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REVIEW

Microtubules in cultured plant protoplasts

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CONTENTS

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INTRODUCTION

Microtubules (MTs) have an important role in plant morphogenesis because they participate in regulating cell-shape and determining the plane and site of cell division. As cells proceed through the cycle of growth and division, the MTs undergo a series of changes taking on a variety of different assemblies. In higher plants the MTs are organized cortically during interphase, as preprophase bands (PPBs) and perinuclear microtubules before or during prophase, as mitotic spindles during metaphase and anaphase, and as phragmoplasts during telophase. As microtubules are polymers of highly conserved proteins which form more or less standard substructures, it is believed that their cellular patterns are regulated spatially and temporally by MT nucleating-sites which are to a large extent under genetic control. Spatial control can be further modulated at the subcellular level by many factors including calmodulin, Ca^{2+} and Mg^{2+} concentration and MT-associating proteins. Furthermore, MT organization can be affected by intercellular factors acting across the cell wall and through plasmodesmata (molecular, hormonal, ionic and electrical gradients) and by environmental factors (temperature, light, gravity, pressure) (see reviews Gunning and Hardham 1982; Lloyd 1987; Derksen *et al.* 1990).

The main advantage of studying the MT organization in protoplasts is that the intercellular influences are eliminated and the environment can be controlled. Extracellular

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conditions can be modified systematically to study chemical and physical effects on MT organization. In addition, the absence of the cell wall removes another stratum of possible controls as very little is known of the MT-plasma membrane-cell wall associations. The influence of the cell wall on MT stability or organization can be studied by the changes occurring after it is removed. The role of cortical MTs on microfibril orientation can be studied in the absence of influence from the old wall. This review of MT organization in protoplasts is restricted to higher-plant species.

FRESHLY ISOLATED PROTOPLASTS

The MTs visualized in freshly isolated protoplasts may be affected by a number of factors including wall-degrading enzymes (Lee *et al.* 1989), conditions during protoplast isolation, source-material condition (Eriksson 1985; Bhojwani & Razdan 1986) and processing the plant material for MT labelling (Simmonds *et al.* 1983). In using the term MT(s), the inference is MT strand(s) because the difference between one MT or a strand of several MTs cannot be resolved by means of fluorescence microscopy.

Cortical microtubule organization

Randomly oriented cortical-MT arrays were observed in protoplasts derived from cell-suspension cultures of soybean (Wang *et al.* 1989a) and maize (Wang *et al.* 1989d), and from embryogenic cultures of white spruce (Fowke *et al.* 1990) and larch (Staxen *et al.* 1990). Although the cortical-MT arrangements in cells from the above cultures have not been reported, others have attempted to correlate the cortical-MT organization of protoplasts and the cells from which they were derived. Elongated cells from suspension cultures of carrot and tobacco showed parallel cortical-MT arrays (Lloyd *et al.* 1980; Hasezawa *et al.* 1988). Complete removal of cell walls from these elongated cells resulted in protoplasts with randomly oriented cortical-MTs. The general conclusion from such studies was that protoplast formation disorganizes cortical-MTs. Different results were obtained however, with a *Vicia hajastana* suspension culture, a mixed cell-population which contained cells with ordered cortical-MT arrays as well as cells with various degrees of disorder (Simmonds *et al.* 1989). The cortical MTs of the protoplasts derived from this culture were ordered in 45% of the protoplasts and disordered in the other 55% (Fig. 1) (Simmonds & Setterfield 1986). Similarly, the cortical-MT network was randomly oriented in the majority of mesophyll protoplasts of *Vitis*, but also organized parallel-arrays were frequently observed (Lee *et al.* 1989). These conflicting results may reflect variations in cortical-MT stability resulting from differences in species, suspension-culture conditions, protoplast-isolation protocols or a combination of these and other factors. Long exposure of cells to wall-degrading enzymes resulted in preparations with random cortical-MT arrangements in all protoplasts of *Vitis* (Lee *et al.* 1989). This indicates that conditions during protoplast isolation can disorganize ordered cortical-MT arrays. Furthermore, cortical MTs were found to be very sensitive to extracellular Ca^{2+} concentration during protoplast isolation (Wang *et al.* 1989d). Maize protoplasts were isolated in the presence of 0, 1, 10, 40 and 80 mM Ca^{2+}. High Ca^{2+} (10–80 mm) retained an extensive cortical-MT network in the protoplasts, whereas zero or low Ca^{2+} (0–1 mM) retained only a few or no MTs. Calcium stabilizes the plasma membrane which may help to stabilize the cortical MTs (Wang *et al.* 1989d and references therein). The extracellular concentration of 4·5 mm Ca^{2+} and mild enzyme-treatment used for protoplast isolation in *V. hajastana* (Simmonds & Setterfield 1986) may have been sufficient to stabilize the

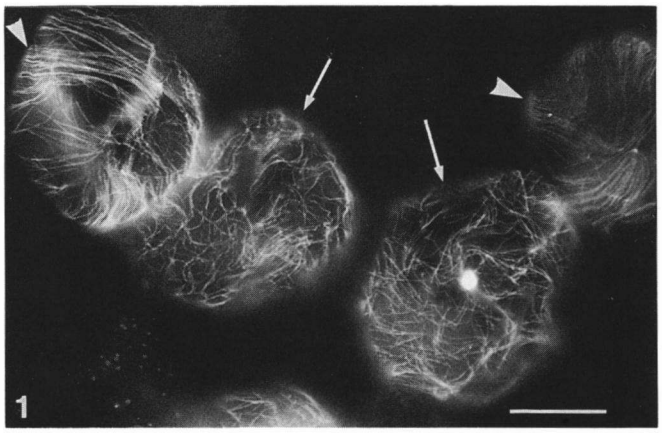


Fig. 1. Freshly isolated protoplasts of *V. hajastana* fixed and stained for MTs by immunofluorescence. In two protoplasts, strands of cortical MTs are organized in parallel (arrowheads) and in two protoplasts, the cortical MTs are randomly oriented (arrows). × 625; bar = 20 μm.

ordered cortical-MTs. Protoplast-isolation protocols coupled with lower Ca^{2+} concentrations of carrot (3 mm Ca^{2+}) and tobacco (0 mM Ca^{2+}) could not sustain cortical-MT order (Lloyd *et al.* 1980; Hasezawa *et al.* 1988). Taxol, a MT-stabilizing drug (Schiff & Horwitz 1980; Falconer & Seagull 1985), has been used prior to and during isolation of protoplasts from the growing region of the pea epicotyl (Melan 1990); both the protoplasts and the cells from which they were derived displayed cortical-MTs organized in parallel arrays. If however, the protoplasts were isolated without taxol but in the presence of 10 mM Ca^{2+}, the cortical MTs were disarranged. This work shows that 10 mM Ca^{2+} alone, was not adequate for stabilizing the order of the cortical-MT arrays in this protoplast system. It appears that MT stabilization after wall removal is a complex process. The maintenance of protoplast-cortical MTs in the state of orientation similar to their cells or origin may have different requirements in the various protoplast systems. The inference is that the plant cell-wall must have a role in stabilizing cortical MTs. The degree of MT stabilization exerted by the wall would probably differ with the structure and composition of the wall as well as the cell type (e.g. origin of cell, *in situ* or *in vitro*; plant species and genotype, organ, tissue and their physiological states; if *in vitro*, the cell-culture conditions).

Cortical MTs in intact protoplasts vs. membrane ghosts

Cortical MTs can be examined in intact protoplasts or on membrane ghosts of protoplasts which are burst after attachment to polylysine coated glass-coverslips. The MTs visualized on membrane ghosts do not comprise the entire cortical-MT network of intact protoplasts, but rather the MTs anchored to the plasma membranes directly or through bridging to other cell-constituents. Non-anchored MTs are washed away after protoplast lysis. The membrane ghosts of mesophyll protoplasts from *N. plumbaginifolia*, *N. tabacum*, and *Petunia hybrida* revealed randomly-oriented microtubules when derived from immature leaves and were devoid of MTs when derived from mature leaves. Because the protoplasts from mature leaves divided only after cortical MTs were re-established, it was suggested that the presence of a cortical-MT network was required for protoplast division (Hahne & Hoffman 1985). It is important to note that this study compared the

stability of MT-plasma membrane associations rather than the presence of cortical MTs. Cortical MTs were present in intact mesophyll-protoplasts isolated from mature leaves of several *Medicago* species (Meijer & Simmonds 1988a). Protoplasts of most species however, did not divide. The species amenable to division developed a dense cortical-MT network prior to division, whereas the species recalcitrant to division failed to do this. Maize-leaf protoplasts did not divide even after a cortical-MT network developed in the cultured protoplasts (Wang *et al.* 1989c) and *Larix* fraction-1 protoplasts did not enter division although a well-developed cortical-MT network was present (Staxen *et al.* 1990). Therefore, although a well developed cortical-MT network is a prerequisite to protoplast division (Hahne & Hoffman 1984, 1985; Fowke *et al.* 1985; Meijer & Simmonds 1988a; Dijak & Simmonds 1988) its presence does not guarantee division.

Division in protoplasts lacking a cell wall

The current interest in the cortical-MT network in protoplasts exists because these MTs are involved with cell-wall formation and because some early protoplast-culture studies have indicated that protoplasts did not divide unless they had regenerated a cell wall (Pearce & Cocking 1973; Schilde-Rentschler 1977). Others have shown that protoplasts can undergo karyokinesis but not cytokinesis if wall regeneration is inhibited either by culturing on a saline medium (Meyer 1974; Herth & Meyer 1978) or with 2,6-dichlorobenzonitrile (Meyer & Herth 1978). Furthermore, freshly isolated protoplasts of *V. hajastana* were found in all stages of mitosis which indicated that division was in progress not only after the wall-digesting enzymes were removed but also during digestion (Simmonds & Setterfield 1986). It is likely that division continued in the presence of enzymes because this was a very vigorous and rapidly-dividing cell-suspension culture (Simmonds *et al.* 1983). This shows that cells without walls can undergo mitosis. Protoplasts isolated from slow-growing cultures or from differentiated tissues would normally regenerate a wall before entering division (Meijer & Simmonds 1988a, b). It has been shown that the absence of a wall during division leads to numerous cytological abnormalities (Simmonds & Setterfield 1986). This is discussed below, in the section on Division Anomalies.

CULTURED PROTOPLASTS

Cell-wall regeneration

Initiation of cellulose-microfibril deposition on the surface of cultured protoplasts varies with the protoplast source, species and the culturing conditions, and probably reflects the metabolic activity of the original cells (Fowke 1978). Using the high resolution of the electron microscope, it was shown that protoplasts derived from the rapidly growing *V. hajastana* suspension-culture initiated microfibril deposition after 10–20 min in culture (Williamson *et al.* 1977) while mesophyll protoplasts of tobacco required 8–24 h (Grout 1975; Willison & Cocking 1975; Burgess *et al.* 1978).

Co-alignment of MTs with newly synthesized microfibrils was not detectable in protoplasts with randomly oriented cortical-MTs (Hahne & Hoffman 1985; Meijer & Simmonds 1988a). Microfibrils and/or other wall material however, first appeared on protoplast surfaces which co-localized with areas abundant with MTs and not on areas devoid of MTs (Meijer & Simmonds 1988a). Melan (1990) used Taxol to stabilize transverse arrays of cortical MTs during protoplast preparation of elongating pea-epicotyl

tissue. The protoplasts released from these tissues showed parallel arrays of MTs and were devoid of Cellufluor stained wall-material. Protoplasts allowed to regenerate wall for 2 h showed parallel arrays of Cellufluor stained fibrils. The nature of the fibrils is unknown as Cellufluor stains callose, cellulose and possibly other material. This study does show however, that the cortical MTs may have a role in orientating these fibrils. This does not rule out that a third cytoskeletal component such as microfilaments may also be involved (Hasezawa *et al.* 1989).

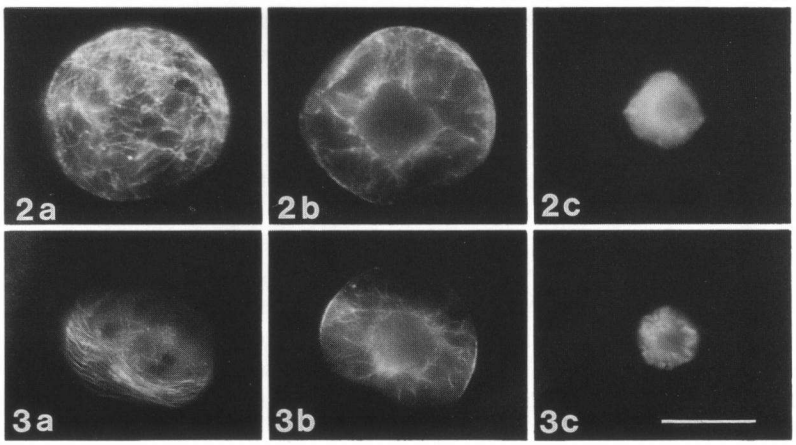
Cortical microtubule organization

A time-course study of cortical-MT organization in cultured tobacco-protoplasts showed that although the ordered cortical-MTs of the suspension-culture cells were lost during protoplast isolation, the parallel cortical-MT arrangement began to return within 1 day in culture (Hasezawa *et al.* 1988). The parallel MT-arrays returned as the cells changed shape indicating that a cell wall was being regenerated or conversely the cell wall may have helped to stabilize the order of the MTs. This suggests that the 'memory' of the spatial organization of cortical MTs is not lost during protoplast isolation even though the cortical MTs are temporarily disordered. A similar ordering of initially disorganized cortical-MTs into parallel arrays was observed in *M. sativa* mesophyll protoplasts after 2 days in culture (Dijak & Simmonds 1988). If however, the protoplasts received 17 h of electrical treatment immediately after isolation, the cortical MTs did not organize into parallel arrays, but instead, a dense network of randomly oriented cortical-MTs developed. During the 17 h of electrical treatment the *M. sativa* protoplasts regenerated a cell wall. It was suggested that the electrical stimulation may have dislocated the putative nucleating-sites or other spatial organizing factors and the newly regenerated walls stabilized the MTs at the prevailing location. These cells with disordered cortical-MTs subsequently divided asymmetrically and some developed further intro embryos whereas the non-electrically treated protoplasts developed ordered arrays, divided symmetrically and developed into callus tissue. It appears that this initial stabilization of disoriented cortical-MTs may have participated in altering the developmental pathway of the protoplasts from callus formation to embryogenesis. On the other hand, the cortical-MTs organized in parallel arrays in protoplast cultures derived from an embryogenic culture of white spruce (Fowke *et al.* 1990). Developmental pathways were not altered as both the cell-suspension culture and the protoplasts derived from it were embryogenic.

Cell division

Division peculiarities associated with protoplast culture is considered in this section. Mitosis in higher-plant cells has been reviewed recently (Baskin & Cande 1990).

Prophase. Mitotic figures were not observed in freshly isolated mesophyll-protoplasts of *N. tabacum* or *M. sativa* (Meijer & Simmonds 1988b). Divisions were seen after 36–38 h in culture in *N. tabacum* and after 48 h in *M. sativa*, by which time a uniform cell-wall had been synthesized. In these cultured mesophyll-protoplasts, prophase was associated with PPBs, PNF (perinuclear fluorescence) and PNR (perinuclear-radiating MTs). The nuclear prophase stages, as judged by chromatin condensation and nucleolar morphology, were not well synchronized with the PPB morphology. A PPB was observed in most of the prophase cells (97%). Both wide and narrow PPBs were observed in early prophase while late prophase was generally associated with narrow PPBs. The coupling was even looser in *V. hajastana* where wide and narrow PPBs were present during late prophase and the PPB



Figs 2 and 3. Protoplasts of *M. sativa* cultured for 3 days. The specimens were fixed and simultaneously stained for MTs by immunofluorescence (Figs 2a,b and 3a,b) and DNA with Hoechst 33258 (Figs 2c and 3c). × 625; bar = 20 μm. Figure 2 shows the random arrangement of cortical MTs at the surface of the protoplast (a) indicates no PPB organization, yet the optical mid plane (b) shows PNF with long radiating MTs (PNR), signs of early prophase. Chromatin condensation is not evident (c). Figure 3 shows early prophase as judged by chromatin condensation (c) with two optical planes of labelled MTs showing a surface view (a) of PPB, and the nuclear mid plane (b) with the PNF and the MTs of PNR extending to the PPB.

was present in only 50% of the prophase cells (Simmonds 1986). However, as the PPB can be initiated in early G_2 or even at the end of the S-phase of the cell cycle in wheat root-tip cells (Gunning & Sammut 1990), this lack of coupling is not surprising. Soybean suspension-cultures however, appear to have a much more tight-coupling of nuclear and cytoplasmic events as the PPBs appear in interphase and narrow progressively as the nuclear chromatin condenses (Wang *et al.* 1989a).

In *V. hajastana*, binucleate and multinucleate protoplasts had either one PPB per cell or each nucleus was associated with one PPB (Simmonds & Setterfield 1986). In soybean-protoplast cultures however, the majority of multinucleates contained only one PPB which led the authors to suggest that PPB regulation was under cytoplasmic control (Wang *et al.* 1989b).

The second divisions in protoplast cultures are often synchronous and the PPBs in the newly-formed daughter-cells are found in a variety of orientations relative to each other: parallel, perpendicular or in intermediate orientations (Meijer & Simmonds 1988b; Wang *et al.* 1989a).

Strands of MT are closely associated with the surface of the nuclear envelope during prophase. The PNF is faint and diffuse during early prophase and develops into spindle poles, perpendicular to the PPB, in late prophase (Simmonds 1986; Simmonds & Setterfield 1986; Meijer & Simmonds 1988b; Wang *et al.* 1989a). In soybean-protoplast cultures the PNF develops only after the PPB has formed (Wang *et al.* 1989a) as is the case in onion root-tip cells (Wick & Duniec 1983). This is in contrast to *M. sativa* mesophyll-protoplasts in which the PNF often develops before the PPB does (Fig. 2) (Meijer & Simmonds 1988b).

A different mechanism of spindle-pole establishment has been documented in endosperm cells, which do not form PPBs; the PNF develops multiple converging-centres of MTs which eventually merge to form two converging-centres at opposite poles of the nucleus (De Mey *et al.* 1982; Schmit *et al.* 1983). This process of spindle-pole formation

was also observed in the *V. hajastana* cultured protoplasts which did not have a PPB (Simmonds 1986). However, as PPBs and PNF multiple converging-centres were present simultaneously in late prophase of *M. sativa*, it seems that in some protoplast cultures either one, or an interaction between the two mechanisms may determine the orientation of the spindle poles (Meijer & Simmonds 1988b). Multiple converging centres were not observed in soybean-protoplast cultures (Wang *et al.* 1989a; 1991).

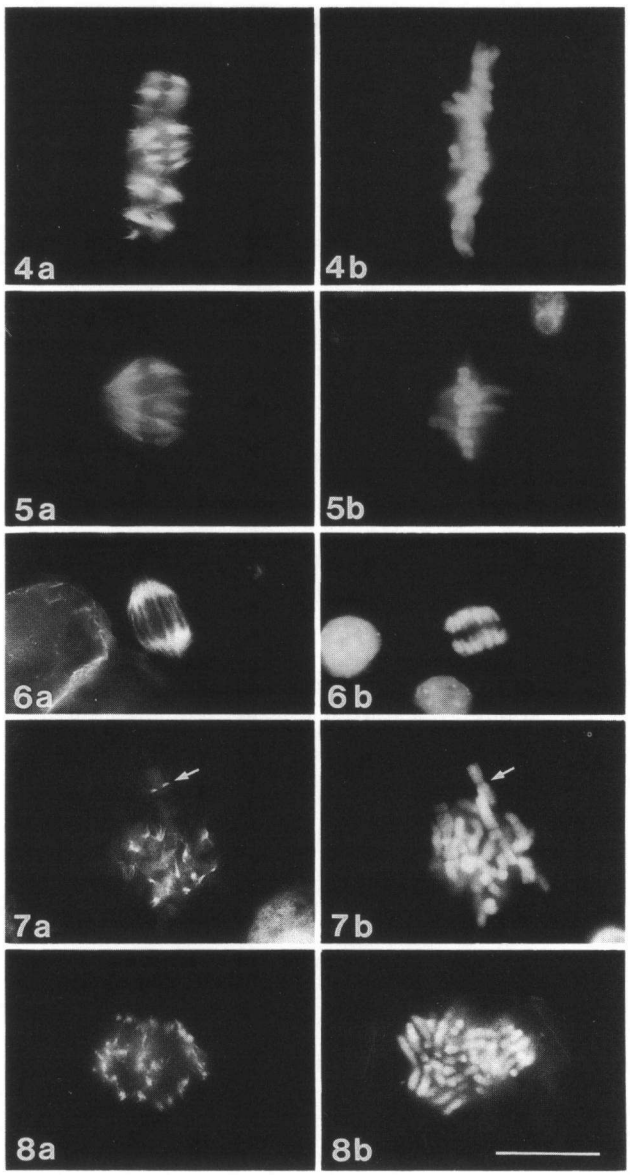
Short radiating-strands of MTs are frequently associated with the PNF (Simmonds 1986; Simmonds & Setterfield 1986; Wang *et al.* 1989a). However, very prominent strands, the PNR, were observed in the plane of the PPB in protoplast cultures of *N. tabacum* and *M. sativa* (Meijer & Simmonds 1988a). These PNR strands appear to be bridging the nuclear envelope and the PPB (Fig. 3). It was suggested that the PNR may be a functional component in nuclear relocation or reorientation which may be associated with dedifferentiation of cultured mesophyll-protoplasts prior to entry into mitosis (Meijer & Simmonds 1988b). The PNR was also involved with protoplast budding which may have resulted in some minor abnormalities in the mesophyll-protoplast cultures (Meijer *et al.* 1988).

Metaphase and anaphase. The morphology of mitotic spindles was variable in regenerating protoplast cultures of *V. hajastana* (Simmonds & Setterfield 1986), *N. tabacum* and *M. sativa* (Meijer & Simmonds 1988a,b). The polar region at metaphase ranged in breadth from broad to fairly focused poles (Figs 4 and 5). Kinetochore MTs of broad-pole metaphases were short and very prominent (Fig. 4). Mid-zone MTs were evident at metaphase (Figs 4 and 5) but more conspicuous at anaphase (Fig. 6). The polar regions were much more prominent at anaphase (Fig. 6) than they were at metaphase (Figs 4 and 5). All of the spindles in cultured soybean-protoplasts were however, the broad-pole and short-kinetochore MT variety (Wang *et al.* 1991).

The variation in morphology of mitotic spindles in protoplast cultures may be the result of a number of factors including: (a) spatial constraints of the cultured protoplasts; (b) variations in chromosome number (e.g. the polar regions in polyploid cells were extremely broad as shown in Fig. 4); (c) non-physiological or unnatural conditions of cells in a protoplast culture.

The origin of the metaphase spindle appears to be a controversial topic.It has been suggested by some workers that it is derived directly from the prophase spindle (De Mey *et al.* 1982; Schmit *et al.* 1983; Wick & Duniec 1984) while others have proposed that the prophase spindle is broken down and the metaphase spindle is formed *de novo* (Kubiak *et al.* 1986; Wang *et al.* 1991). Wang *et al.* (1991) have observed prometaphase figures in soybean-protoplast cultures which were devoid of prophase polar-spindles but in which kinetochore MTs had formed. They suggested that this stage preceded the *de novo* formation of the metaphase spindle with broad poles, which were morphologically very different from the prophase spindle with the more focused poles. Similar prometaphase spindles have been observed in cultured *M. sativa* and *N. tabacum* protoplasts, but these figures were usually associated with interpolar-MT bundles (E. G. M. Meijer & D. H. Simmonds, unpublished observations). As these apparently non-polar prometaphases seem very prevalent in protoplast cultures, further work is required for stabilized cells to elucidate prophase–metaphase transitions.

Telophase. The phragmoplast appears at the termination of anaphase and is associated with cell-plate formation. The majority of phragmoplasts were normal in cultured



Figs 4–8. Mitotic figures in cultured protoplasts of *V. hajastana* and *M. sativa*. Protoplasts were fixed and simultaneously stained for MTs by immunofluorescence (Figs 4a–8a) and DNA with Hoechst 33258 (Figs 4b–8b). ×625; bar = 20 μm. Figure 4 shows metaphase of *V. hajastana* with (a) broad spindle-poles, prominent bundles of kinetochore MTs, and MTs in the mid-zone region. The large number of chromosomes (b) indicate that the cell is most probably hyperploid. Figure 5 is a metaphase of *V. hajastana* showing (a) focused spindle-poles (as compared to broad poles of Fig. 4), bundles of kinetochore MTs, and MTs in the mid-zone area. The chromosome number (b) appears to be much lower than that of Figure 4b. Figure 6 is an anaphase of *M. sativa* showing (a) focused spindle-poles, conspicuous mid-zone MTs and (b) segregating chromosomes. Figure 7 is a metaphase of *V. hajastana* showing (a) kinetochore MTs (arrow). The highly condensed chromosomes (b) are not aligned at the equatorial plate. Figure 8 is an abnormal anaphase of *V. hajastana* showing (a) pairs of kinetochore MTs. Polar MTs were not detectable in this protoplast. The sister chromosomes (b) had separated but they are situated in a disorganized configuration of prometaphase, i.e. they are not aligned at the equatorial plate. (Figs 7 and 8 are from Simmonds, D.H. and Setterfield, G., *Planta* **167:** 460–468, 1986. With permission of Springer-Verlag.)

mesophyll-protoplasts of *M. sativa* and *N. tabacum* (Meijer & Simmonds 1988a,b). Multinucleate protoplasts of soybean that had shown only one PPB had, however, multiple phragmoplasts which indicated that phragmoplasts were under nuclear control whereas PPBs were under cytoplasmic control (Wang *et al.* 1989b). Phragmoplasts were abnormal in many *V. hajastana* divisions which occurred during the first 24 h of culture, whereas the majority of phragmoplasts were normal in divisions occurring at 48 h and later (see section on Division Anomalies) (Simmonds & Setterfield 1986).

Phragmoplasts of embryogenic-protoplast cultures were of particular interest. In cultures of embryogenic protoplasts of *Larix*, Y-shaped phragmoplasts occurred very frequently and appeared to be dividing the mother cell into three daughter-cells, two with a nucleus and one without a nucleus (Staxen *et al.* 1990). As these latter cultures had normal PPBs, this observation indicates that the PPBs and phragmoplasts were not tightly coupled. Enucleate cells were observed in the proembryos derived from electrically treated protoplasts of *M. sativa* (Dijak & Simmonds 1988; and D. Simmonds, unpublished observations). Unless selected nuclei can be destroyed, this suggests that PPBs and phragmoplasts were uncoupled in prior divisions. These data indicate that embryogenic differentiation is associated with partitioning off large sections of cells and that the phragmoplast may be involved with regulating this process. It would follow then that the location of the end of the phragmoplast at the parental wall may be regulated by factors other than the prior location of the PPB.

DIVISION ANOMALIES IN PROTOPLAST CULTURES

V. hajastana protoplast cultures regenerate callus very vigorously. However, although as many as 97% of the protoplasts undergo first division, 20–40% of these colonies in which divisions had been initiated, stop growing and eventually die. A protoplast plating efficiency greater than 50% is considered to be very high; plating efficiencies of 0·1–1% are not unusual. Nevertheless the puzzling high death-rate of young colonies prompted a study of division and growth patterns in *V. hajastana* protoplast cultures (Simmonds & Setterfield 1986). Spindle and phragmoplast abnormalities resulted in daughter nuclei containing different amounts of DNA and incomplete cytokineses accompanied by abnormal walls.

The spindle abnormalities observed in the *V. hajastana* study included multipolar spindles as well as spindles without poles. The spindles without poles consisted of chromatid pairs, with short kinetochore-MTs (Fig. 7). Such figures were distinguished from normal prometaphases where interpolar MTs were present, although the poles were not clearly defined. Wang *et al.* (1991) observed prometaphase figures lacking polar MTs in soybean-protoplast cultures and suggested that this may be a normal stage of prometaphase. The chromosomes of all prometaphase figures of *V. hajastana* however, did not subsequently line up at the equatorial plate or if they did, they were released from the ordered arrangement prior to anaphase. This is supported by early anaphase figures in which the chromosomes had undergone disjunction, chromosome separation had not yet begun because the kinetochore-MT bundles lay in pairs, the interpolar MTs were absent and the chromosomes were situated in a disordered prometaphase configuration rather than at the equatorial plate (Fig. 8). Evidence that abnormal mitosis had occurred is provided by cytofluorimetric analysis of sister nuclei in telophase, which showed unequal DNA-content thus indicating that the chromosomes had not segregated normally. Furthermore, as such spindle abnormalities occur sporadically after the initial 3–4 days

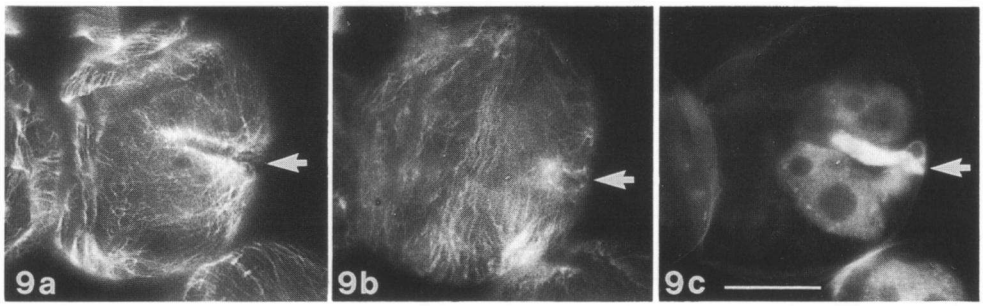


Fig. 9. *V. hajastana* cultured protoplasts fixed and simultaneously stained for MT by immunofluorescence (a,b), DNA with Hoechst 33258 (c) and cell walls with Calcofluor (c). Cell in interphase shows that cytokinesis had not been completed. Two optical planes (a and b) show cortical MTs at both cell surfaces. The cleft (arrow) divides only part of the cell, allowing the cortical MTs to traverse the undivided region (b). An incomplete wall separates sister nuclei different in size (c). × 620; bar = 20 μm. (From Simmonds, D.H. and Setterfield, G., *Planta* **167**: 460–468, 1986. With permission of Springer-Verlag.)

of protoplast culture, this suggests that these anomalies are a peculiarity of the early culture-phase.

Phragmoplast abnormalities were identified in *V. hajastana* protoplast cultures. These included displaced and generally malfunctioning phragmoplasts as indicated by incomplete cross-walls after termination of cytokinesis (Fig. 9). Furthermore, there was an association between incomplete cross-walls and unequal nuclear-divisions. After 5 days of protoplast culture, 91% of the divisions with incomplete cross-walls contained nuclei of unequal size whilst conversely, 78% of divisions with properly formed cross-walls contained nuclei of equal size. These data indicate that the normal spatial orientation and functioning of MTOCs (MT-organizing centres) or MT-nucleating sites of spindles and phragmoplasts in cultured *V. hajastana* protoplasts had been lost, or that the malfunctioning one MT-array (e.g. spindles) may affect the orientation of the following MT-array (e.g. phragmoplasts). It appears that when one abnormality occurs in the MT organization it may be very difficult to prevent subsequent abnormalities. This was demonstrated by 100% abnormal second-divisions (as defined by incomplete cross-walls and unequal nuclear divisions) following abnormal first-divisions.

The frequency of division abnormalities in *V. hajastana* were greatest during early-culture periods. These abnormalities include abnormal spindles, unequal DNA-content of telophase sister-nuclei, malfunctioning phragmoplasts and incomplete cross-walls. The second divisions which were completed by 48 h were all abnormal and appear to be the products of early first-division mistakes. Such colonies are undoubtedly responsible for lowering plating-efficiency as they would stop dividing after one or two more divisions and compounding of abnormalities.

First divisions were normal much more frequently if they occurred after 24–48 h of protoplast culture. The inference is that before normal divisions can take place a 'normal' cell-wall must be regenerated. As mentioned above, *V. hajastana* protoplasts begin regenerating a cell wall as early as 10 min after culture initiation (Williamson *et al.* 1977) and by 6 h of culture the newly deposited wall is clearly detectable with Calcofluor staining. However, the initial walls regenerated by protoplasts have been shown to be different from walls synthesized at later culturing periods (Klein *et al.* 1981). Evidently the initial wall does not support normal cell-divisions whereas the more mature wall does.

Table 1. Frequency of mitotic abnormalities occurring in protoplast cultures of *Vicia hajastana, Nicotiana tabacum* and *Medicago sativa* during the first 2, 3 and 4 days of culture, respectively. Data are based on the results from three different experiments in which c. 100 metaphases were scored for each species and divisions were scored in 550 cells of *V. hajastana* and 3000 cells in each of *N. tabacum* and *M. sativa*

	V. hajastana	*N. tabacum*	*M. sativa*
First mitosis	continuous	day 1–2	day 2–3
Abnormal mitotic-spindles	47·3%	17%	8%
Incomplete cross-walls	55·6%	0·1%	0·2%

Division abnormalities can be greatly reduced if early divisions can be prevented. Mesophyll protoplasts which are derived from differentiated tissue, require a period of readjustment in culture before division begins. Divisions in *N. tabacum* and *M. sativa* are initiated after 1–2 and 2–3 days in culture, respectively. During this period a substantial cell-wall can be regenerated. A comparison of division abnormalities in mesophyll protoplasts, where division begins after a wall is synthesized, and cell-suspension derived protoplasts, where division occurs before an adequate wall is regenerated, is shown in Table 1. Abnormal division-frequencies are exceptionally low in mesophyll protoplasts as compared to suspension-derived protoplasts.

CONCLUSION

The importance of the plant cell-wall in stabilizing MTs, MTOCs and/or nucleating sites is the underlying theme in this review. The removal of the wall destabilizes the cortical MTs. This is evident in freshly isolated protoplasts where cortical-MT order is lost unless MT-stabilizing factors are used (e.g. DMSO, Taxol, high Ca^{2+} concentration). In the absence of a 'normal' wall, protoplast divisions proceed with the formation of abnormal spindles, phragmoplasts and irregularities in chromosome separation and cell-plate development. These irregularities are greatly reduced in protoplasts cultured for a few days because during this time a more 'normal' wall can be regenerated. What constitutes a 'normal' wall is unclear. It is known, however, that the initial wall regenerated by protoplasts is different from the previous and the subsequent cell-wall.

Very little is known about the interactions between the plant cell-wall and the cytoskeletal components. The use of plant protoplasts has indicated that the disruption of such interaction results in chaos; it follows therefore that these interactions must play a significant role in modulating cell-shape and division. The elucidation of MT-cell wall interactions will be a challenge.

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