Spatial limitations induce spindle tilting and result in oblique phragmoplasts in *Vicia faba* L. root tip cells, but do not result in oblique cell walls

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SUMMARY

Chromosome and phragmoplast positioning proceed differently during mitosis and cytokinesis in short and long root tip cells of *Vicia faba* L. and has been studied previously (Oud & Nanninga 1992, 1994). No correlations, however, were made with the microtubular cytoskeleton. Here this correlation is investigated using longitudinal sections of hydroxy-urea synchronized root tip cells. Microtubules were labelled with anti α-tubulin, chromosomes were stained with propidium iodide, and both were visualized using confocal scanning laser microscopy. Preprophase bands of microtubules were always positioned in the midplane of both short and long cells, and they were perpendicular to the length axis of the files of cells in the root. Also spindle formation started in a similar way in short and long cells, but from meta- to anaphase, the spindle axis in short cells increasingly tilted, due to spatial constraints. While chromosomes separated, the spindle axis acquired a position inclining to a diagonal of the cell, thus giving rise to the earlier observed oblique chromosome positions in anaphase plates. In short cells oblique phragmoplasts/cell plates expanded in oblique division planes. However, after karyokinesis, oblique cell plates rotated towards the transversal plane and the final site of wall connection was not eccentric, but at the site of the earlier preprophase band. We conclude that the suggestion that, in *Vicia faba* L., oblique walls are due to oblique anaphase plates (Oud & Nanninga 1992), has to be corrected. Even when chromosomal alignment is offset and

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Abbreviations: FITC fluorescein isothiocyanate; HU hydroxy-urea; MSB microtubule stabilizing buffer; Mt microtubule; PI propidium iodide; PPB preprophase band; CSLM confocal scanning laser microscopy.

oblique cell plates are formed in cramped cells, transverse preprophase bands still predict transverse division planes.

Key-words: cell division, cell plate orientation, cytoskeleton, microtubules, oblique spindles, phragmoplast, *Vicia faba* L.

INTRODUCTION

The positional control of the plane of cell division is one of the key factors in plant morphogenesis (Barlow & Carr 1984; Lintilhac 1984; Lloyd 1991). To exert this control, various physical factors such as pressure, size and shape of the mother cell and thermodynamical considerations of minimal surface area have been suggested (e.g. Thompson 1945). Later, it was understood that cells also play a more active role in achieving a specific cell plate orientation. In this respect, the role of the preprophase band (PPB) was investigated. At early stages of symmetrical cell division the PPB, a central ring of microtubules (Mts) in the cortical cytoplasm of the cell, is formed (Picket-Heaps & Northcote 1966; Gunning & Sammut 1990), replacing the interphase cytoskeletal array of Mts. In general the PPB, which is visible from G_2-phase to early prophase, marks the site at which the phragmoplast/cell plate complex meets the parental wall during cytokinesis (Gunning 1982; Gunning & Wick 1985; Palevitz 1986; Flanders *et al.* 1990; Mineyuki & Gunning 1990; Wick 1991a). In this manner PPBs determine the position of the new cell walls.

In various cells, the mitotic apparatus is often tilted or distorted (Wick 1991a; Oud & Nanninga 1992; Palevitz 1993). In guard mother cells of onion cotyledons most spindles are oblique (Palevitz & Hepler 1974; Palevitz 1986; Mineyuki, Marc & Palevitz 1988) and similar tilting of spindles is described for guard mother cells of other species (Cho & Wick 1989; Cleary & Hardham, 1989). Fusiform cambial initials (Bailey 1920) and rib meristem cells (Wada 1965) are other examples of aberrant spindle positions. Usually, however, the phragmoplast-cell plate position is corrected, and transversal division in the midplane (i.e. at the site of the original PPB) takes place. Nevertheless few exceptions exist, where the influence of the PPB is not absolute. For *Triticum* leaf epidermal cells Galatis, Apostolakis & Katsaros *et al.* (1984a) described a positional inconsistency between PPB Mts and the final cell plate position during triangular subsidiary cell and atypical hair cell formation. Here strong morphogenetic factors overrule the influence of the PPB in determining the position of the cell wall. Absence of PPBs as in some *Arabidopsis* mutants results in absence of cell files in roots (Traas *et al.* 1995).

Root cortex cells of *Vicia faba* show many oblique divisions. For these cells Oud & Nanninga (1992, 1994) described a progressive increase in the tilting of the spindle axis by measuring the obliqueness of chromosome orientations and daughter nuclei from prophase to telophase. They concluded that in small cells a tilted spindle axis causes an oblique cell wall. They did not, however, correlate these data with data on cytoskeletal changes. We have reinvestigated the phenomenon of tilted spindle axes in *Vicia faba* in relation to cell size, using a combined approach of both chromosomal and cytoskeletal visualization. Special attention was paid to the location of the PPB and the microtubular arrays during spindle tilting in relation to the cell length. Although oblique spindles and cell plates were often observed in dividing *short* cells, *Vicia* roots are characterized by

regular files of cells with transversal cell walls. We therefore further questioned how cytokinesis proceeded in cells with oblique spindles and focussed on the site of cell plate fusion.

We show that preprophase bands in short cells do not anticipate to oncoming spatial limitations of the spindle and are always perpendicular to the cell file axis and in the midplane of the cell. From our observations we must conclude that the previous suggestion, that oblique cell walls in *Vicia faba* result from oblique phragmoplasts (Oud & Nanninga, 1992), must be reconsidered. Obviously, spindle tilting affects phragmoplast orientation to a great extent, but essentially does not affect the orientation of the final cell wall.

MATERIALS AND METHODS

Plant material and conditioning of the cell cycle

Commercially obtained seeds of the broad bean (*Vicia faba* L.) were immersed in tap water for 1 day and germinated on wet filter paper within 2 days at 23°C in a humid atmosphere. Seedlings were cultured on aerated 0·5 × Hoagland solution (Gamborg & Wetter 1975) for 1–3 days. When the primary roots were 2–4 cm long, they were incubated in aerated 0·5 × Hoagland medium, containing 2·5 mM hydroxy-urea (HU), a drug which blocks the cell cycle at the transition of G_1 to S-phase (Doležel *et al.* 1992). After 12–14 h the roots were washed for a few minutes in a flow of tap water to remove HU, and the plantlets were cultured further on HU-free medium at 23°C. Using this treatment, the mitotic index increased from 15% cells in mitosis (without HU) to maximally 90% of cells in mitosis (with HU). At 12, 14, 16 and 18 h after the release of the block, some root tips were squashed and stained with acetocarmin to determine the percentage of cells in mitosis and the phase of mitosis. When many mitotic stages were observed, similar root tips were processed for immunolabelling.

HU-treated roots, subcultured for 12 h after HU removal, did not exhibit cell divisions, indicating both the blocking efficiency of HU and also the necessity of a chase longer than 12 h for the transition from G_1-S phase to mitosis. When root tips were sampled at 14 or 16 h after the removal of the drug many metaphase configurations were found and, when sampled at 18 h after HU treatment, telophase and early interphase configurations were most common. Under optimal growth conditions a mitotic index of 90% was achieved after HU treatment. The absence of aeration, and growth temperatures below 18°C during the pulse and chase of HU, lowered the mitotic index to less than 10%. Microtubular configurations did not differ in HU treated cells, when compared to non-treated cells. Also cell shapes and cell dimensions were comparable, and HU treated plantlets developed normal roots and phenotypes.

Fixation and embedding

Root tips (0·5 cm) were harvested for immunolabelling at 14 h and 16 h after the release of HU and immediately fixed for 1 h at room temperature in 4% (w/v) paraformaldehyde and 0·01% (v/v) glutaraldehyde in microtubule stabilizing buffer (MSB: 100 mM PIPES, pH 6·9, 5 mM EGTA, 5 mM $MgSO_4$) containing 0·1% (v/v) purified Triton X-100 (Surfact-Amps X-100, Pierce). Thick primary roots were incised longitudinally to improve the penetration of the fixative into the stele and cortex. Root tips were embedded in polyethylene glycol (PEG) according to Van Lammeren (1988),

but 100% PEG 1500 was used instead of a 3:2 mixture of PEG 1500 : PEG 4000, this to enable sections of 10 to 30 μm thickness. For cryosectioning, root tips were infused stepwise with 10% (30 min), 15% (30 min) and 20% (overnight) sucrose (w/w) in 50 mM phosphate buffer, pH 7·2, quickly frozen in liquid nitrogen and mounted in Tissue-Tek (Agar Scientific Ltd, Essex, GB). For optimal root tissue morphology and to study the site of cell plate connection, root tips were fixed in 3% paraformaldehyde with 2% glutaraldehyde in MSB. They were dehydrated, infused and polymerized in Technovit 7100 according to the manufacturer's procedure. Sections of 2 μm were stained with 1% toluidine blue in water.

Sectioning and immunolabelling

Longitudinal sections (15–20 μm thickness) were made of PEG embedded root tips using a Microm HM 340 rotary microtome. Frozen longitudinal sections (20 μm thickness) were prepared using a Microm HM500 OM cryomicrotome at −18°C. Sections were affixed to slides that had been coated with 2% (v/v) organosilane. Cell walls were digested with 1% (w/v) hemicellulase (Sigma H2125) for 10 min, and subsequently extracted in a mixture of 1% (v/v) purified Triton X-100 (Surfact-Amps X-100, Pierce), 2 mM EGTA, 0·2 mM phenylmethylsulphonyl fluoride (PMSF) in MSB for 10 min. The sections were blocked with 1% (w/v) bovine serum albumin (BSA, Fraction V, Serva, Heidelberg, FRG) for 10 min, followed by incubation with 0·1% (w/v) acetylated BSA (BSA-c, Aurion, Wageningen, NL) for another 10 min. Indirect immunolabelling of Mts was done using a monoclonal mouse-IgG anti-α-tubulin (clone DM1a, Sigma) and goat anti-mouse-IgG-Bodipy®-FL (Molecular Probes, Oregon) or GaM-FITC (Sigma). Each antibody was diluted 1/300 in PBS containing 0·1% (w/v) BSA-c, and incubations were performed at room temperature for 2 h. After the first antibody incubation, extensive washings were done with 0·1% BSA-c in PBS (six times 5 min) and after the secondary antibody, washings were done in PBS (six times 5 min). DNA was counterstained by incubating the slides for 7 min in freshly prepared 0·3 μg/ml propidium iodide (PI) in 0·05 M phosphate buffer, pH 7·8. To reduce fading of both fluorochromes, sections were mounted in Citifluor in glycerol (Citifluor Ltd., London) and slides were stored in the dark at 4°C.

Microscopy and image analysis

Fluorescent Mts and chromosomes or nuclei were visualized with a Nikon Microphot epifluorescence microscope at various mitotic stages and at the end of cytokinesis. The fluorochromes FITC and Bodipy-FL 503/512 were visualized using excitation filter 450–490 nm, dichroic mirror DM 510 and barrier filter BP 520–560 nm. Propidium iodide images were obtained with excitation filter 510–560, dichroic mirror DM 580 and barrier filter 590 nm. Further analysis was done with a Bio-Rad MRC 600 Confocal Scanning Laser Microscope (Bio-Rad, Hertfordshire, UK) equipped with an argon–krypton laser on a Nikon Labophot inverted microscope. Cytoskeletons and chromosomes were recorded by dual channel imaging and Kalman filtering.

   Confocal BioRad PIC files (378*512, 8 bits) were converted to TIFF format using Confocal Assistant software (BioRad). Light micrographs imaged with a Panasonic wv-E550 3-CCD camera were digitized in TIFF format using a 756*536 (24 bits) Prysm framegrabber (Synoptics Ltd, Cambridge, UK). Files were contrast-enhanced in Adobe Photoshop and printed using a Kodak XLS 8600 dye sublimation printer.

Statistics

In five separate experiments roots were harvested at high mitotic index and for each mitotic stage 10 primary roots were sectioned. Over 30 000 cells were labelled for Mts. More than 6000 cells were properly longitudinally sectioned and labelled well. For each mitotic stage more than 300 cells were studied with the CSLM and a selection of 20–40 *short* and *long* cells were recorded.

RESULTS

The results described here are based on the simultaneous staining of chromosomes with propidium iodide (PI) and immunolabelling of microtubules (Mts). Irrespective of cell length, interphase cells in the root meristem showed transverse cortical Mts. At preprophase, these cortical Mts gradually disappeared alongside the upper and lower parts of the longitudinal walls, and a preprophase band (PPB) appeared. Two rings of PPB Mts were sometimes seen temporarily (arrowheads in Fig. 1e). PPBs progressively narrowed and were always positioned in the midplane of the cell, almost transverse to the length axis of the root (Fig. 1a,e). The increase of the mitotic index with hydroxy-urea allowed observing Mt configurations during mitosis in many cells. From each mitotic phase at least 300 cells were observed after labelling. Special attention was given to the position of the PPB in *short* cells. Of all PPBs observed 95% deviated less than 10° from the midplane. The position of PPBs could deviate up to 15° from the midplane, corresponding with a distance of 2–3 μm from the mid-transversal plane. These slight aberrations occurred in less than 5% of all cells observed and correlated with avoidance of four-way junctions (Flanders *et al.* 1990).

During PPB formation, chromatin condensed and individual chromosomes could be discerned (Fig. 1a′,e′). When the length or the width of a cell was a limiting factor, nuclei were not spherical but oval shaped (e.g. Fig. 1e′). Many Mts were formed at the surface of the nuclei, especially at the poles, and some of them ran towards the PPB (pole-to-PPB Mts, Fig. 1a,f, arrows). At prometaphase, PPBs and pole-to-PPB Mts had disappeared, but arrays of Mts emanating from the poles of the nucleus now ran towards the opposite pole and a dense network of fine Mts encaging the condensing chromosomes was formed (Fig. 1b). When the pole-to-PPB Mts had disappeared, the spindle axis was slightly tilted in those cells where the spindle poles were in the vicinity of the transversal walls (not shown). At metaphase these cells exhibited oblique spindles (Fig. 1g). The shorter the cell, the more the spindle axis tilted. Simultaneously, the metaphase plane with chromosome centromeres tilted and remained perpendicular to the spindle axis (Fig. 1g′). Chromosome arms were never inside the oval-shaped spindle, leaving a free central area. Instead chromosomes extended from and bent around the spindle in *short* and *flat* cells (Fig. 1g′). However, they ran parallel to the length axis of the cell in *long* and *slender* cells (not shown).

General phenomena observed during anaphase were the increase of the pole-to-pole distance, the persistence of the pole-to-pole Mts, the shortening of the centromere Mts and the simultaneous separation and movement of the chromatids to the spindle poles. Most anaphase spindles in *long* cells remained parallel to the length axis of the cell file (Fig. 1c). *Short* cells mostly exhibited oblique anaphase spindles, varying up to a fully diagonal position (Fig. 1h). Occasionally, oblique spindles twisted or bended, to gain
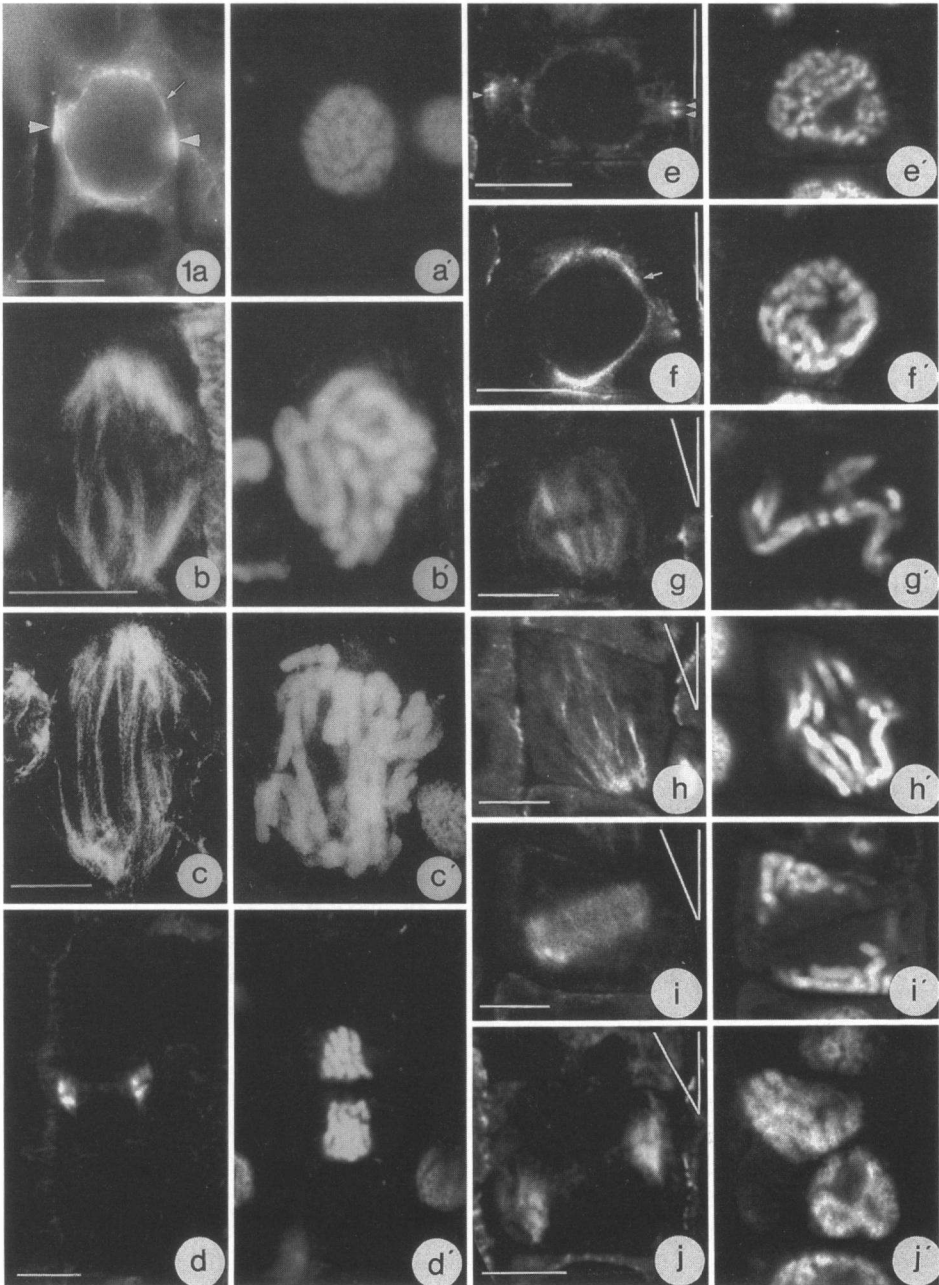
length. In *long* cells spindle tilting was observed incidentally, but only when the presence of large vacuoles at both sides of the spindle restricted the cytoplasmic space (not shown).

At telophase separated chromatids condensed, and a phragmoplast was formed. A cylinder of dense Mts appeared, which expanded laterally in the midplane, meanwhile depositing a cell plate perpendicular to the former spindle axis. Thus in *long* cells, the growing cell plate was mostly transversal and centrally positioned (Fig. 1d). Cytokinesis completed by fusion of the cell plate with the parental wall, forming two daughter cells stacked in the file. In *short* cells with oblique spindles, the phragmoplast also developed in the plane perpendicular to the spindle axis (Fig. 1i), giving rise to an oblique cell plate. When an oblique cell plate approached the lateral parental wall, phragmoplast-Mts aligned parallel to that cell wall, and cell plate edges buckled towards it (Fig. 1j), causing the cell plate to become sigmoidal. To follow the curvature and positioning of such cell plates during cytokinesis we not only used labelled sections, but also toluidine blue stained Technovit sections (Fig. 2). An oblique cell plate (Fig. 2a, upper cell) became more curved at its edges (Fig. 2b, upper cell) or developed a sigmoidal shape (Fig. 2c) as it expanded. Such sigmoidal cell plates, however, did not fuse eccentrically with the lateral wall. In longitudinal Technovit sections 60 *short* cells were examined at late telophase, all exhibiting oblique cell plates with curved edges. Instead, prior to wall fusion, the flexible cell plate oriented from oblique towards the midplane of the cell (Fig. 2a, lower cell), stretched and fused perpendicularly with the lateral wall (Fig. 2b, lower cell), even in *short* cells of only 15 µm heights. Figure 3 shows a schematic summary of the Mt arrays during mitosis in *long* and *short* cells of the root cortex. The correction of a tilted phragmoplast towards the midplane, as in *short* cells prior to cytokinesis, is represented in Fig. 3g′–i′.

DISCUSSION

Increasing tilting of the plane of chromosomes coincided with an increasing spindle tilting in successive stages of mitosis. We questioned if the *short* cells anticipated

Fig. 1. Dual channel confocal images of fluorescent microtubules (Mts) (a–j) and DNA (1a′–j′) at successive stages of mitosis in root tip cells of *Vicia faba*; aa′–dd′ long cells; ee′–jj′ short cells. Pictures are mounted in the same orientation as cells were positioned in the cell files. Bars represent 10 µm for all panels. (a) Preprophase in long cell with PPB Mts (arrowheads) and Mt formation at the nuclear poles. Arrow points to pole-to-PPB Mts. Chromosomes condense (a′). (b) Late prophase with Mts extending from the poles and surrounding the condensed chromosomes (b′). (c) Anaphase with pole-to-pole Mts and dense Mts from centromeres to pole. While centromeres are near a pole, chromosome arms still reach to the middle plane (c′). (d) Late telophase showing phragmoplast, perpendicular to the length axis of the cell. Note that the daughter nuclei are positioned in line with the long axis of the cell (d′). (e) Prophase in short cell showing spindle Mts radiating from the poles, some connecting with the PPB (arrow). Arrowheads point to the two rings of the PPB. The poles are in line with the cell file. Condensed chromosomes position at the nuclear periphery (e′). (f) Prophase in short cell showing Mts radiating from two poles on the nucleus. Pole-to-PPB Mts are indicated by an arrow. (g) Metaphase with centromere Mts and pole-to-pole Mts. Note the slightly oblique position of the spindle and chromosomes (g′). Centromeres are in the middle plane and chromosome arms extend from here and surround the spindle. (h) Anaphase with tilted spindle in diagonal position and tilted chromosome (h′). The pole at the lower right-hand side is in focus, whereas the pole at the upper left hand side is not, due to tilting. (i) Early telophase showing an oblique phragmoplast/cell plate and daughter chromosomes in diagonal, opposite corners of the cell (i′). Arrows indicate the position of the cell plate. (j) Late telophase with an oblique phragmoplast/cell plate marked by phragmoplast Mts at its periphery. Newly formed daughter nuclei are still in oblique position (j′).
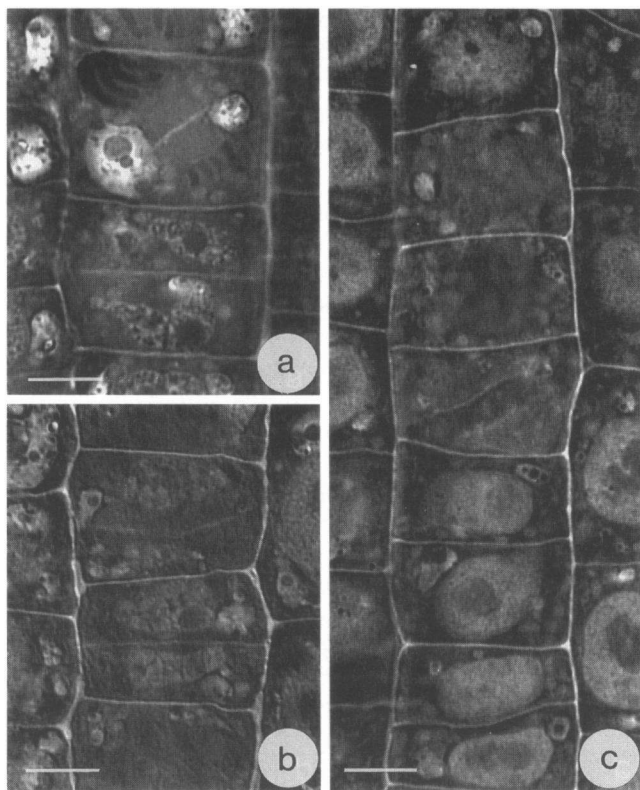
Fig. 2. Selection of light micrographs of longitudinal sections of Technovit embedded root tips of *Vicia faba*, showing short cells stained with toluidine blue. Bars represent 10 µm. (a) Cell file showing one cell at telophase with an oblique cell plate bordered by vacuoles. Below, a pair of daughter cells separated by a cell plate now in transversal position. (b) Two pairs of cells just after division. The upper pair shows a bent cell plate not yet attached to the parental wall. The lower pair exhibits the final stage of cytokinesis with a straight transversal cell plate. (c) File of cells within central position a cell during cytokinesis showing a curved cell plate extending towards the lateral walls. The pair of cells lower in the file exhibits a slightly tilted and curved cell wall.

oncoming spacial limitations by forming oblique or curved PPBs. However, PPBs were positioned close to the transversal midplane both in *long* and in *short* cells and, consequently, spindle initiation always started perpendicular to the PPB. During prophase, Mts radiated from the surface of the nucleus into the cytoplasm, mainly from the nuclear poles. Pole-to-PPB Mts are expected to function in positioning the nucleus in the plane marked by the PPB, and in fixing the spindle pole positions (Mineyuki, Marc & Palevitz *et al.* 1991). Indeed, as long as this anchorage existed, spindle poles remained in position. At prometaphase the PPB and the pole-to-PPB Mts disappeared, and the spindle was formed. As soon as the spindle elongated and its poles pushed against the transversal walls, spatial limitations occurred, spindle poles glided towards diagonal cell corners and chromosome tilting occurred. Therefore, we conclude that chromosome tilting is caused by spindle tilting, which in its turn is caused by spatial limitations. Similarly, Mineyuki *et al.* (1988) suggested spatial limitation of the spindle to explain tilting in dividing guard mother cells of *Allium*. The morphological plasticity of the mitotic apparatus is evident (Palevitz 1993).
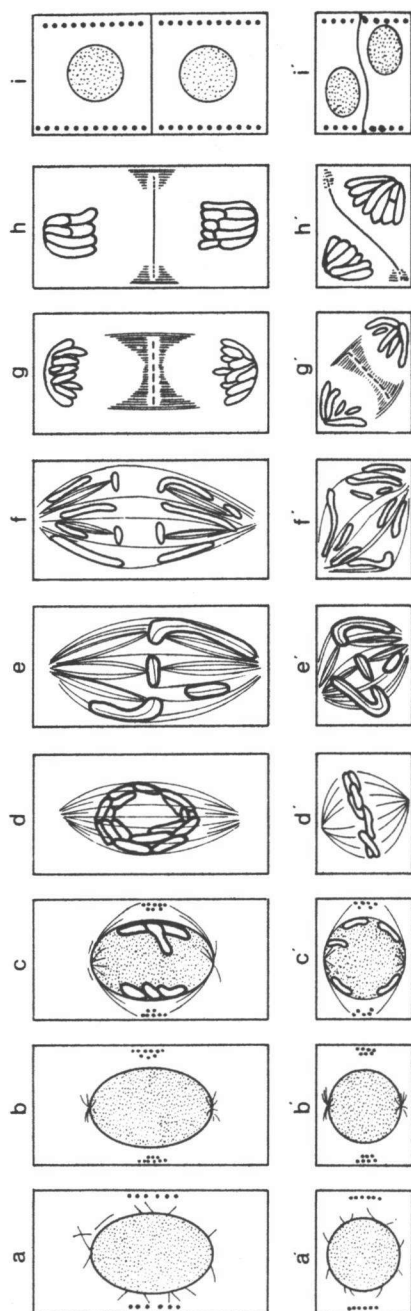
Fig. 3. Schematic representation of Mts and chromosome positions in long (a–i) and short (a'–i') cells during mitosis. (a,a') Early prophase with PPB formation and Mts radiating from nucleus. (b,b') Mid prophase with established PPB and Mts radiating from nuclear poles, which are in line with the cell file. (c,c') Late prophase with pole-to-PPB Mts. (d,d') Prometaphase pole-to-PPB Mts disappeared, pole-to-pole and pole-to-centromere Mts were formed, and spindle tilted in short cell and chromosomes were at the periphery of the nucleus (d'). (e,e') Metaphase with centromeres in equatorial plane and chromosome arms extending towards the poles (e) or lateral sides (e'). (f,f') Early anaphase with centromeres close to the equatorial plane and chromosome arms pushed towards the poles (f) or spindles in diagonal position (f'). (g,g') Early telophase with phragmoplast extension and cell plate formation in the former equatorial plane (g,g') and in diagonal position (g'). (h,h') Late telophase with a transversal cell plate (h) or a sigmoidal cell plate (h'). (i,i') Completed cytokinesis with a transversal cell wall both in long (i) and in short cells (i'). In short cells after division a slight curvature of the young cell wall can sometimes still be seen.

Wada (1965) has described an increasing chromosome tilting in *Triticum* when the chromosome number enlarged from diploid to hexaploid varieties. In general, in species with relatively large chromosomes, such as in *Allium cepa, Crepis capillaris* and *Vicia faba*, tilting of the plane of chromosomes is frequently observed (Oud & Nanninga 1994). These data indicate that both the number and the size of chromosomes can contribute to lack of space and increase of steric hindrance. Large *Vicia faba* chromatids extended from the spindle (Fig. 1g′), as was also observed in *Allium* by Palevitz (1986) and in the alga *Oedogonium* by Schibler & Pickett-Heaps (1987). The position of the chromatid arms in *Vicia* was laterally extended in wide cells and longitudinal in long-and-slender cells, demonstrating a direct influence of space limitation on chromosome positioning (see also Oud, Nickless & Rowland 1995). The Mts connected to chromosomal arms might influence their movement during mitosis, as observed by McIntosh & Pfarr (1991) in animal cells.

Tilting of the equatorial plane is correlated with cell size and increases from metaphase to telophase. In 85% of all telophases, equatorial planes deviated more than 5° from the midplane and in 70% equatorial planes deviated over 20° from the midplane (Oud & Nanninga, 1992). Although we observed such oblique phragmoplasts in more than 300 cells, we never saw the fusion of cell plates with parental walls in oblique position. Even in very *short* cells (length<20 µm), the edge of the cell plate fused with the parental wall in the middle of the cell (Fig. 2b). Cell plate fusion was preceded by cell plate bending. The stretching of the sigmoidal cell plates, prior to fusion, is reminiscent to the flattening and stiffening observed in cell plates of dividing *Tradescantia* stamen hair cells (Cleary *et al.* 1992). Apparently, there is an interaction of the growing cell plate edge with the zone of the prior PPB (Galatis *et al.* 1984b). The correction of oblique cell plates towards the transversal plane, explains the ordered pattern of cells in a file, present in the root cortex of *Vicia faba*. Oud & Nanninga (1992) observed oblique walls and hypothesized that they were the outcome of oblique divisions. When we sectioned longitudinally through the middle of files with many *short* cells, all pairs of daughter cells were separated by transversal walls varying less than 10° from the perpendicular plane; oblique walls were occasionally observed in sections. Since files are roofing over each other in the root cortex, the percentage of oblique walls quickly rose when the root bent or when sections were not parallel to the longitudinal axis of the root. We therefore hereby adjust the model for oblique cell division that was hypothesized by Oud & Nanninga (1992).

Since we have found both PPBs and newly formed walls in transversal position, the position of the PPB has probably determined the division plane. This concept is generally accepted (Wick 1991a,b). We did not study the actin cytoskeleton, but it is known that microfilaments play a role in cell division (Kakimoto & Shibaoka 1987; Lloyd & Traas 1988; Cleary *et al.* 1992; Liu & Palevitz 1992; Mineyuki & Palevitz 1990; Staehelin & Hepler 1996). The role of actin and its associated proteins in the controlled positioning and fusion of the cell plate remains to be elucidated.

ACKNOWLEDGEMENTS

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