Microtubular configurations during endosperm development in *Phaseolus vulgaris*

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Microtubular cytoskeletons in nuclear, alveolar, and cellular endosperm of bean (*Phaseolus vulgaris*) were analyzed immunocytochemically and by electron microscopy to reveal their function during cellularization. Nuclear endosperm showed a fine network of microtubules between the wide-spaced nuclei observed towards the chalazal pole. Near the embryo, where nuclei were densely packed, bundles of microtubules radiated from nuclei. They were formed just before alveolus formation and functioned in spacing nuclei and in forming internuclear, phragmoplast-like structures that gave rise to nonmitosis-related cell plates. During alveolus formation cell plates extended and fused with other newly formed walls, thus forming the walls of alveoli. Growing wall edges of cell plates exhibited arrays of microtubules perpendicular to the plane of the wall, initially. When two growing walls were about to fuse, microtubules of both walls interacted, and because of the interaction of microtubules, the cell walls changed their position. When a growing wall was about to fuse with an already existing wall, such interactions between microtubules were not observed. It is therefore concluded that interactions of microtubules of fusing walls influence shape and position of walls. Thus microtubules control the dynamics of cell wall positioning and initial cell shaping.

Key words: cell wall, cellularization, endosperm, microtubule, *Phaseolus vulgaris*.

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Afin de mettre en lumière leur fonction durant la formation des cellules, les auteurs ont utilisé l'immuno-cytochimie et la microscopie électronique pour analyser les cytosquelettes de l'endosperme nucléaire, alvéolaire et cellulaire du haricot (*Phaseolus vulgaris*). L'endosperme nucléaire montre un fin réseau de microtubules entre des noyaux largement espacés, localisés vers le pôle chalazal. Près de l'embryon, où les noyaux sont densément regroupés, les faisceaux de microtubules s'irradient à partir des noyaux. Ils apparaissent juste avant la formation des alvéoles et agissent en espaçant les noyaux et en formant entre les noyaux, des structures ressemblant à des phragmoplastes, lesquelles donnent naissance à des plaques cellulaires non-reliées à une mitose. Pendant la formation des alvéoles, les plaques cellulaires s'étendent et se fusionnent avec d'autres parois nouvellement formées, constituant ainsi les parois des alvéoles. Au départ, les marges pariétales en croissance des plaques cellulaires montrent des ensembles de microtubules perpendiculaires au plan de la paroi. Lorsque les parois en croissance sont sur le point de fusionner, les microtubules des deux parois interagissent et, sous l'influence de l'interaction des microtubules, les parois cellulaires changent de position. Lorsqu'une paroi est sur le point de fusionner avec une paroi déjà existante, on n'observe pas de telles interactions entre les microtubules. Les auteurs concluent que les interactions des microtubules des parois en voie de fusionnement influencent la forme et la position des parois. Ainsi les microtubules contrôlent la dynamique du positionnement des parois cellulaires et la définition initiale de la forme des cellules.

Mots clés : paroi cellulaire, formation des cellules, endosperme, microtubule, *Phaseolus vulgaris*.

[Traduit par la rédaction]

Introduction

In angiosperms with nuclear endosperm, the transition from nuclear endosperm to cellular endosperm was investigated in *Helianthus annuus* (Newcomb 1973), *Triticum aestivum* (Mares et al. 1977; Morrison and O'Brien 1976; Morrison et al. 1978; Fineran et al. 1982; Van Lammeren 1988), *Haemanthus Katherinae* (Newcomb 1978), *Arabidopsis thaliana* (Mansfield and Briarty 1990), *Phaseolus vulgaris* (Brown 1917; Weinstein 1926; Yeung and Cavey 1988), *Nitraria sibirica* (Li et al. 1992), *Glycine max* (Dute and Peterson 1992), and *Ranunculus sceleratus* (XuHan and Van Lammeren 1993). Questions, however, still remain as to how nuclear endosperm cellularizes. The alveolus has been proposed to be the functional unit in the cellularization process (Fineran et al. 1982; XuHan and Van Lammeren 1993). However, from a mathematical point of view, the number of mitosis-established cell walls (Fineran et al. 1982) does not agree with the number of walls needed to complete cellularization. Thus, additional questions arise whether wall ingrowths (Pate and Gunning 1972) contribute to cellularization, new cell walls branch to give rise to additional cell walls, or other mechanisms cause the formation of the nonmitosis-related walls. In alveolar endosperm it was observed that freely growing wall edges (GWE) are always associated with microtubules (MTs) (Van Lammeren 1988; XuHan and Van Lammeren 1993). Therefore the freely GWE of the anticlinal wall was defined as the continuum of the original phragmoplast that continuously grows until cellularization is completed (XuHan and Van Lammeren 1993). However, how lateral walls of alveoli fused with each other was not investigated in detail.

Here we question to what level MTs do determine cellularization of endosperm and how MTs influence the site, shape, and size of alveoli. Especially we questioned when and how cell wall fusion was influenced by MTs. *Phaseolus vulgaris* is especially suited to study the dynamics of the microtubular cytoskeleton in the process of endosperm cellularization because it exhibits nuclear, alveolar, and cellular endosperm simultaneously from the early globular embryo stage until the cotyledonary stage. We present the results of changing microtubular configurations in developing endosperm, obtained by transmission electron microscopy and by light microscopic observations on excised fertilized embryo sacs and sectioned material.

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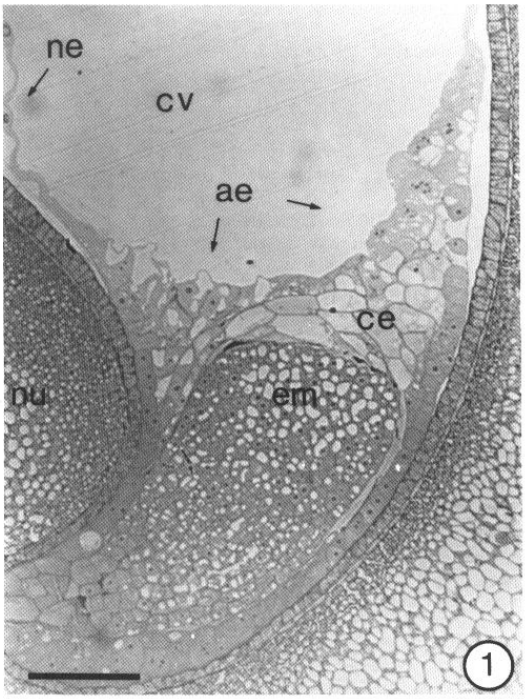
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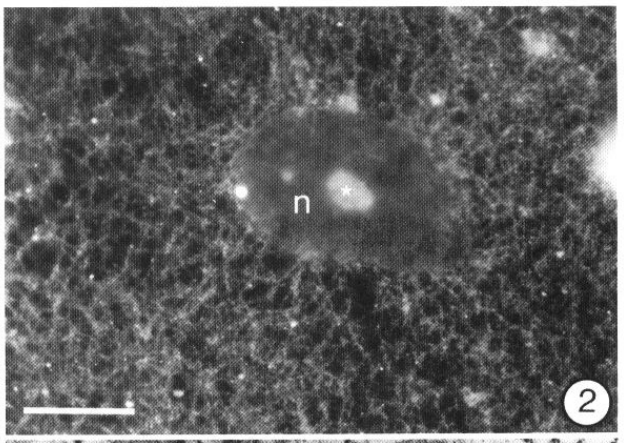
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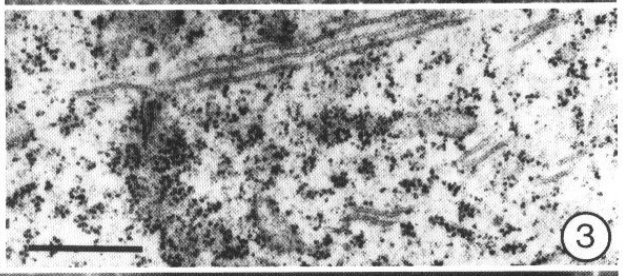
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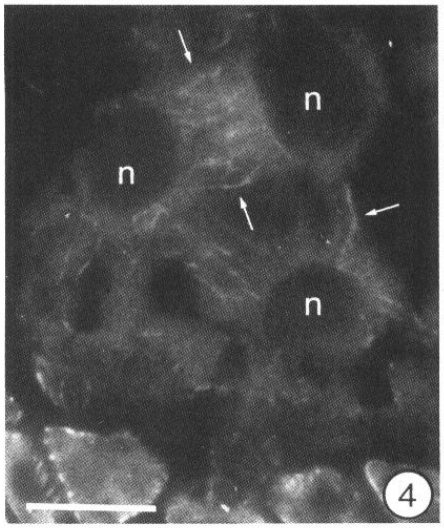
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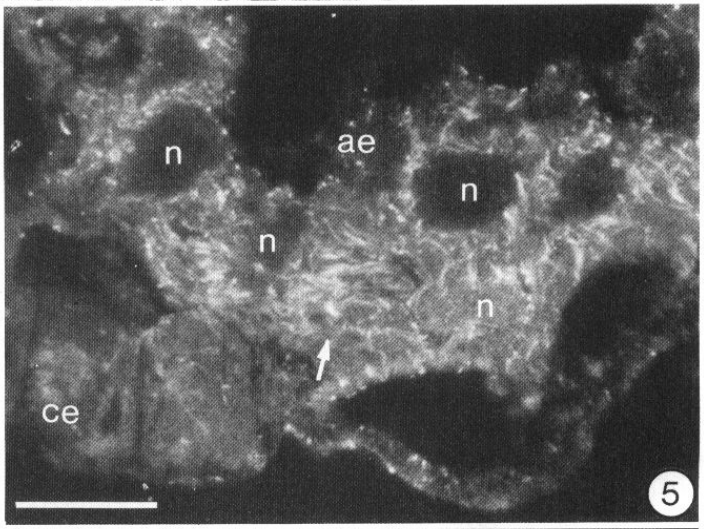
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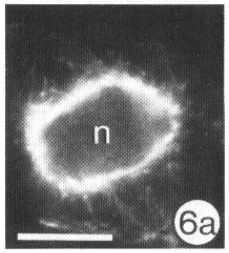
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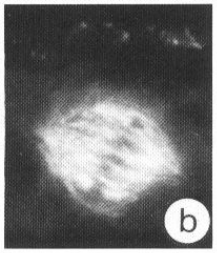
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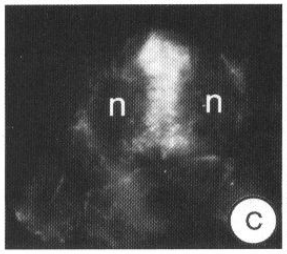
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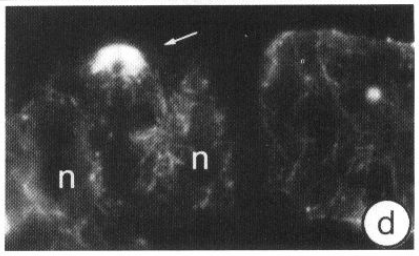
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Material and methods

Plants of *P. vulgaris* L. var. Groffy were grown under greenhouse conditions (23:18°C, light:dark). For the immuno-cytochemical staining of MTs, developing seeds were excised, immersed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.0) with 0.1% Triton X-100, and integuments were peeled off for the greater part, using a binocular microscope. Isolated embryo sacs were further fixed for 24 h, dehydrated in a graded series of ethanol, and embedded in polyethylene glycol (PEG) for the preparation of semithin (1- to 2-μm) sections. Fixation in 4% paraformaldehyde, the immuno-cytochemical staining of MTs with polyclonal antitubulin raised against calf brain tubulin (Van Lammeren et al. 1985), and the labelling with a second antibody conjugated with fluorescein isothiocyanate (FITC) (GaR-FITC Nordic, The Netherlands) were similar to the procedures described elsewhere (Van Lammeren 1988). Immunolabelling of whole mounts of endosperm was done after dissection of parts of the nuclear and alveolar endosperm. Additional treatment of the endosperm with 1% Triton X-100 for 1−2 h preceded immunolabelling to improve penetration of antibodies. The application of monoclonal anti-α-tubuline (Sigma Chemical Co., St. Louis, Mo.) revealed comparable configurations of fluorescent MTs, although the faint diffuse fluorescence, regularly observed in nuclei and nucleoli after labelling with the polyclonal antibody, was absent in such labelling experiments. Omission of the first antibody and application of preimmune serum served as controls. There was no fluorescence in such sections. Images were recorded with conventional fluorescence microscopy.

For electron microscopy, fixation with 2% paraformaldehyde, 2.5% glutaraldehyde, and 1 or 2% osmium tetroxide, embedding in Spurr's resin, ultrathin sectioning, and post-staining were the same as reported previously (XuHan and Van Lammeren 1993).

Results

Microtubular configurations in nuclear, alveolar, and cellular endosperm

When the embryo was at the globular to elongated stage, the endosperm already had nuclear, alveolar, and cellular parts (Fig. 1). Microtubules were observed in all the tissues of the seed at this stage. In the nuclear endosperm at the chalazal part of the embryo sac MTs formed a fine reticulate network (Fig. 2). Some MTs radiated from nuclear envelopes. They were interwoven with other MTs in the internuclear area. The arrays of the network consisted of bundles of parallel MTs as was revealed by electron microscopy (Fig. 3). In control experiments in which preimmune serum was applied or in which the first antibody was omitted, no such configurations were observed. In the nuclear endosperm near the embryo, in which alveolus formation was about to start, bundles of MTs radiated from neighbouring nuclei. They were interwoven and formed arrays of bundles between pairs of nuclei (Fig. 4). Such arrays of internuclear MTs (INMTs) would ultimately give rise to phragmoplast-like structures and cell plates by which the alveolar cell walls developed.

The alveolar endosperm was restricted to the region near the apical part of the embryo (see Fig. 1). Alveoli often exhibited irregular shapes because the elongating embryo pushed the endosperm forward. Microtubules were abundantly present in the alveolar endosperm (Fig. 5). They ran from nuclei through the cytoplasm.

In cellular endosperm karyokinesis was always followed by cytokinesis (Fig. 6). Pre-prophase bands (PPBs) were found in dividing cells whereas PPBs were never observed in the nuclear endosperm. Hereafter, metaphase, anaphase, and telophase MT configurations appeared, and at interphase an MT network was present throughout the whole cytoplasm again (Fig. 6).

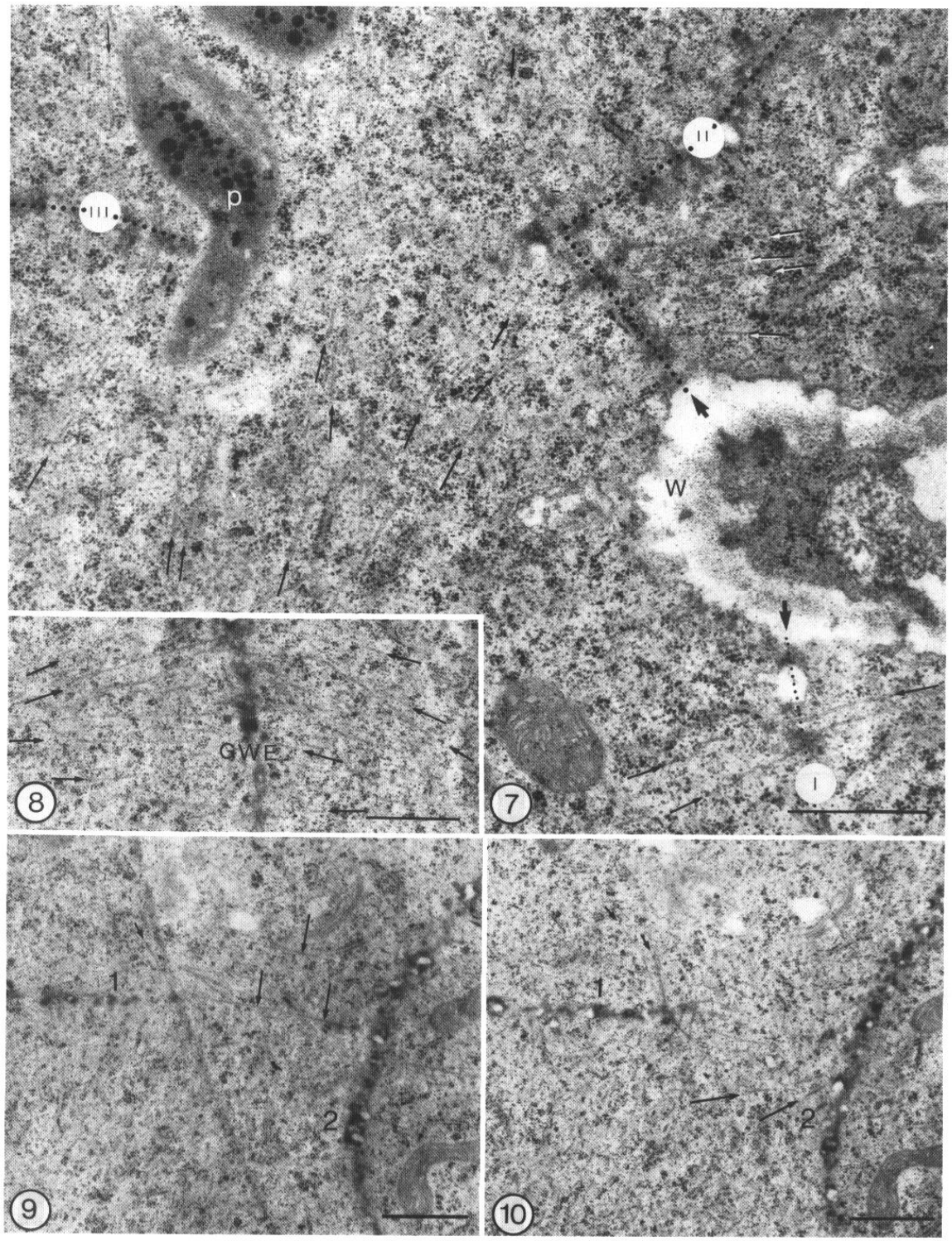
Configurations of GWE−MTs

In the alveolar endosperm, cell plates were formed in the equatorial planes of the phragmoplast-like structures. The GWEs of cell plates expanded to all sides. The inner GWE grew towards the centre of the embryo sac. The outer GWE grew towards and finally connected to the existing embryo sac wall or to the profiles of the sac wall ingrowths. Lateral GWEs connected to other adjacent endosperm walls. Wall ingrowths themselves never gave rise to anticlinal wall formation.

Microtubules, associated with the GWE that fused with the embryo sac wall or with an already thickened endosperm cell wall, showed a perpendicular orientation to the growing wall plane. They maintained this configuration during cell wall fusion (Fig. 7). We regularly observed cortical MTs near thickened endosperm cell walls or near the wall of the embryo sac. They were not involved in the process of cell wall fusion.

A different MT configuration was observed when GWEs grew towards and connected to recently formed cell walls or cell plates that still had associated MTs and in which vesicles were still fusing (Figs. 9, 10). Just before fusion, the GWE and the recently formed cell plate both exhibited MTs in perpendicular orientations. Then the MTs of the approaching GWE as well as those of the approached plate gradually changed their orientations. The original perpendicular orientation of MTs changed into an oblique orientation while the fusion proceeded (Fig. 8). In the fusion area the MTs of the approached plate parted away from either side of the approaching GWE (Figs. 9, 10). The MTs of the approaching GWE formed rather small acute angles to the GWE but maintained mirror symmetric configurations to the GWE. Simultaneously the approached plate bent and formed a bulge towards the approaching GWE, and MTs at the fusion site attained parallel orientations to the bisectors of the angles between the plates.

FIG. 1. Bright-field light micrograph showing a longitudinal semithin section of the micropylar side of the embryo sac of bean with embryo (*em*) and endosperm. The endosperm consisted of nuclear (*ne*), alveolar (*ae*), and cellular endosperm (*ce*). Embedded in PEG. *nu*, nucellus. Scale bar = 100 μm. FIG. 2. Immunofluorescence micrograph of a whole-mount preparation of endosperm showing fluorescent arrays of MTs in the nuclear endosperm in the region towards the chalazal pole. Note the reticulate network of numerous bundles of MTs, some of them running from the nuclear envelope. The faint diffuse labelling in the nucleus is due to fluorescence of out of focus MTs. The labelling in the nucleolus (*) is due to nonspecific binding. *n*, nucleus. Scale bar = 20 μm. FIG. 3. Electron micrograph showing bundles of internuclear MTs in the nuclear endosperm. Scale bar = 500 nm. FIG. 4. Immunofluorescence micrograph of semithin PEG section of nuclear endosperm near the embryo showing thick internuclear bundles of MTs (INMTs, arrows). *n*, nucleus. Scale bar = 10 μm. FIG. 5. Immunofluorescence micrograph of semithin PEG section with cellular (*ce*) and developing alveolar (*ae*) endosperm. Compared with Fig. 4, INMTs increase in number and will give rise to phragmoplast-like structures between nuclei (arrow). *n*, nucleus. Scale bar = 10 μm. FIG. 6. Fluorescent MTs in semithin PEG sections of cellular endosperm showing successive stages of normal cytokinesis. (*a*) Prophase cell with MTs surrounding the nucleus. (*b*) Metaphase cell with spindle of MTs. (*c*) Telophase cell with phragmoplast. (*d*) Late telophase cells showing nearly completed cytokinesis. The phragmoplast still existed at the side towards the central vacuole (arrow). At the right-hand side an interphase cell is partly depicted. *n*, nucleus. Scale bar = 10 μm.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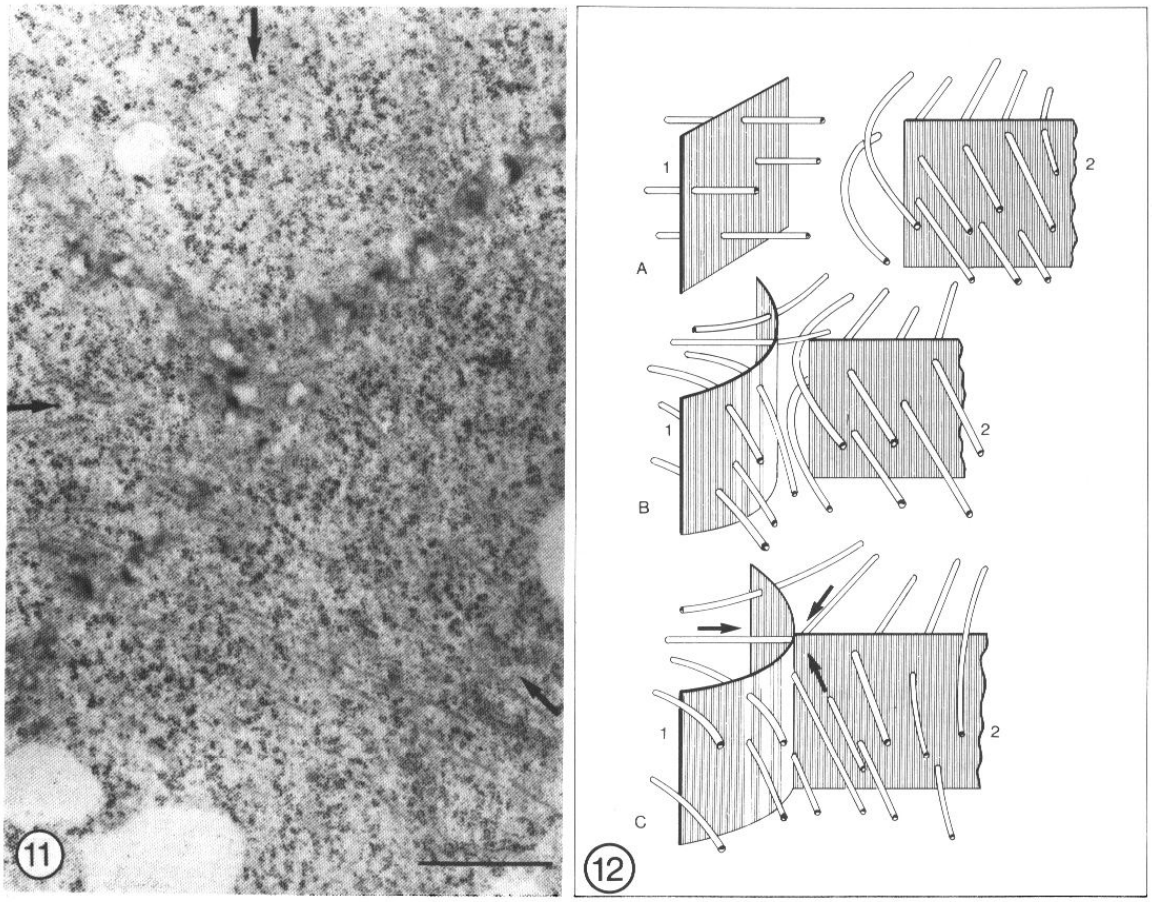

FIG. 11. Electron micrograph of the vertex area of the wall juncture. Note that MTs attain orientations parallel to the future bisectors (arrows). Scale bar = 1 μm. FIG. 12. A diagrammatic illustration of a wall coalescence area showing the changes in microtubular configurations when two growing walls or plates fuse at a right angle. (A) When the GWE of plate (2) is still far away from the recently formed wall or plate (1), each population of MTs exhibits mirror symmetric orientations to each plane of the walls or plates. (B) When the two walls or plates are about to fuse, both populations of MTs interact and change their orientations in the fusing area. As a result, wall or plate number 1 changes its shape. (C) When fusion has finished, the three walls and the MTs are separated by the walls or plates in the three alveoli and form Y-shaped crosses but the MTs stagger by half an angle to the wall or plate Y-shaped cross. The MTs on both sides of a common wall or plate are in mirror symmetric configuration. They will disappear when the wall or plate matures. Arrows indicate the bisectors in each newly formed alveolus.

At the end of the fusion, a Y-shaped cross of cell plates or walls was formed (Fig. 11). The MTs near the vertex of the Y-shaped cross were parallel to the bisectors of the angles and they formed a Y-shaped configuration, too. Throughout our observations, wall branching processes were not observed.

Discussion

Microtubular configurations in nuclear, alveolar, and cellular endosperm

Compared with the previous work reviewed in the introduction, the present work shows remarkable differences in MT

FIG. 7. Electron micrograph showing endosperm wall joinings. On the lower right and upper right GWEs (I and II) attach to a thickened endosperm wall (*W*) at the sites indicated by thick arrows. Dotted lines indicate the position of the growing walls. Note that GWE−MTs (small arrows) are perpendicular to the planes of the growing wall edges I and II. On the upper left, a GWE (III), also associated with MTs, approaches. Microtubules in the central area are either associated with GWE II or run in front of GWE III. Scale bar = 1 μm. FIG. 8. Electron micrograph of bean endosperm showing a GWE that is near the fusion site with an other young cell wall still associated with MTs. MTs (arrows) gradually change orientation from perpendicular to the GWE away from the fusion site (lower side) to oblique to the GWE, i.e., parallel to the bisectors of the wall angles near the fusion site (upper side). Orientation of MTs is indicated by arrows. Scale bar = 1 μm. FIGS. 9 and 10. Electron micrographs of two serial sections of a stage of wall fusion between a GWE (1) and a recently formed wall or plate (2) in the alveolar region of bean endosperm. Both cell walls exhibit associated MTs. MTs from wall 2 part away from the approaching wall 1 as is indicated by the long arrows at the upper side (Fig. 9) and the long arrows at the lower side (Fig. 10) of the approaching GWE. Note that the recently formed wall or plate (2) exhibits a bulge towards the approaching GWE. Its associated MTs overlap the MTs of the GWE 1 (short arrows) that are pushed back. Scale bar = 1 μm.

configurations in nuclear, alveolar, and cellular endosperm. In the nuclear endosperm with wide-spaced nuclei, MTs run throughout the cytoplasm forming a reticulate network. In nuclear endosperm that is about to form alveoli, MTs radiate from nuclei, and bundles of INMTs become prominent. In the alveolar endosperm GWE–MTs form the most remarkable MT arrays. Finally in the cellular endosperm, cortical, PPB, spindle, and phragmoplast MTs appear in a way common to somatic cells.

Microtubular configurations and cellularization

Microtubules function in cellularization in various ways. Pre-prophase bands (Pickett-Heaps and Northcote 1966) determine new wall planes by fixing the plane of division (Gunning 1982) and spindle, phragmoplast, and GWE–MTs control the position of the cell plate and the direction of cell plate extension in alveolar endosperm (XuHan and Van Lammeren 1993). The configurations of the MT arrays observed in the nuclear endosperm near the embryo of bean evoke considerations as to the function of these arrays.

The first MT configurations related to cellularization were observed in the nuclear endosperm just before alveolus formation. The MTs radiating from the nuclei probably function in the regular spacing of the nuclei first, and later, in the development of cell plates around each nucleus. Those cell plates are restricted to equatorial planes initially and expand from the centre to the periphery following a development as commonly seen during cytokinesis.

Cell plates between daughter nuclei are formed in the phragmoplast after karyokinesis. The other cell plates are formed in the phragmoplast-like structures, developed from the INMTs between nondaughter nuclei and are thus independent from karyokinesis. Thus, cell plates are formed in all the internuclear areas in the alveolar endosperm, and nuclei become surrounded by cell walls at their lateral sides, not at the sides directed towards the wall of the central cell and towards the central vacuole of the central cell. Since alveoli are only formed when nuclei are near each other, induction of cytokinesis apparently depends on the distance between the nuclei in the endosperm. Phragmoplast-like structures were not described for *Nitraria sibirica* by Li et al. (1992) who only stated that phragmoplasts were a result of normal mitosis. Dute and Peterson (1992) described a different formation of anticlinal walls, rather projecting from the wall of the central cell into the endosperm cytoplasm. This might, however, be the result instead of the onset of anticlinal wall formation.

The extension of the cell plate occurs in three directions, i.e., the GWEs grow towards the embryo sac wall, towards the inner side of the embryo sac, and in lateral directions. The GWEs that grow towards the embryo sac wall fuse with that wall or with profiles of wall ingrowths from the embryo sac in a random fashion and then do not grow further. Thus, it is concluded that the anticlinal walls are formed by cell plates that grow and connect to wall profiles or to the embryo sac wall. Existing wall profiles themselves do not form anticlinal walls. The inner GWEs of the anticlinal walls continuously grow towards the central vacuole, forming the walls of the alveoli and the file of cells behind. The lateral sides of the cell plate grow towards the other cell plates or to existing anticlinal cell walls and fuse, forming the alveoli. Wall branching, as suggested in *Arabidopsis thaliana* (Mansfield and Briarty 1990), was never observed in either this species or in *R. sceleratus* (XuHan and Van Lammeren 1993). We observed that anticlinal walls of the bean endosperm are often disturbed in position in the region near the tip of the embryo because the embryo presses against the endosperm. Thus, walls become oblique.

In our studies, as well as in those on soybean (Dute and Peterson 1992), extensive arrays of MTs are associated with GWEs, independent of the directions of growth or of the fusion of the GWEs with other walls. Growing wall edges show the same ultrastructure as normal cell plate edges (Hepler and Jackson 1968) and should therefore be considered as a continuum of the phragmoplast. When GWEs grow, GWE–MTs form approximate mirror symmetric configurations as seen in mitotic phragmoplasts. These results are in agreement with those of our previous investigation in *R. sceleratus* (XuHan and Van Lammeren 1993).

Function of GWE–MTs

During the cellularization of bean endosperm, GWE–MTs control the direction of wall growth and wall fusion. An interesting phenomenon was observed when the GWE of a growing wall was about to fuse with another recently formed wall. According to mechanics and the interaction of MTs not taken into consideration, the approaching GWE could have pushed the recently formed wall. However, the recently formed wall bends towards the approaching GWE. This can be explained by the model shown in the diagrammatic illustration (Fig. 12). Here it is suggested that the dynamic configuration of the MT framework plays a role in cell wall fusion and the changing shape of the walls. First the MTs of the two fusing walls do not interact (Fig. 12A). Then the MTs of the approaching wall push aside the MTs of the opposite wall. By this action the opposite wall bends because the associated MTs change position (Fig. 12B). Ultimately this results in the rearrangement of the fusion site with angles between the walls approaching 120° each (Fig. 12C). Microtubules of adjacent walls interfere, and special configuration of MTs then provide an equilibrium in the area of wall junction as was analyzed previously in *R. sceleratus* (XuHan and Van Lammeren 1993). The present results, however, revealed the origin of such configurations. Since TEM studies were not done in the analysis of endosperm cellularization in wheat (Van Lammeren 1988), it might well be that comparable interactions of cell plate associated MTs lead to the hexagonal shape of the alveoli observed in that study.

Acknowledgments

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