Specific patterns of cortical and endoplasmic microtubules associated with cell growth and tissue differentiation in roots of maize (Zea mays L.)

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Summary

Indirect immunofluorescence using α-tubulin antibodies applied to sections of maize roots prepared using low melting point wax was found to give excellent visualisation of both cortical and endoplasmic microtubule (MT) arrays. This allows, for the first time, these arrays to be investigated in cells of the different tissues at various stages of their growth and development. Many cells in the zone between the meristem and the cell elongation region develop a highly ordered transversal bundling of cortical MTs, which we suggest is related to vacuolation of the cytoplasm and which may also be indispensable for the ensuing rapid cell elongation. On the other hand, there are subtle differences between the cells of the individual tissues regarding the arrangement of their cortical MTs in this zone. The possible physiological significance of these tissue-specific MT arrays is discussed. Endoplasmic MTs were seen to encircle and to connect the nucleus with the cortical MT arrays in both dividing and elongating cells. Even the G1 phase nuclei of the slowly dividing cells of the quiescent centre were encircled by endoplasmic MTs. The continuity of the two MT systems may provide the cell with an important signalling system whereby mechanical and physiological information is relayed from the exterior of the cell to the nucleus.

Key words: cell growth, cytoskeleton, differentiation, microtubules, roots, Zea mays.

Introduction

Most of our knowledge concerning the spatial and temporal organization of microtubular arrays in plant cells has been obtained from electron microscopy (Gunning et al. 1978; Hardham and Gunning, 1979) as well as by indirect immunofluorescence light microscopy of isolated plant cells (Lloyd et al. 1980; Wick et al. 1981; Simmonds et al. 1983). The principal conclusion reached from these studies is that microtubules (MTs) represent an integrated and dynamic intracellular system which can alter its arrangement in response to environmental and developmental signals.

The changing appearance of the MT cytoskeleton during the development and differentiation of tissues is difficult to follow using the electron microscope. Similar restrictions apply to immunological studies made on cells dissociated from tissues by maceration. To overcome the limitations of both these methods, other immunofluorescence microscopic techniques have been elaborated to visualise developmental changes in the MT cytoskeleton. For example, Roberts et al. (1985) analyzed MT arrays in peels of epidermal tissue of pea stems and, more recently, Flanders et al. (1990a) used strips of stem tissue excised from internodes of Datura stramonium. After staining by indirect immunofluorescence and viewing with a confocal scanning laser microscope, the latter authors were able to observe MT arrays that could not have otherwise been recognized. Marc and Hackett (1989) introduced yet another method enabling immunofluorescence observations to be made of intact cell layers at the surface of a shoot apex in their original three-dimensional state by digesting away all unwanted cell layers. All these methods, however, are limited to studying MTs of only the external tissues of plant organs, since the internal tissues are necessarily sacrificed.

In order to visualize the complex of MT networks as it exists within the various tissues of an organ, immunofluorescence of sectioned material is unavoidable. Although the MT arrays of adjacent cell layers, or even arrays within different parts of the cell, can hinder the interpretation of microscopic images (Cho and Wick, 1989), use of the recently developed confocal microscope can mitigate this to some extent (e.g. see Flanders et al. 1990a; Lloyd et al. 1992). Interference from other tissues can also be overcome, at least partially, by a judicious choice of section thickness, or
by reconstructing images from serial sections (Hogetsu and Oshima, 1986; Tiwari et al. 1984; Gubler, 1989). Immunofluorescent staining of sections of embedded material thus represents an alternative to techniques that use peeled or isolated cells. It is also especially useful for revealing the internal endoplasmic MT arrays, which would otherwise be obscured by the more abundant cortical MTs and for this reason remain less well known.

We have successfully applied a recent technique devised by Brown et al. (1989), which makes use of an ethanol-soluble, low melting point wax (Steedman’s wax) for embedding and sectioning plant material in order to visualise MTs by immunofluorescence. By this means, changes in the patterns of cortical and endoplasmic MT arrangements in various tissues of the maize root have been examined during the course of the growth and differentiation of their respective cells. The primary root of maize was chosen for study because much is known about the pattern of cell growth and tissue differentiation in this system (Erickson and Sax, 1956; Barlow and Macdonald, 1973; Barlow, 1982, 1987; Baluška et al. 1990; Kubica et al. 1991). Moreover, observations of MTs in the undisturbed root tip provide a baseline against which MT changes in experimental situations can be assessed (Baluška et al., unpublished data).

Materials and methods

Plant material

Grains of Zea mays L. (cv. LG 11) were germinated in moist vermiculite at 20°C in darkness. Seedlings with straight primary roots, 15-20 mm long, were transferred to an aerated Hoagland's nutrient solution placed in a growth room at 20 (±0.2)°C. Light was excluded from the roots by means of black beads floating on the surface of the culture solution under a mesh supporting the seedlings.

Fixation and embedding

After 48 h of growth, apical segments (6-8 mm) of primary roots were excised into 4.6 ml of MT-stabilizing buffer (MTSB: 50 mM PIPES buffer, 50 mM MgSO₄, 50 mM EGTA, pH 6.9) mixed with 0.5 ml of dimethyl sulphoxide (DMSO). They were incubated for 15 min at room temperature and then fixed with 4% paraformaldehyde in the MTSB and DMSO mixture for 1 h at 20°C. Following a brief rinse in MTSB, they were dehydrated in a graded ethanol series diluted with phosphate buffered saline (PBS). The wax (known as Steedman’s wax) for embedding the tissue was prepared from PEG 400 distearate and 1-hexadecanol mixed in proportions 2:1, 1:1 and 1:2 (v/v) at each step, followed by three changes of pure wax under vacuum to remove the final traces of ethanol from the tissues. The infiltrated segments were then embedded by allowing the wax to polymerize at room temperature.

Indirect immunofluorescence microscopy

Longitudinal sections were cut at thicknesses between 4 and 8 μm, but mainly at 5 μm as this was the optimum for our investigations. Median sections were mounted on slides coated with poly-L-lysine (1 mg ml⁻¹, Mₙ > 3 x 10⁵) (Sigma, Poole, Dorset, UK) or Mayer’s albumin. Sections were incubated with mouse monoclonal antibody raised against chick brain α-tubulin (Amersham, Buckinghamshire, UK), diluted 1:200 in PBS for 60 min at 37°C. After another rinse with PBS, they were stained with fluorescein isothiocyanate-(FITC-)conjugated anti-mouse IgG raised in goat (Sigma, Chemical Co., St. Louis, MO, USA), diluted 1:20 in PBS for 60 min at 37°C. Nuclear DNA was counterstained with 4,6-diamidino-2-phenylindole (DAPF) (1 μg ml⁻¹) for 2 min. After rinsing in PBS, the stained sections were treated with 0.01% Toluidine Blue in PBS for 10 min to diminish the autofluorescence of the tissues. Sections were finally mounted in anti-fade mountant containing p-phenylenediamine (Johnson and Nogueira Araujo, 1981). Fluorescence was examined using a Zeiss photomicroscope III equipped with epifluorescence and standard FITC exciter and barrier filters (BP 450-490, LP 520). The various arrangements of cortical and cytoplasmic MTs were examined in all tissues of the root throughout the growing zone. Eight categories of MT arrays were recognised (see Table 1), each category being specific to a given cell, and their frequencies were evaluated in each region of interest by scoring a total of 200 cells (4 roots, 50 cells per region per root). Differences between individual roots were insignificant. Photographs of fluorescent images were taken on Kodak T-Max 400 film at 400 ASA and developed in Diafine (Acufine Inc, Chicago, Ill, USA).

Results

Cortical and endoplasmic MTs (CMTs and EMTs) were recognizable in all tissues throughout the growing zone of the primary root apex (root cap, quiescent centre,

Table 1. Categories of microtubule arrays in cells of the growing zone of the primary root apex of maize

<table>
<thead>
<tr>
<th>Category</th>
<th>Description and orientation of MT arrays</th>
</tr>
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<tbody>
<tr>
<td>I</td>
<td>Dense and random</td>
</tr>
<tr>
<td>II</td>
<td>Dense and transverse with some branched and overlapping MTs</td>
</tr>
<tr>
<td>III</td>
<td>Less dense and transverse</td>
</tr>
<tr>
<td>IV</td>
<td>Less dense and forming transverse bundles</td>
</tr>
<tr>
<td>V</td>
<td>Dense and oblique</td>
</tr>
<tr>
<td>VI</td>
<td>Less dense and oblique</td>
</tr>
<tr>
<td>VII</td>
<td>Less dense and longitudinal</td>
</tr>
<tr>
<td>VIII</td>
<td>Less dense and random</td>
</tr>
<tr>
<td>IX</td>
<td>MTs absent</td>
</tr>
</tbody>
</table>

The arrangements refer to the cortical MTs; endoplasmic MTs are present to varying degrees in all categories (except IX).
epidermis, outer cortex, inner cortex, pericycle, stelar parenchyma and late metaxylem). The various arrangements of CMTs fell into eight distinct categories (listed in Table 1); EMTs were present to varying degrees in each of these patterns. As these eight categories could be recognized without undue ambiguity, the frequency of each was evaluated in the various tissues (Figs 1-3). A ninth category recorded the absence of MTs from the cell. The categories in Table 1 are referred to by Roman numerals (I-IX) in the text that follows and are illustrated in Figs 4 and 5.

**Root cap**

The frequencies of the various MT arrays (I-IX) formed in cap cells are recorded in Fig. 1. Interphase cells of the meristematic calyptrogen exhibited dense transverse arrangements (II) of CMTs with many branched or criss-crossed MTs (Fig. 1A). Just distal to the meristem, CMTs were oriented predominantly transversal to the cell axis (III) (Figs 1B,C, 4B) and sometimes showed a slightly bundled appearance (IV). This arrangement gradually shifted to an oblique, longitudinal, or even a random pattern (V-VIII) in cells located in the centre of the cap and close to its periphery (Fig. 1D). Cells detached from the cap lacked a discernable MT cytoskeleton (IX) (Figs 1G,H, 4C). However, mature, peripheral cap cells which remained in close contact with the flank of the root exhibited well preserved arrays of CMTs (Fig. 1F).

EMTs connected the nucleus with the cell periphery and showed a bright fluorescence in all cells. Their images strongly indicated that EMTs and CMTs represent a continuous system. However, EMTs, like the CMTs, were absent in detached cap cells and fluorescence was confined to small clumps of material around the outer cell membrane and the nucleus (Fig. 4C).

**Quiescent centre**

Most of this region of the root had a random distribution of dense CMTs (I) (Figs 1E, 4A). Some cells located near, or at, the basipotic border of the quiescent centre (QC) showed a more organised appearance (II) with the CMTs aligned predominantly transversely in relation to the main axis of cell elongation. Occasionally, cells with highly ordered MT configurations characteristic of cell division (pre-prophase band (PPB), mitotic spindle, phragmoplast) appeared among cells with randomly distributed CMTs. EMTs not only encircled the nuclei of QC cells (which are mainly in G1 phase), but also radiated from the perinuclear region towards the CMTs (Fig. 4A).

**Tissues of the root proper**

The frequencies of the various MT arrays found in cells of the root proper are recorded in Figs 2 and 3. The region 0-1500 μm from the root cap junction (RCJ) mainly comprises the apical meristem (Figs 2A-C, 3A-
Here, the CMTs of most interphase cells showed a dense transversal distribution (II) (Fig. 4D). The CMTs were frequently branched and/or criss-crossed. Individual tissues in this segment of the root did not differ conspicuously in the arrangement of their CMTs. However, CMTs of developing late metaxylem elements were grouped together thereby forming clear, but not very prominent, parallel bundles (IV) (Figs 3C, 4E). EMTs were distinguishable in all interphase cells (Fig. 4F). The MTs fanned out from points on the nuclear envelope towards the cell periphery where they seemed to merge with the system of CMTs.

In the segment 1500-3000 μm from the RCJ (Figs 2D-F, 3D-F) the majority of cells have ceased mitotic division but continue to widen; most cells have not yet started to elongate rapidly. The transverse arrangement of CMTs (II, III) was more evident in this region of the root due to a diminution of the branching and criss-crossing of the MTs. Concomitantly, the CMTs lost their rather even distribution along the edges of the cell and acquired a bundled appearance (IV) (Fig. 4G). This bundling showed a definite tissue-specificity: bundled CMTs were very prominent in cells of both the outer cortex cells (Figs 2E, 4G) and late metaxylem (Fig. 3F), whereas epidermis, pericycle and stelar parenchyma exhibited only weak and rather infrequent MT bundles at the cell periphery (Figs 2D,E, 3D,E, 4H).

Despite the absence of cell divisions, the EMTs showed mostly the same continuity with the CMTs as was found in the more distal meristematic region of the root (Fig. 4I). The only exception was the late metaxylem whose elements were already commencing elongation growth due to their earlier cessation of mitotic activity. Here, the EMTs often showed a discontinuous distribution which seemed to be related in some way to the inability of the nucleus to take up a position against the outer cell membrane at one side of the cell (Fig. 5A). However, irrespective of nuclear position, the system of EMTs lost its integrity within all rapidly elongating cells of the late metaxylem in this segment of the root. This was accompanied by an accumulation of fluorescent material around, and apparently within, the metaxylem nuclei both as short irregular rods (MTs) and as a diffuse material that might consist of free tubulin (Fig. 5B). Both types of fluorescence could appear associated with one and the same nucleus.

In the segment 3000-4500 μm from the RCJ (Figs 2G-I, 3G-H), all the cells of the root are in a phase of rapid elongation accompanied by the development of an extensive vacuome. Widening of the cells has already
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Fig. 3.

pericycle
stelar parenchyma
late metaxylem

4500-6000
3000-4500
1500-3000
0-1500

Arrangement of cortical MTs

Discussion

The embedding technique employing Steedman's wax was effective in permitting observation of the MT
Fig. 4. Various microtubular arrays in cells of the root cap and root proper. The distances of cells in the root proper from the root cap junction is given in $\mu$m. The basico-apical axis of each cell runs from top to bottom of the page.

(A) Quiescent centre. Note the presence of EMTs and random distribution (I) of CMTs. (B) Maturing root cap cells located in the centre of the cap with dense oblique arrays of CMTs (V). (C) Detaching root cap cell losing the integrity of its MT cytoskeleton. (D) Meristematic cells of epidermis and outer cortex ($600 \mu$m). Their CMTs have a dense transversal distribution (II) and EMTs connecting the nuclear envelope with the cell periphery are clearly recognisable in all cells. (E) CMTs in late metaxylem elements that have ceased to divide and are in the stage of post-mitotic 'isodiametric' growth ($1000 \mu$m). The dense transverse arrangement of the CMTs shows the first signs of bundling (II or IV). (F) Post-mitotic late metaxylem elements located in the transition region exhibit a very prominent network of EMTs ($1500 \mu$m).

(G) Transverse bundles of CMTs (IV) in cells of the outer cortex ($3000 \mu$m). (H) Post-mitotic stelar parenchyma cells ($2500 \mu$m) show a rather homogeneous distribution of their less-dense transverse CMTs (III). (I) EMTs in post-mitotic cells of epidermis and hypodermis ($1800 \mu$m). Bar, $10 \mu$m.

cytoskeleton in sectioned cells in all tissues of the growing region of the maize root. All the MT arrays, some of which have been described in studies by other workers, were observed in an excellent state of preservation, except in tissues where they were obviously disintegrating as part of the ontogenetic programme. Moreover, EMTs connecting the nucleus with the cell periphery (Kubiak and Tarkowska, 1987) - which have hitherto been only poorly described, and then mostly only in dividing cells - were clearly
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Fig. 5. Various microtubular arrays in cells of the root. (A) Elongating cells of the late metaxylem (*) rapidly lose the continuity and integrity of their EMT cytoskeleton (2500 µm). (B) Disassembly of EMTs in an elongating late metaxylem element is associated with an accumulation of fluorescent material around/or within the nucleus (3500 µm). (C) Nucleus of elongating cortical cell (2800 µm) in close contact with the EMTs, which show apparent continuity with the CMT arrays. (D) Stelar parenchyma nucleus enclosed within a MT basket. The orientation of these perinuclear MTs is quite different from that of the CMTs, which can also be seen (though the latter are not in focus) (7800 µm). (E) Outer stele cells show transverse bundles of CMTs (IV) at the end of the elongation region (7500 µm). (F) Less-dense oblique CMTs (VI) in a cortical cell ceasing elongation growth (7500 µm). (G) At the end of the elongation region, some pericycle cells show prominent random organisation of their CMTs (VIII) (8000 µm). Orientation and distances (µm) are as referred to in the legend to Fig. 4. Bar, 10 µm.

recognisable in both meristem and elongating cells proximal to the meristem.

Our observations show that meristematic cells have arrays of CMTs that are less ordered during interphase than are the arrays in cells preparing for, or actively engaged in, rapid elongation. Here, the cells showed predominantly transverse arrays which frequently formed distinct bundles. A similar spatial sequence of CMTs arrays was reported for roots of *Pisum sativum* (Hogetsu and Oshima, 1986), leaf mesophyll cells of wheat (Jung and Wernicke, 1990), and seed hairs of *Cobaea* (Quader et al. 1986). In contrast to the situation in these cells, which are rapidly and preferentially growing in one direction, the slowly and isotropically growing cells at the pole of the root (i.e. cells of the QC) had randomly arranged CMTs. These observations accord with the widely accepted view that the orientation of the CMTs determines the direction of cell growth (Gunning and Hardham, 1982) via their supposed control over the orientation of cellulose microfibrils in the cell wall. Previous studies (Baluška et al. 1990; Kubica et al. 1991) of the pattern of cellular growth in the maize root apex, together with the present observations of CMTs, give further support to this concept.

Cessation of cell division in the root apex is not accompanied by the immediate onset of rapid cell elongation. Rather, the cells continue to grow in both length and width, and thus accomplish post-mitotic ‘isodiametric’ cell growth. In the more proximal zone of the root, however, the onset of rapid cell elongation is associated with cessation of cell widening (e.g. see Baluška et al. 1990; Barlow et al. 1991). The post-mitotic ‘isodiametric’ growth thus represents a transitional phase in cell ontogeny where there is a shift in the arrangement of the CMTs from a rather homogeneous distribution, often with branched or criss-crossed MTs, characteristic of dividing cells, to one with more clearly ordered transverse arrays with more or less pronounced bundling. Similar adjustments to CMT arrays after the termination of cell divisions have been recorded by Falconer and Seagull (1985), Quader et al. (1986), Marc...
et al. (1989) and Jung and Wernicke (1990), though these authors did not specify the interrelationship between the degree of CMT bundling and the relative amounts of growth in width and length. Certain indirect evidence suggests that gibberellins may be one possible candidate for regulating the ordering and bundling of CMTs in the growth zone of the root (e.g. see Simmonds et al. 1983; Tanimoto, 1987). The relationship between gibberellins, MTs and their orientation, and the growth of maize root cells will be discussed in a subsequent publication (Baluška et al., unpublished data).

Bundled CMTs are often regarded as characteristic of developing xylem cells (Falconer and Seagull, 1985), but we find that they occur in other tissues. However, since these tissues do not show the same sort of sculpturing of their secondary walls as does the xylem, the MT bundles may not be permanently fixed to any given region in the cell but constantly shift position (perhaps because of the cells' faster elongation rate) and so give no opportunity for localised wall deposition to occur. This would be particularly true of cells in the rapidly elongating zone. Metaxylem development, by contrast, starts in the meristem where cell growth is slower, which in turn could favour localised wall deposition.

Bundling of the MTs coincides with the development of the vacuome, which breaks up the continuity of the cytoplasm. Consequently, EMTs and their associated actin filaments (Derkson et al. 1986; Seagull et al. 1987) can extend from the nucleus to the cell cortex only along certain radii and hence meet the CMTs at localised points. The endoplasmic component of the cytoskeleton could thus gather the CMTs into clusters, which are then seen as bundled arrays. This view in agreement with the greater prevalence of bundling in tissues that show a greater degree of vacuolation: for example, xylem and cortex have more rapid rates of MT deposition.

The deceleration of cell elongation was accompanied by a shift of the arrangement of CMTs from a transverse to an oblique, or even longitudinal, orientation. Similar transformations have been observed in other plant cells ceasing elongation (Traas et al. 1984; Hogetsu and Oshima, 1986; Laskowska, 1990). Our observations suggest that these shifts in orientation are only an incidental accompanying feature of decreasing elongation because obliquely oriented CMTs did not appear simultaneously in all cells at the same distance from the root tip as would be expected if MT orientation was intimately coupled with a change in the growth rate. Traas et al. (1984) also reported that the shift in CMT distribution accompanying the decline in cell elongation was cell-specific and that adjacent cells could have different MT orientations.

Oblique MT arrays found in cells ceasing elongation were often accompanied by a lower density of CMTs (as already mentioned, the pericycle is an exception). This contrasts with the dense transverse MT arrays observed at the onset of rapid cell elongation, suggesting that, at least in maize roots (and also in roots of radish; Traas et al. 1984), the interpolation of new MTs during cell elongation is not as efficient as it is, for example, in roots of Azolla pinnata (Hardham and Gunning, 1979).

Whether the disintegration of CMTs and EMTs that was observed in metaxylem cells occurs in other tissues requires further study. So, too, does the fate of the MT components. The nuclei of differentiating metaxylem were diffusely fluorescent after anti-tubulin staining, suggesting that free monomeric tubulin could be associated with the chromatin (cf. Michieux et al. 1986). Depolymerisation of the MT cytoskeleton in chilled maize roots is accompanied by a similar accumulation of anti-tubulin-stainable fluorescent spots in/or around their nuclei (Baluška et al., unpublished data). Whether the fluorescent spots and strands seen closely associated with the xylem nuclei were actually within the nuclei or simply appressed to its invaginated surface could not be established. The former possibility cannot be ruled out, in view of Barnett's (1991) observation of MTs within cambial nuclei of Aesculus hippocastanum. In any case, the disintegration of the MT cytoskeleton in metaxylem and root cap cells seems to be part of their development towards a programmed death.

Until recently, the EMts in plants have been described mainly in undifferentiated, mitotically dividing cells (Wick, 1985a,b; Kubiak and Tarkowska, 1987), or in vacuolated cells preparing for a mitosis induced by wounding (Bakhuizen et al. 1985; Flanders et al. 1990b; Lloyd et al. 1992). We found that all
meristematic cell nuclei in the maize root apex were invariably associated with arrays of EMTs. Even the predominantly G₁ nuclei of the slowly dividing QC cells (Barlow and Macdonald, 1973) were encircled by a dense MT network. This latter finding contrasts with the report of Hasezawa et al. (1991) that EMTs are absent from G₁ cells of BY-2 tobacco suspension cultures. However, these G₁ cells were either non-cycling, stationary phase cells, or were cells under the influence of chemical synchronising agents and therefore may not reflect the typical G₁ phase of a cycling population. Elongating and non-growing vacuolated plant cells are also generally considered to be devoid of nucleus-associated EMTs (Wick, 1985a,b; Flanders et al. 1990b). The presence of EMTs in elongating cells of the root apex is at variance with this, although in some cells these MTs are not immediately recognisable, due to extensive vacuolation of the cytoplasm and the proximity of the nuclei to the cell walls. The picture of a continuous cytoskeleton, consisting of nucleus-associated EMTs and CMTs agrees with other observations, suggesting that the nuclear envelope represents a MT-orientation. Furthermore, if CMTs are arranged in long helices cross-bridged both with each other and with the plasma membrane (Lloyd, 1984; Flanders et al. 1989), their nucleation at the nuclear envelope represents a plausible alternative to MT initiation at discrete sites along the cell edges (Gunning et al. 1978). Rather, these edge sites help to organise the subsequent spatial distribution of CMTs that is crucial for the oriented deposition of cellulose microfibrils and for the consequent orientation of cell growth.

In conclusion, our observations suggest that the EMT and CMT arrays form an integral unit in maize root cells. It is tempting to speculate that this continuous MT cytoskeleton senses and responds to various endogenous and exogenous factors and, as a result, provides the cell with an important channel for the exchange of information between the cell wall and the nucleus.

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References
change in orientation follows the initiation of growth rate decline. *Planta* 181, 44-52.


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