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Microtubule organization, mesophyll cell morphogenesis, and intercellular space formation in *Adiantum capillus veneris* leaflets

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Summary. Mesophyll cells (MCs) of *Adiantum capillus veneris* are elongated and highly asymmetric, bearing several lateral branches and forming a meshwork resembling aerenchyma. Young MCs are polyhedral and display oppositely arranged walls and transverse cortical microtubules (Mts). Their morphogenesis is accomplished in three stages. At first they become cylindrical. Intercellular space (IS) canals, containing PAS-positive material, open through their junctions and expand laterally. During the second stage the cortical Mts form a reticulum of bundles, externally of which an identical reticulum of wall thickenings, containing bundles of parallel cellulose microfibrils, emerges. MCs do not grow in girth in the regions of wall thickenings, where constrictions form and new ISs open. Thus, MCs obtain a multi-lobed form. At the third morphogenetic stage MCs display a multi-axial growth. During this process, additional Mt rings are assembled at the base of cell lobes accompanied by similarly organized wall thickenings-cellulose microfibrils. Consequently, cell lobes elongate to form lateral branches, where MCs attach one another, while the IS labyrinth broadens considerably. Colchicine treatment, destroying Mts, inhibits MC morphogenesis and the concomitant IS expansion, but does not affect IS canal formation. These observations show that: (a) MC morphogenesis in *A. capillus veneris* is an impressive phenomenon accurately controlled by highly organized cortical Mt systems. (b) The disposition of Mt bundles between neighbouring MCs is highly coordinated. (c) The perinuclear cytoplasm does not appear to be involved in cortical Mt formation. Cortical sites seem to participate in Mt bundling. (d) Although extensive IS canals open before Mt bundling, the Mt-dependent MC morphogenesis contributes in IS formation.

Keywords: *Adiantum capillus veneris*; Cell morphogenesis; Intercellular space formation; Microtubules.

Abbreviations: EM electron microscopy; ER endoplasmic reticulum; IS intercellular space; MC mesophyll cell; MSB microtubule stabilizing buffer; Mt microtubule; PBS phosphate buffered saline.

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Introduction

The role of the cortical Mt cytoskeleton in plant cell morphogenesis is widely accepted (for reviews, see Gunning and Hardham 1982, Robinson and Quader 1982, Hepler 1985, Lloyd 1987, Seagull 1989, Williamson 1991). Recent work has shown that cortical Mts are involved in the development not only of regular cell forms but also of asymmetric ones, like the variable and highly lobed epithem cells of *Pilea cadierei*. The organization of a network of Mt bundles underneath the plasmalemma results in the transformation of polyhedral cells into lobed ones (Galatis 1988). External to Mt bundles, wall thickenings enclosing identical bundles of cellulose microfibrils are deposited. The latter prevent an increase in cell girth locally, leading to the formation of cell constrictions with the simultaneous development of ISs (Galatis 1988, Apostolakos et al. 1991).

Since then, the role of cortical Mts in the shaping of other lobed cell types has been further shown in the mesophyll of *Triticum aestivum* (Jung and Wernicke 1990) and *Zea mays* (Apostolakos et al. 1991). In the latter plant MC morphogenesis also keeps pace with IS formation. Mts also participate in "semi-lobed" cell morphogenesis in *Adiantum capillus veneris* (Panteris et al., in prep.).

In this work the role of cortical Mts in MC morphogenesis as well as in IS formation is investigated in *A. capillus veneris* leaflets by conventional light microscopy, tubulin immunofluorescence and electron microscopy (EM). The leaflets of this fern develop highly elongated asymmetric MCs bearing several lateral

branches and forming a cellular mesh resembling aerenchyma (Wylie 1948, 1949). The role of cortical Mts in morphogenesis of such asymmetric cell types of higher plants has not been studied so far.

Materials and methods

Light and electron microscopy

Small pieces of *Adiantum capillus veneris* leaflets at various stages of differentiation were prepared for light and electron microscopy according to a procedure described in Panteris et al. (1991). Very young leaflets of *A. capillus veneris* plants grown for 7 days in small dishes containing cotton wetted with a 0.08% colchicine solution in Hoagland were also processed for light and electron microscopy. Thin sections of embedded tissue, double-stained with uranyl acetate and lead citrate, were examined with a Philips 300 EM (Philips, Eindhoven, The Netherlands). Semithin sections of the embedded material were stained with 1% toluidine blue in 1% sodium tetraborate solution and examined with an Axioplan microscope (Carl Zeiss, Oberkochen, Federal Republic of Germany).

The insoluble polysaccharides were demonstrated on semithin sections of embedded material with the periodic acid-Schiff's reaction (PAS) according to the protocol described by Galatis et al. (1978). Lipophilic substances were localized on semithin sections of the same material with a saturated solution of Sudan black B in 70% ethanol at 60 °C.

Immunofluorescence microscopy

Differentiating as well as mature leaflets of *A. capillus veneris* plants grown in a controlled environment were prepared in accordance with a protocol similar to that of Jung and Wernicke (1990). Squashing of the tissue is avoided by this procedure so that cell shapes are optimally preserved. Leaflet pieces, about 1 × 1 mm, were incubated in 5% dimethyl sulfoxide in Mt stabilizing buffer (MSB; 50 mM PIPES, 5 mM EGTA, 5 mM $MgSO_4$) for 30 min at room temperature. Fixation was then performed in 8% paraformaldehyde in MSB for 45 min. Afterwards, the pieces were rinsed three times with MSB and digested for 1–3 h in 1–2% pectinase (Sigma Chemical Co, St. Louis, MO) in MSB. After careful washing with MSB the samples were forced through a Pasteur pipette to release the cells. Equal

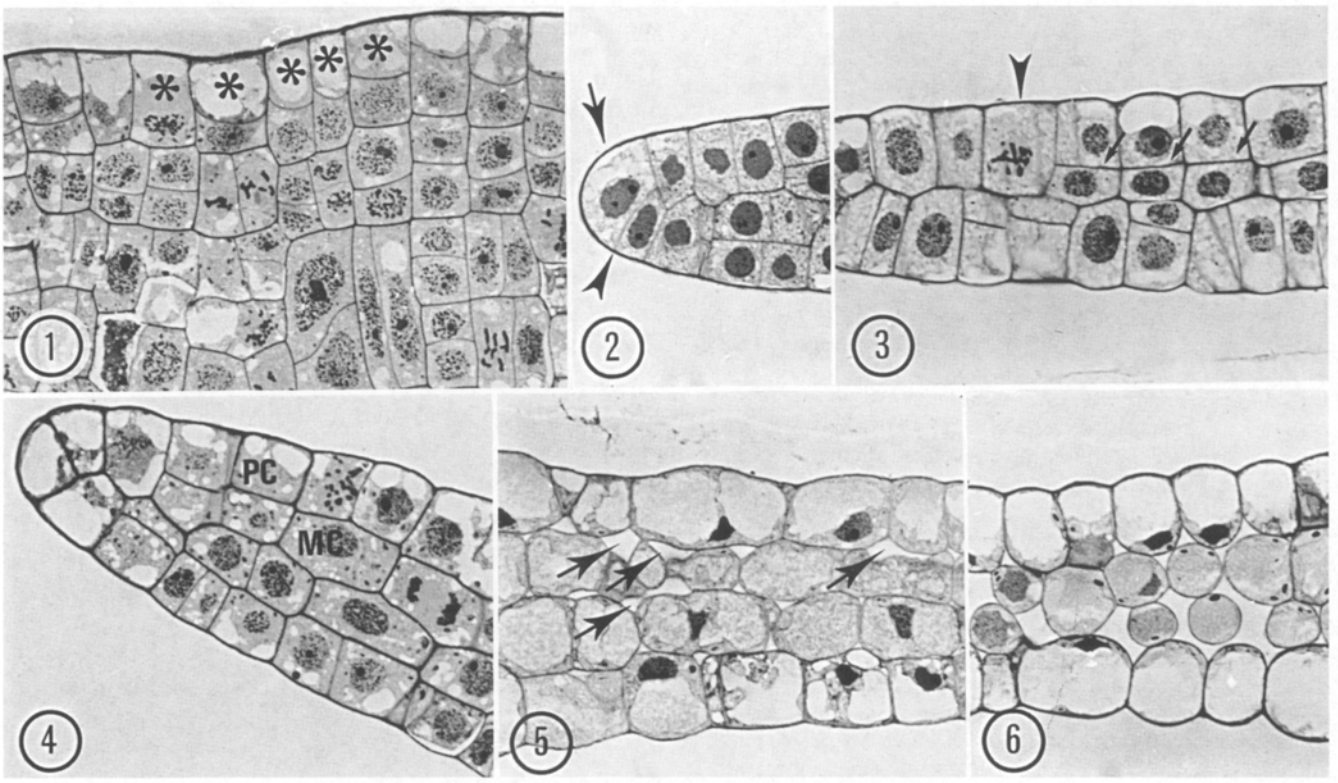


Fig. 1. Paradermal section through the meristematic leaflet area. The marginal meristematic cells (asterisks) form a continuous row. × 560

Fig. 2. Longitudinal transverse section of the meristem. The marginal meristematic cell (arrow) has just divided. Arrowhead indicates the daughter wall. × 560

Fig. 3. Longitudinal transverse section of the meristem. The periclinal walls separating protodermal cells and MCs tend to be opposite (arrows). The cell shown by arrowhead is dividing periclinally. × 560

Fig. 4. Longitudinal transverse leaflet section near the margin, which consists of four cell layers. *PC* Protodermal cell, *MC* mesophyll cell. × 560

Fig. 5. Longitudinal transverse profile of a leaflet at a region where IS canals (arrows) have opened. The cells are partially detached. × 560

Fig. 6. Transverse leaflet section perpendicular to the veins at a region similar to that shown in Fig. 5. Note MC form. × 560

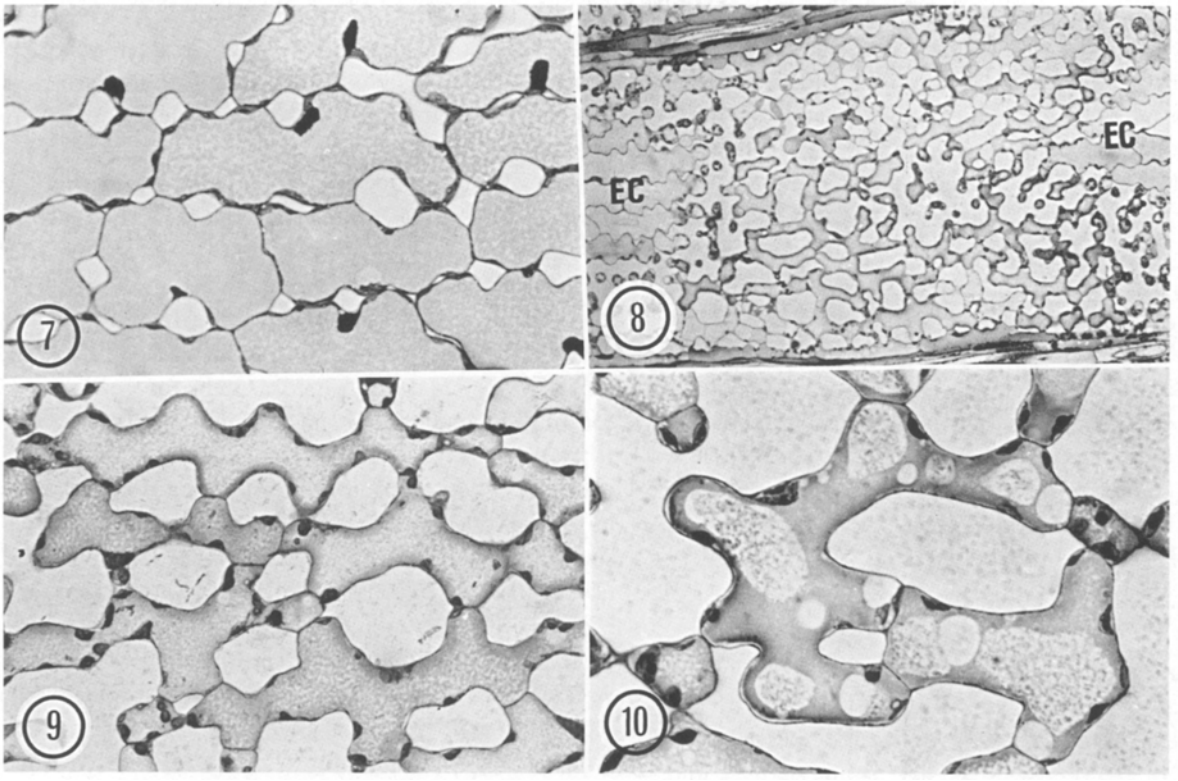


Fig. 7. Paradermal section of lobed MCs. ISs are opened at their constrictions. MCs remain attached at their lobes. × 560

Fig. 8. Paradermal view of differentiated mesophyll. The tissue strongly resembles aerenchyma. *EC* Epidermal cell. × 110

Fig. 9. Higher magnification of differentiated MCs. Note the branches and broad ISs. × 360

Fig. 10. Paradermal section of a peculiarly-shaped MC. × 560

amounts (10 µl) of cell suspension and ultra low temperature-gelling agarose (Sigma, type IX) were mixed and allowed to gel at 4 °C. All subsequent steps were carried out in these aliquots. The cell walls were first digested with 2% cellulase "Onozuka" R-10 (Yakult Honsha Co Ltd., Tokyo, Japan) and 1% macerozyme R-10 (Yakult Honsha) in MSB for 20–30 min. Then, the cells were extracted with 1–2% Triton X-100 in phosphate buffered saline (PBS) for 1 h and thoroughly washed in PBS.

Incubations with antibodies took place at room temperature overnight as follows: First with a monoclonal rat anti-α-tubulin (YOL 1/34, Seralab) diluted 1 : 150 in PBS containing 1% bovine serum albumine and second with FITC-conjugated anti-rat IgG (Sigma Co.) 1 : 20 in the same buffer. In some experiments cellulase digestion was not performed and after the antibodies the cellulose microfibrils were stained with 0.001% Calcofluor white M2R (Tinopal LPW, Sigma) in PBS. Finally, the agarose blocks were transferred on microslides and mounted in a solution of PBS-glycerol 1 : 1 containing 0.1% p-phenylenediamine.

Immunofluorescence preparations were viewed with a Zeiss Axioplan microscope and photographed with T-MAX 400 film (Eastman Kodak, Rochester, NY) rated at ASA 1600.

Results

General remarks

The leaflet of *Adiantum capillus veneris* grows by a marginal meristem consisting of a single row of cells, which in longitudinal transverse sections display triangular profiles (Figs. 1 and 2). Every marginal meristematic cell divides asymmetrically in two planes parallel to its internal faces (Fig. 2). In this way a bi-layered meristem is formed, from which all leaflet tissues are derived (Figs. 2 and 3). More developed leaflets grow by several groups of marginal meristematic cells and not by a continuous cell row. These cells proliferate by symmetrical anticlinal divisions perpendicular to the leaf margin. Protodermal cells and MCs are separated by a single polarized periclinal division of each immediate derivative of the marginal meristematic cells (Fig. 3). In this way two MC layers are generated in

the intercostal areas (Fig. 4). It is noteworthy that the walls laid down by periclinal divisions are of opposite disposition (Fig. 3). Afterwards, both protodermal cells and MCs undergo anticlinal divisions in two planes perpendicular to each other (Fig. 1). The cell walls formed by these divisions tend to be oppositely arranged.

Differentiated mesophyll consists of highly-vacuolated elongated cells showing various sizes and shapes in different leaflet areas (Figs. 8–10). The long cell axis tends to be parallel to the veins, which are dichotomously arranged. Each MC bears several lateral branches, which join similar branches extended by adjacent cells on the leaflet plane (Figs. 8 and 9). MCs join their upper and lower cells by branches shorter than the lateral ones. Thus, differentiated mesophyll resembles aerenchyma (Fig. 8).

Early MC morphogenesis

MCs in the meristematic region are relatively small and polyhedral. Their long axis is usually parallel to the veins. Their organization is typical of meristematic cells, except for the elevated dictyosome activity and the presence of an extensive endoplasmic reticulum (ER) system, a large part of which is smooth and tubular (Figs. 11 and 13). Dictyosomes consist of several cisternae and release numerous large vesicles (Fig. 13). They remain active throughout MC morphogenesis. Cortical Mts are randomly distributed along the walls, usually oriented transversely to the cell axis (Fig. 17 a). The cellulose microfibrils follow the same orientation (Fig. 18). At this as well as at later stages of differentiation, interphase MCs lack perinuclear and generally endoplasmic Mts (see Figs. 20 b, 26, and 28 d). This fact was also confirmed by EM observations (data not shown).

In this leaflet region electron dense intra-wall material is localized in the cell corners (Fig. 12 inset). A particular activity in the cytoplasm adjacent to these regions was not observed, except for some tubular ER elements lining the plasmalemma and images suggesting exocytosis of dictyosome vesicles (Fig. 12). Afterwards, the intra-wall material disintegrates and small ISs open in all cell corners (Fig. 12).

As MC differentiation commences cell divisions diminish, while cell size increases mainly by development of the vacuolar system (Fig. 5). At this stage ISs extend through cell junctions where usually four cells meet, forming IS canals traversing the mesophyll (Figs. 5, 6, 14, and 15). They extend laterally between every tetrad of adjacent walls (Fig. 6). Initiating canals always contain significant quantities of material positive to PAS staining and negative to Sudan black B (Fig. 14 and upper inset), which disintegrates as they broaden (Fig. 15). This material exhibits rather fibrillar texture, structurally resembling that contained in dictyosome vesicles (Fig. 14 lower inset). Examination of transverse sections reveals that MCs assume a more or less cylindrical form, still remaining laterally attached to each other at median wall regions (Figs. 5 and 6).

MC lobe formation

While IS canals open, the cortical Mts start to form bundles in most or all cell faces. Mts of forming bundles are focused in cell edges (Fig. 19). Some Mt bundles form complete rings (Fig. 20 a, b), while others line part of the cell surface. Some Mt bundles tend to be normal to the cell axis while others are diagonal. They seem by immunofluorescence to form an integrated reticulum (Fig. 20 a), although independent Mt rings may be detected. Mt bundles between neighbouring MCs tend to have an opposite disposition (Fig. 20 a, b; see also

Fig. 11. Median paradermal view of a MC in the meristematic leaflet region. The cell exhibits polyhedral shape. *N* Nucleus, *C* chloroplast, *V* vacuole. × 2,300

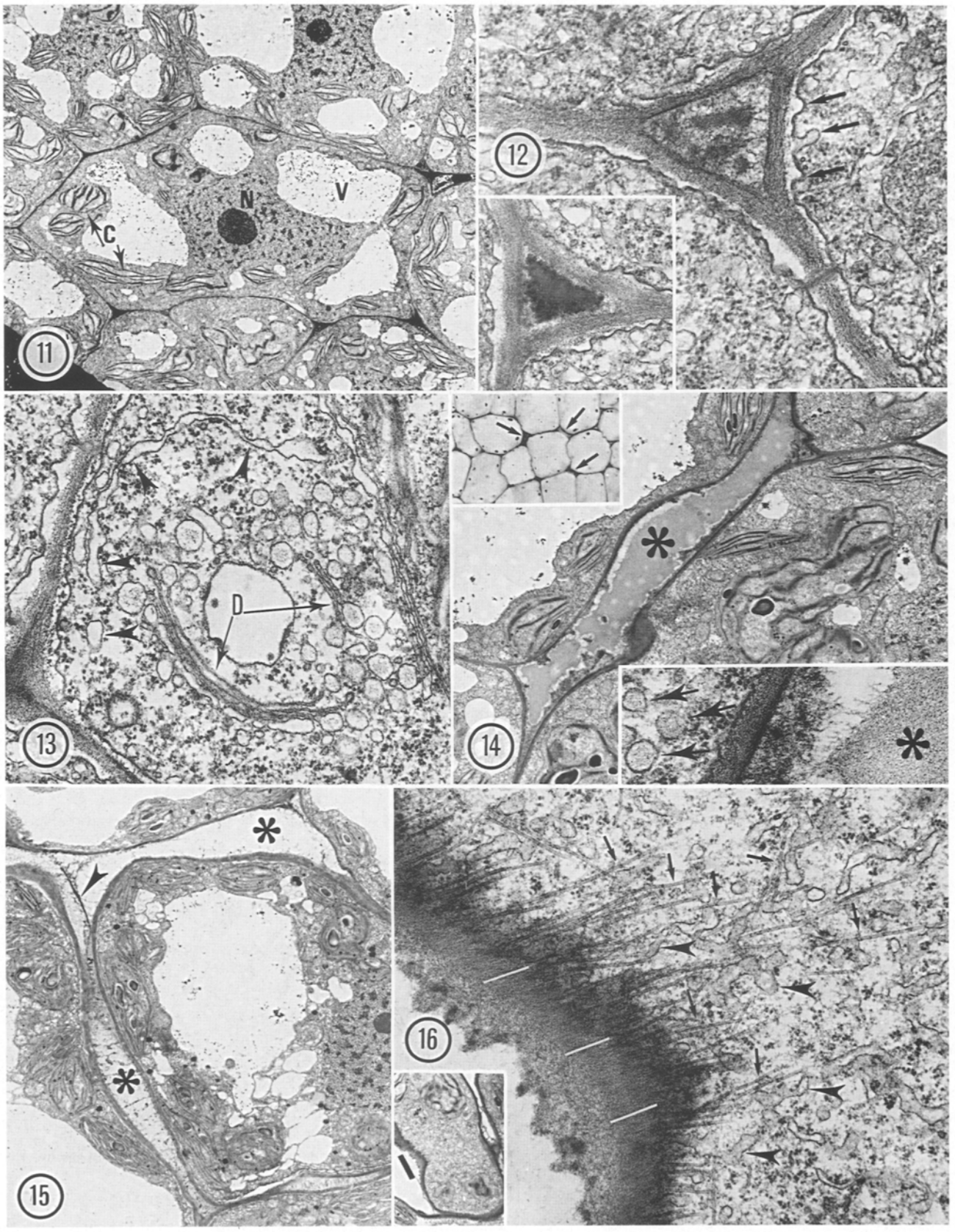
Fig. 12. Initiating IS. The intra-wall material is partially disintegrated. Note the humped regions (arrows) of the plasmalemma. × 24,000. **Inset** MC wall junction. Electron dense intra-wall material is localized in the initiating IS. × 11,000

Fig. 13. Active dictyosomes (*D*), dictyosome vesicles and portions of smooth ER (arrowheads) in a meristematic MC. × 34,500

Fig. 14. IS canal (asterisk) in a paradermal mesophyll section, containing large amounts of material. × 4,100. **Upper inset** Transverse leaflet section stained with PAS. Positively stained material is localized in IS canals (arrows). × 560. **Lower inset** Higher magnification, showing the texture of the IS canal material (asterisk) and its similarity with the dictyosome vesicle (arrows) content. × 45,000

Fig. 15. Paradermal section through IS canals (asterisks). Their content has disintegrated. The arrowhead points to remnants of the middle lamella. × 3,300

Fig. 16. Longitudinal section of a Mt bundle. × 29,000. **Inset** MC region, defined by the bar, from where Fig. 16 was taken. × 1,900. Cellulose microfibrils (white lines) are parallel to Mts (arrows). Tubular ER portions (arrowheads) lie in the proximity of Mts and the plasmalemma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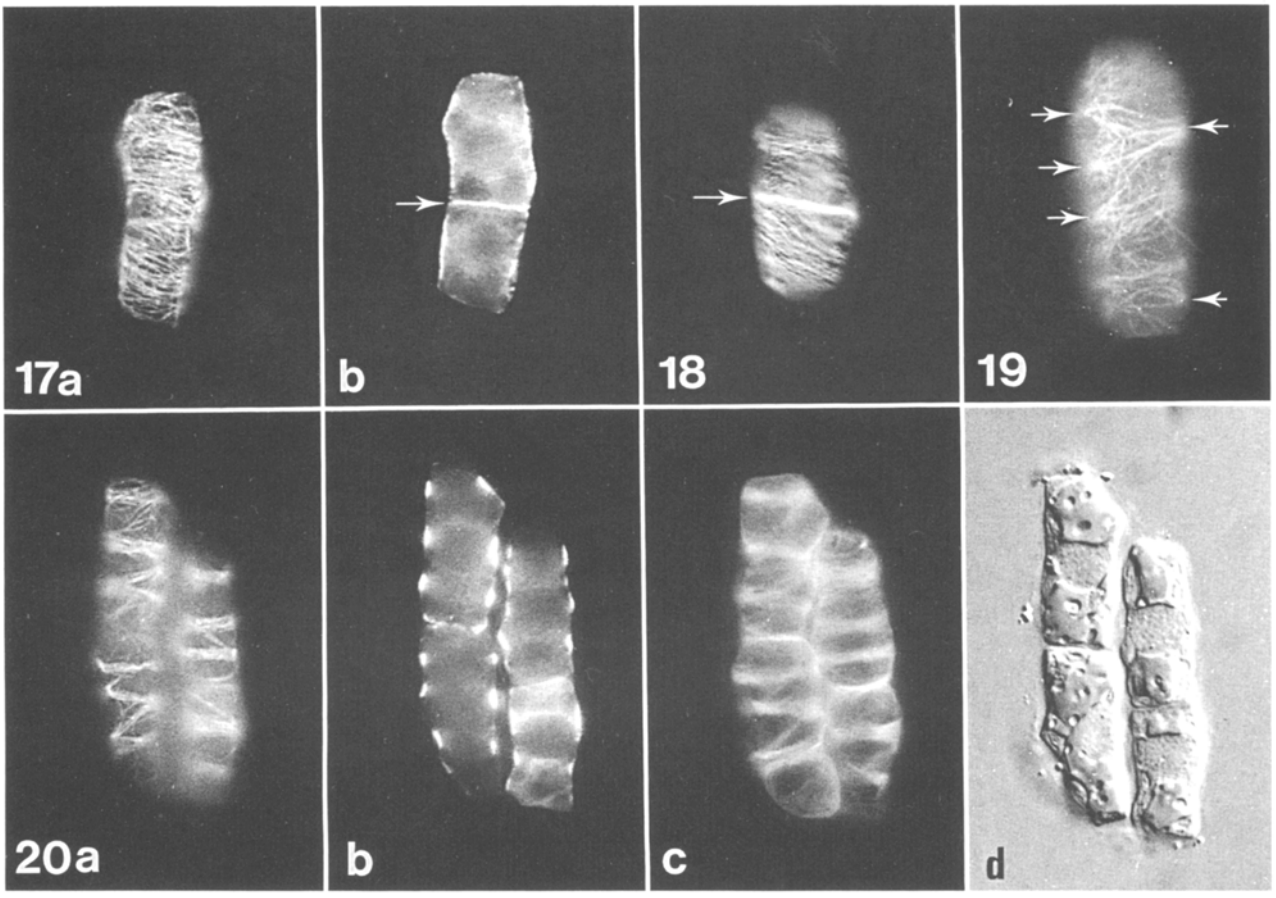


Fig. 17 a, b. Immunofluorescence micrographs of a pair of meristematic MCs at two focus levels. **a** Transverse cortical Mts line the surface of both cells. **b** The same cells in an inner focus plane. Arrow indicates their common wall. × 830

Fig. 18. Calcofluor staining of a pair of meristematic MCs. Cellulose microfibrils show same arrangement as the Mts in the cells of Fig. 17 a. Arrow points to their common wall. × 830

Fig. 19. MC organizing Mt bundles. Sites of Mt convergence (arrows) can be observed in cell edges. × 830

Fig. 20 a–d. A group of four MCs with organized Mt bundles. **a** Mt bundles at cell surface, either normal or diagonal to cell axis, appear interconnected. Few Mts exist between Mt bundles. **b** Same as in **a** but in inner focus plane. Mt bundles appear as fluorescence spots at the cell periphery. **c** Calcofluor staining reveals identity of cellulose microfibril bundle network with that of Mt bundles (compare with **a**). **d** Nomarski micrograph of these cells. × 830

Fig. 21. A pair of MCs displaying organized Mt bundles. The cell at the right exhibits Mts fanning out from the existing Mt bundles. It probably represents a stage of new Mt bundle organization (see text). Note the opposite disposition of Mt bundles with the cell at the left part of the figure, which exhibits a different pattern of Mt organization. × 690

Fig. 22 a, b. Immunofluorescence (**a**) and Calcofluor staining (**b**) of a lobed MC. Mt bundles are interconnected (**a**) and so are the wall thickenings (**b**). This cell is at a later morphogenetic stage than that of the cells depicted in Fig. 21. × 690

Fig. 23. Immunofluorescence image of a pair of MCs at a morphogenetic stage similar to that of Fig. 22. Mt bundles as well as new Mt interconnections display an opposite arrangement between them. × 690

Fig. 24. Calcofluor staining of a pair of MCs, at a morphogenetic stage similar to those shown in Fig. 23. The wall thickenings are oppositely arranged between them. × 690

Fig. 25 a, b. Immunofluorescence images of two external focus planes of a lobed MC, forming new Mt rings. Images of Mts converging on certain Mt bundle sites (arrows) can be observed. × 690

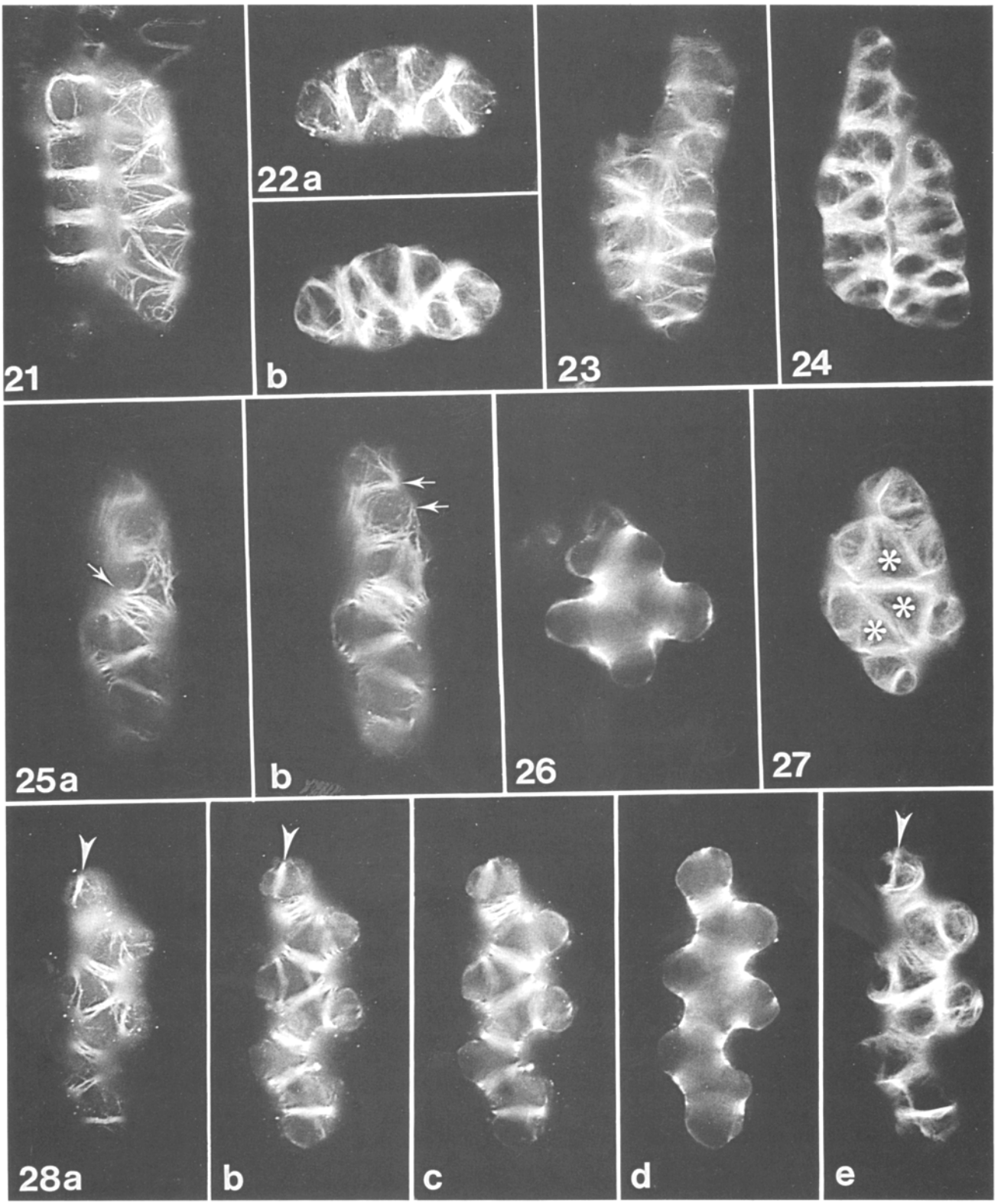
Fig. 26. Immunofluorescence image of an almost cross-shaped MC. Intense fluorescence at lateral lobe bases represents Mt rings at an inner focus plane. Endoplasmic Mts do not exist. × 690

Fig. 27. Calcofluor staining of a peculiarly lobed MC, showing the complexity of the wall thickening network. In certain sites meshes are formed (asterisks), which will give rise to short branches (see text). × 690

Fig. 28 a–e. Immunofluorescence (**a–d**) and Calcofluor (**e**) images of a more or less typically lobed MC with fully organized Mt bundle network, which appears continuous and integrated. Mt rings surround the bases of lateral lobes. Several lobes exist, which can be distinguished in different focus planes (**a–d**). The upper cell lobe displays a diagonal Mt bundle-wall thickening (arrowheads in **a, b,** and **e**). No endoplasmic Mts are present (**d**). The pattern of cell wall thickening (**e**) is identical to that of Mt bundles. × 690

Fig. 23). Few Mts exist along the wall areas which are free of Mt bundles (Fig. 20 a). During this process MC shape does not change detectably (Fig. 20 d).

Narrow local thickenings emerge at the wall regions adjacent to Mt bundles (Fig. 20 c). Tubular ER portions are juxtaposed with the plasmalemma adjacent to the rising thickenings (Fig. 16). Cellulose microfibrils in the thickenings are parallel to Mts (Figs. 16 and 29).



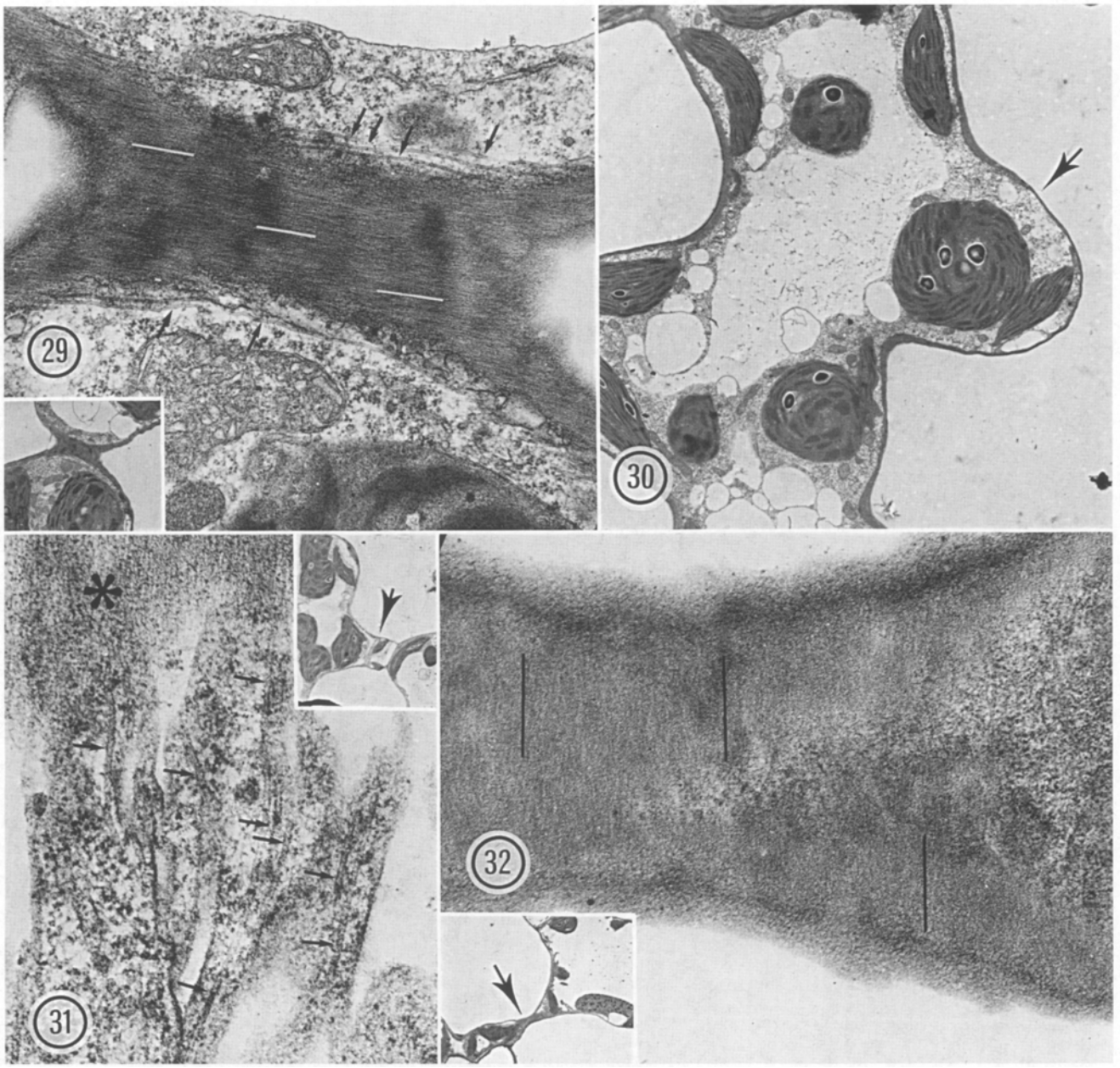


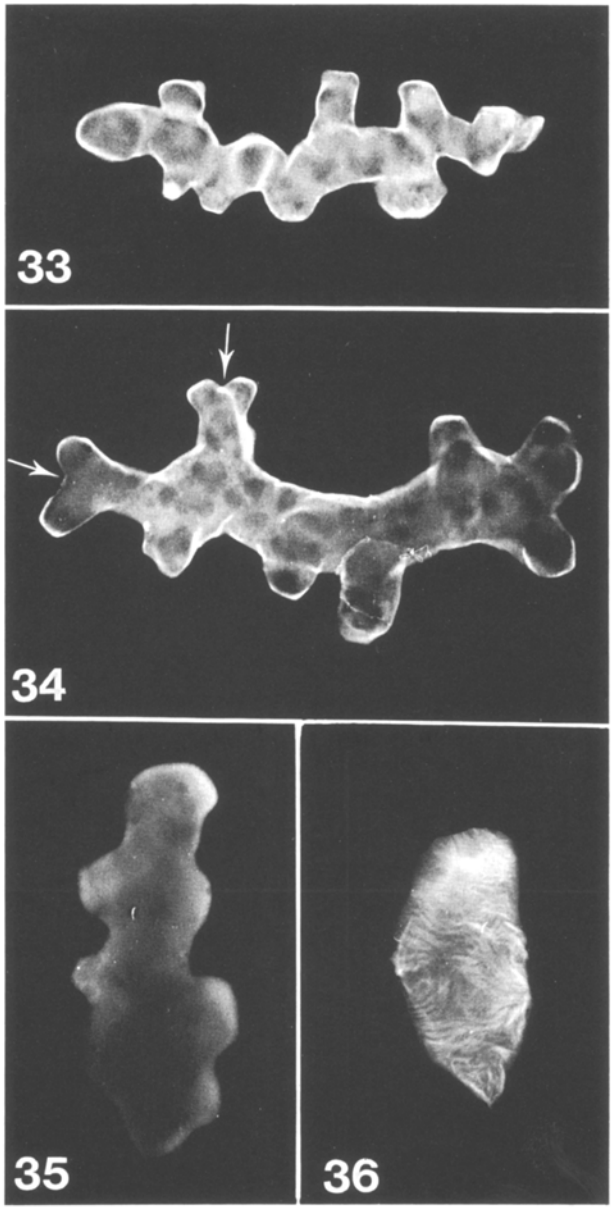
Fig. 29. Surface section of a wall thickening taken from a MC constriction (**inset**). Cellulose microfibrils (white lines) are parallel to Mts (arrows). × 24,000; inset, × 1,300

Fig. 30. Short lateral branch (arrow) of MC formed at a final morphogenetic stage. Cell wall is thinner at its tip. × 3,300

Fig. 31. Lateral branch region (arrow in **inset**). Cortical Mts (arrows) are normal to the branch axis. Cellulose microfibrils at the wall site marked by the asterisk are parallel to Mts. × 53,500; inset, × 1,100

Fig. 32. Surface section of a lateral MC branch cell wall (arrow in **inset**). Cellulose microfibrils are perpendicular to the branch axis. Black lines show microfibril orientation. × 34,500; inset, × 660

Calcofluor staining makes evident that the pattern of wall thickenings and of the constituent bundles of cellulose microfibrils copies with surprising accuracy that of Mt bundles (Fig. 20 c; compare with Fig. 20 a). Similarly to the latter, the local wall thickenings of neighbouring cells are opposite (Fig. 20 c; see also Fig. 24). MCs gradually assume a lobed form and separate further at the thickened wall regions, where isthmi appear (Figs. 7 and 29 inset). In this way, new ISs form (Fig. 7) by detachment of the walls at their thickened regions,



Figs. 33 and **34.** Calcofluor staining of two differentiated branched MCs. Note their irregular shape. In the cell depicted in Fig. 34, some forked-branches (arrows), probably arisen from one lobe each, are also observed. × 440

Fig. 35. Colchicine treated MC, showing no Mts at all by immunofluorescence. Cell lobes, formed before colchicine treatment, exist but no Mt bundles can be observed. × 690

Fig. 36. Calcofluor staining of a MC affected by colchicine. The wall thickening pattern appears disturbed. × 690

interconnecting with the initial IS canals. The newly-formed ISs contain material similar to that found in IS canals. In most cases two adjacent cells participate in every IS opening (Fig. 7). MC morphogenesis seems to begin in the intercostal mesophyll regions and to proceed laterally. At the end of this morphogenetic stage MCs have become highly vacuolated, elongated, and lobed, remaining attached at their lobes (Fig. 7).

Final MC morphogenesis

The lobed form obtained by MCs is temporary and cell morphogenesis continues. New cortical Mts appear to fan from the existing Mt bundles (Fig. 21). They extend to form new bundle interconnections and/or new Mt rings at the MC surface. Thus, a more complex reticulum of interconnecting and anastomosing Mt bundles is organized (Figs. 22 a and 23). Identically to Mts new wall thickenings are deposited (Figs. 22 b and 24). The arrangement of Mt bundles and wall thickenings between neighbouring cells remains opposite (Figs. 21, 23, and 24). Although complex, Mt organization complicates further with new formation of Mt rings at the base of cell lobes, especially those parallel to the leaflet plane (Figs. 26 and 28 a–c). Mts are observed to converge in sites of the existing Mt network, which still persists (Fig. 25 a, b). These rings tend to be parallel to the MC axis and appear generally connected with the pre-existing Mt bundles, forming an integrated Mt system (Fig. 28 a–c). Cortical Mts are absent from the dome of cell lobes (Fig. 28 b, c). Endoplasmic Mts were not observed (Figs. 26 and 28 d).

The cell wall is now reinforced by cellulose microfibrils deposited in directions determined by the Mt bundle system (Figs. 27 and 28 e). Simultaneous tubulin immunolabelling and calcofluor staining as well as EM observations reveal the existence of a system of wall thickenings-cellulose microfibrils identical to that of the Mt system (Fig. 28 e; compare with Fig. 28 a–c). This pattern of cellulose microfibrils allows the multiaxial growth of the cell. In particular, the cellulose microfibrils at the base of cell lobes parallel to the leaflet plane, in correlation to the rest microfibril system, lead to lateral growth of the lobes, which finally form lateral branches (Figs. 9, 10, 33, and 34). Sometimes, a lobe, in addition to its basal Mt ring, displays a Mt bundle or another Mt ring (Fig. 28 a, b). The deposition of a similar wall thickening (Fig. 28 e) results in the formation of a forked lateral branch (Fig. 34). EM observations show that the extending branches display transverse cortical Mts and similarly aligned cellulose microfibrils along their length (Figs. 31 and 32). Simultaneously, MCs elongate mainly at the regions of cell constrictions, where cellulose microfibrils still display transverse orientation.

During the same process, short branches emerge, not attached to neighbouring cells (Fig. 30). At their base

a Mt ring was not observed. They rise at sites where the bundles of Mts-cellulose microfibril network form meshes (Fig. 27 and 28 a–c, e). The wall at the top of the short branches is thinner than that at their base (Fig. 30).

Differentiated MCs encompass an enormous vacuolar system, the growth of which probably provides the forces bringing about the above changes in the cell shape. These events are accompanied by considerable broadening of the IS system, and mesophyll now resembles an aerenchymatic tissue (Figs. 8–10).

Morphogenesis of colchicine treated MCs

Detailed examination of thin sections and immunolabelled specimens shows that MCs affected by colchicine lack Mts (Fig. 35). In leaflets treated for 7 days all cell divisions are blocked. The inhibition of the periclinal divisions producing protodermal cells and MCs results in the formation of leaflets, the marginal region of which consists of two cell layers (Figs. 37 and 38). IS canals, containing electron dense-PAS-positive material, open between the junctions of their internal periclinal walls and extend very close to the marginal meristematic cells (Figs. 37–39). IS canals containing the same material open in all junctions of the young MCs (Fig. 40).

MC morphogenesis in the affected leaflets is inhibited (Figs. 41 and 43) and very few cells possess lobes, which have been formed before colchicine treatment (Fig. 42). These cells are mainly located in the central area of the mesophyll between the veins. Cells close to the veins are generally not lobed at all (Fig. 42). Calcofluor staining reveals an extensive disturbance in the pattern of the wall thickening-cellulose microfibril orientation in affected MCs (Fig. 36). The IS formation, which is coupled with MC morphogenesis, is also inhibited (Fig. 43). The existing ISs (Fig. 41) probably result from unpatterned IS canal broadening.

Discussion

Early MC morphogenesis

The present work describes the morphogenetic mechanism functioning during the development of the multibranched MCs of *A. capillus veneris* leaflets. MC morphogenesis is achieved in three distinct stages, during which the pattern of cell growth gradually shifts from axial to multi-axial. During the first stage the polyhedral MCs elongate to some extent and become cylindrical, detaching at their junctions and forming IS canals, which extend in the proximal inter-wall areas. Therefore, IS canal formation is involved in early MC morphogenesis. A similar phenomenon has not been observed in the young mesophyll of *Zea mays* (Apostolakos et al. 1991). IS canal opening is carried out in colchicine treated leaflets, a fact implying its independence of Mts. However, the assumption of a cylindrical form by young MCs is based on the existence of transverse cellulose microfibrils, which have been previously deposited in the presence of transverse cortical Mt arrays.

Regarding the mechanism of IS canal formation only hypotheses can be made. Enzymatic dissolution of middle lamella and/or some primary wall material probably occurs. The localization of PAS positive material is in

Figs. 37–43. Colchicine-treated MCs

Fig. 37. EM micrograph of the leaflet region shown in Fig. 38. Electron dense material has been accumulated in IS canals (arrows) formed between the two cell layers. × 1,800

Fig. 38. Light micrograph of the marginal region of an affected leaflet in a longitudinal section. The periclinal divisions are inhibited (compare with Fig. 4) and IS canals (arrows) containing PAS-positive material (Fig. 39) have opened. They extend up to the marginal meristematic cell (arrowhead). × 560

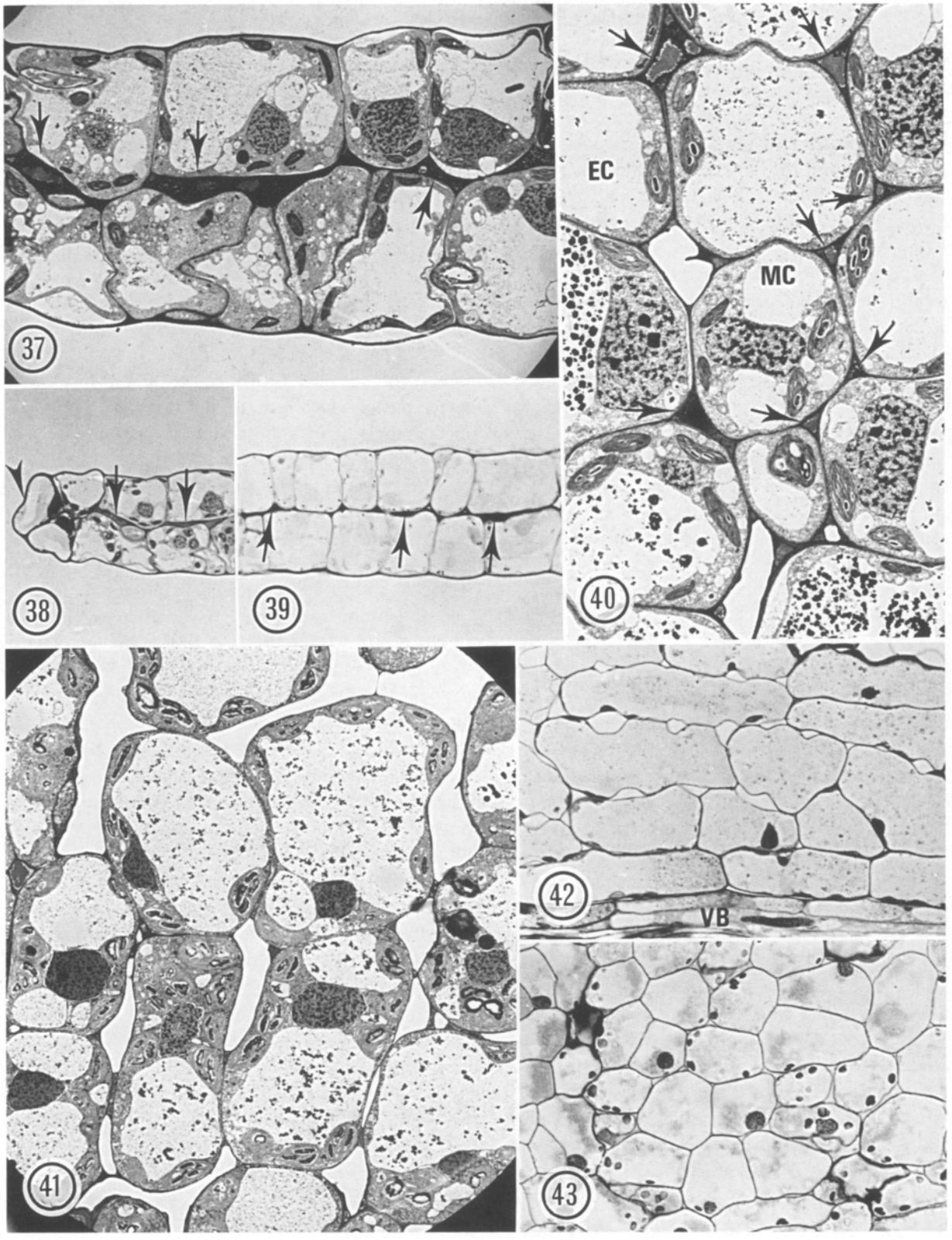
Fig. 39. PAS-staining of a section of the same leaflet area as in Figs. 37 and 38. The arrows indicate the PAS-positive material in IS canals. × 560

Fig. 40. Transverse section of an affected leaflet perpendicular to the veins. ISs at the corners of MCs have opened, some of them (arrows) containing large amounts of electron dense material. *EC* Epidermal cell, *MC* mesophyll cell. × 3,400

Fig. 41. Paradermal section of affected MCs. No lobes or constrictions exist. The ISs between them have been formed by an unpatterned broadening of IS canals. × 1,900

Fig. 42. Paradermal section of affected MCs. Their morphogenesis is inhibited and only minor lobes exist. They have been formed before treatment. *VB* Vascular bundle. × 560

Fig. 43. Paradermal section of MCs, affected at an earlier morphogenetic stage than those of Fig. 42. Morphogenesis is totally inhibited. × 560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accordance with this hypothesis. Signs of degradation have also been detected in initiating ISs in *Z. mays* mesophyll (Apostolakos et al. 1991) and in developing *Pilea cadierei* epithem (Galatis 1988). However, it cannot be deduced on a structural basis whether the content of IS canals is the product of middle lamella and/or primary wall lysis or it represents or contains some other material (see Roland 1978, Jeffree et al. 1986). On the other hand, elevated dictyosome activity in differentiating MCs suggests that the IS canal content could also be the product of directed exocytosis of polysaccharidic materials. Such a phenomenon might facilitate the detachment or loosening of the walls and might also be a response to "cell wounding" from enzymatic IS initiation. A structural feature quite important for initial IS opening is probably the tendency for opposite arrangement of walls in the meristematic leaflet region. This is the result of a definite cell lineage established by the activity of marginal meristematic cells and their derivatives. According to Bloch (1965), when four walls join at a point, the cells exhibit a greater tendency to pull apart at this point, and a larger volume of ISs may be formed than if the walls were alternate to each other.

MC lobe formation

This stage begins with a remarkable shift in the organization of cortical Mts, which form a network of bundles. Local wall thickenings displaying bundles of parallel cellulose microfibrils, rise externally to Mt bundles. Mt organization in *A. capillus veneris* MCs, although far more complicated, resembles that of *P. cadierei* epithem cells (Galatis 1988) and not that of *Z. mays* (Apostolakos et al. 1991) and *Triticum aestivum* (Jung and Wernicke 1990) mesophyll, where Mt annuli with less prominent interconnections are organized. Mt reticuli in both *P. cadierei* epithem cells and MCs of *A. capillus veneris* vary considerably between different cells but are in complete accordance with the emerging individual cell shape.

It is of particular importance that the pattern of Mt bundles is accurately imprinted on that of wall thickenings, the pattern of the latter being disturbed by colchicine treatment. Therefore, cortical Mts control wall differentiation in MCs of *A. capillus veneris* (see also Apostolakos et al. 1991). A similar observation has been made in "semi-lobed" cell types of the same plant (Panteris et al., in prep.). Cortical Mts attaining higher levels of organization are also implicated in cell wall differentiation-cell morphogenesis in some specialized cell types, i.e., guard cells (see Galatis 1980, Galatis and Mitrakos 1980, Palevitz 1982, Cho and Wick 1989, Cleary and Hardham 1989) and tracheary elements (see Hepler 1981, Roberts et al. 1985, Seagull 1989).

As has been discussed more extensively in previous articles (Galatis 1988, Apostolakos et al. 1991), the above network of cellulose microfibril bundles establishes particular expansion properties in MC wall. This wall reinforcement prevents increase in cell diameter at the thickenings but not at intervening wall areas, which bulge by forces generated by turgor. Thus, cell isthmi appear. This relatively simple but elegant mechanism is quite effective and results in cell "lobing". The inability of colchicine affected MCs, the local wall thickenings of which have been disturbed, to obtain a lobed shape supports the above conclusion. Our observations show that this mechanism is of widespread occurrence among higher plants.

The present observations demonstrate that IS canal development in *A. capillus veneris* leaflets is part but not the whole story of IS formation. During MC "lobing" cell walls detach at thickened regions and new ISs open in the same way as in *Z. mays* mesophyll (Apostolakos et al. 1991) and *P. cadierei* epithem (Galatis 1988). Development of *Z. mays* mesophyll and *P. cadierei* epithem in the presence of colchicine results in almost complete inhibition of IS formation, except for some small ISs at cell corners (Apostolakos et al. 1991). In *A. capillus veneris* mesophyll colchicine suppresses IS development to a lesser extent compared with the above plants. It inhibits wall detachment related to MC "lobing", and IS broadening accompanying the development of lateral branches.

All these facts support further the conclusion that cortical Mts play a significant role in IS development as a consequence of cell morphogenesis. Previous EM investigations on roots, hypocotyls, cotyledons, and mesophyll did not mention participation of Mts-wall thickenings in IS formation (Roland 1978, Kollöffel and Linssen 1984, Jeffree et al. 1986). The wall thickenings described by Kollöffel and Linssen (1984) at wall junctions in pea cotyledons, where ISs are initiated, differ from those described here.

MC branch formation

In contrast to *P. cadierei* epithem (Galatis 1988) and *T. aestivum* and *Z. mays* mesophyll (Jung and Wernicke 1990, Apostolakos et al. 1991), the "lobing" of MCs in *A. capillus veneris* is not the end of morphogenesis.

It is an intermediate stage necessary for its continuation. In the highly vacuolated lobed MCs new Mt rings are assembled at lobe bases, while the previous Mt system persists. In this course, the expanding Mt network forms several meshes. A network of cellulose microfibril bundles, copying the Mt pattern, allows the multi-axial growth of MCs and the transformation of the lobes into lateral branches. Besides, short branches rise at meshes formed by the cellulose microfibril reticulum. The sequence of events during final MC morphogenesis, i.e., establishment of new polarity axes, Mt reorganization and concomitant deposition of concentric cellulose microfibril arrays, is similar to that operating during establishment of new growth axes in individual cells and organs, as suggested by Green (1980) (see also Hardham et al. 1980, Hardham 1982, Selker 1990). Mt ring organization at the base of cell outgrowths has also been observed in some lower and higher plant cell types (among others see Kiermayer 1981, Galatis et al. 1986, Apostolakos and Galatis in prep.). However, the simultaneous existence of Mt rings parallel and transverse to cell axis in MCs has not been described in other higher plant cells.

MC branching keeps pace with an enormous IS broadening, the latter being the outcome of the former process. In this way the mesophyll becomes an aerenchymatic type of tissue.

Mt organization

During Mt bundling as well as during Mt reticulum expansion, the perinuclear cytoplasm, which has been suggested to initiate Mts (for reviews see Lloyd 1987, Seagull 1989, Palevitz 1991, Staiger and Lloyd 1991), lacks Mts. As it has been argued for other cortical Mt systems (for reviews see Gunning and Hardham 1982, Lloyd 1987, Seagull 1989, Palevitz 1991, Williamson 1991), in our material the initial Mt bundles form either from lateral displacement of pre-existent Mts and/or new assembly process. The common finding that MC edges in *A. capillus veneris* are sites of Mt convergence is worthy of attention. These sites may nucleate Mts or at least are involved in the spatial arrangement of existing or newly formed Mts. Similar observations from guard cells, root cells, and epidermal cells of *A. capillus veneris* (Galatis et al. 1983; Panteris et al. 1991, in prep.), and from root cells of *Azolla* (Gunning et al. 1978) show that cell edges may play a particular role in cortical Mt organization in ferns.

The finding that at the final MC morphogenetic step new Mt bundles are assembled while the previous ones exist, allows the conclusion that new Mt assembly takes place in the cell cortex (see also Schnepf and Deichgräber 1979; Hardham and Gunning 1979; Seagull 1983, 1986). Images which might represent intermediate stages of Mt bundle formation have also been observed: Unordered Mt bundles grow towards each other from two opposite sites lying at the cortical cytoplasm. These cortical sites, including the adjacent plasmalemma regions, are the most probable candidates for Mt initiation.

The opposite disposition of Mt bundles between adjacent MCs, suggests that the pattern of Mt organization is imprinted by one cell to another, or is induced by a common unknown intercellular factor. Positional Mt co-ordination between neighbouring cells is a frequent phenomenon (see Hardham et al. 1980, Hardham 1982, Selker 1990, Hush et al. 1990). The fact that in colchicine affected leaflets lobed cells, whenever present, are located in the middle of intercostal areas, connotes that the whole phenomenon probably begins in these areas and expands centrifugally. Perhaps some kind of substance(s), functioning as morphogen(s), diffusing from the veins reach(es) a critical concentration in the mid-distance between them. Alternatively, some mechanical factor, e.g., stresses applied by the growing tissue to individual cells, reach in the mid-intercostal region a critical intensity to switch on the whole mechanism (see also Williamson 1991). Whichever the factor(s) and induction mechanism could be, the sequence: Mt bundling – local wall thickening – cell "lobing" – branch formation, seems to start by activation of cortical sites initiating and/or spatially arranging Mts. In conclusion, the widely accepted view that the cell wall diminishes a plant cell's ability to change form is not correct. *A. capillus veneris* MCs provide a convincing example. During morphogenesis they change form continuously, attaining a very complicated one. By the same process, which is largely based on Mts, MCs control not only the wall form but also the formation and size of the IS continuum. However, many questions concerning the cellular and intercellular factors underlying the emergence of Mt cytoskeleton pattern remain to be elucidated.

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