarges by vacuolation, but this enlargement is strongly influenced by the growth of both the nucellus and integument.

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