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Cell-Aging Related Configurations of Cortical Microtubules in Carrot (*Daucus carota* L.) Suspension Cells

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Cytoplasmic microtubules (MTs) are essential for many important cellular processes in higher plant cells. As have been documented by many authors, particular microtubular configurations are associated with certain patterns of cell development both *in vivo* and *in vitro* (Llyod *et al.* 1980, Galway and Hardham, 1986, Hasezawa *et al.* 1988, Derksen *et al.* 1990, Simmonds 1991, XuHan and van Lammeren 1993, also see review Gunning and Hardham 1982). They commonly occur ahead and/or accompany even large-scaled cytomorphological changes and can be, thus, considered as markers or even triggers for the following processes. However, as a plant cell undergoes aging, the aging related microtubular configurations remain unknown. Futhermore, in current *in vitro* culture fields, several callus types have been identified according to their morphology and development (McCain *et al.* 1988). In general, some calli are embryogenic or regenerative, while others are not. Cell aging must play a determinative part in the expression of the totipotency. Unfortunately, the cell aging factor has not been paid enough attention; its related structure is poorly recorded, and no detail information of the aging related configurations of MTs is available up to now.

The data on carrot somatic embryogenesis in our laboratory have been published before (Roustan *et al.* 1989, Roustan *et al.* 1990). In this report we focus on the aging related configurational changes of interphase MTs using the well-established carrot suspension system. The aims of the present research are 1) to reveal the MT changes in cell aging processes, and 2) to search for a marker to distinguish the embryogenic/regenerative cell suspension from non-embryogenic/non-regenerative cells for the selection of proper cell populations for gene transformation and plant regeneration.

Materials and methods

The cell suspension culture was initiated from the hypocotyls of a domestic carrot, *Daucus carota* L. cv. Chantenay (Roustan *et al.* 1989). The cells were cultured in a B5 liquid medium (Gamborg *et al.* 1968) containing 20 g/l sucrose and 1 mg/1 2,4-dichlorophenoxyacetic acid (2, 4-D) (L.B5-20D) at 26°C under a light flux of 100 μEm^{-2} s^{-1} (Osram fluora fluorescent tubes) and a 16/8 hr light/dark photoperiod. For somatic embryogenesis, suspensions of 7 days after culture (dac) in L.B5-20D were transferred to the same but 2,4-D free medium (L.B5-20) at the density of 5×10^4 cells ml^{-1}.

For immunocytochemical staining, individual cells, colonies and/or somatic embryos at 1–30 dac in L.B5-20D or 1–10 dac in L.B5-20 were fixed for 3 hr with 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) at pH 7, plus 2% (w/v) sucrose, 50 mM Pipes, 2 mM $MgSO_4$ and 1% (v/v) Triton X-100. Some samples stored in PBS at 4°C were attached to slides coated with poly-L-lysine, others were embedded in polyethylenglycol (PEG) and sectioned by

3.5-8μm with an LKB 2218 Historange microtome. After washing in PBS, immunofluorescence labelling was performed using a monoclonal anti-β-tubulin (Amersham Intl. plc) as the first antibody at 1 : 5000 dilution for 3 hr, and fluorescein isothiocyanate (FITC) linked sheep anti-mouse IgG (Amersham Intl. plc) as the second antibody at 1 : 50 dilution for 2 hr, both at 30°C. Three-time washes for more than 2 hr in PBS were followed by each labelling. Omission of the first antibody was applied to be the control. Observations and microphotographs were taken on a Leitz Laborlux 12 microscope with a Wild Photoautomat MPS45 exposure system, using Kodak Ektachrome 1600HC and TMAX P3200 films.

For electron microscopy, the cultures were fixed in 4% glutaraldehyde and 2% paraformaldehyde in PBS and post-fixed in 1% osmium tetroxide. Samples were dehydrated, and then embedded in a low viscosity resin (Spurr 1969). Ultrathin sections were stained with uranyl acetate followed by lead citrate and examined with JEM-120 EXII transmission electron microscope operating at 80 kV.

Results

Suspension cell growth

In the cell suspension after a few days of culture in L.B5-20D, several kinds of cells in colonies or individuals were observed:

- Cytoplasm-rich and small isodiametric cells (type I) in colonies, which gave rise to somatic embryos after transferred into L.B5-20 (Fig. 1).
- Vacuolated, slightly enlarged and isodiametric cells (type II) in colonies. When these cells were transferred to L.B5-20, they usually underwent further vacuolation and differentiation of non-embryogenesis (Fig. 2).
- Significantly elongated/enlarged and vacuolated cells (type III). They were most present as individuals and never gave rise to somatic embryo in L.B5-20.

Cell differentiation and microtubular configurations

In the type I cells (Figs. 3, 4), bundles of the cortical MTs formed an extensive framework. In thick sections, in which half-global MT configurations were often observed, cortical MT bundles were found running in various directions underneath the cell wall. They were usually in smoothly-curved thick bundles and often connected to the cytoplasmic MTs and the MTs radiating from the nuclear envelope.

In type II cells (Fig. 5), which were undergoing vacuolation and enlargement, the cortical MT framework lost some MT density. The MT bundles became thin and appeared rather straight compared with that in type I cells in which more than four MTs were usually present in one bundle (Fig. 6).

In the elongating cells, the cortical MTs formed about parallel arrays transversal to the major axis in the elongating part, however, randomly oriented in the cell ends (Fig. 7).

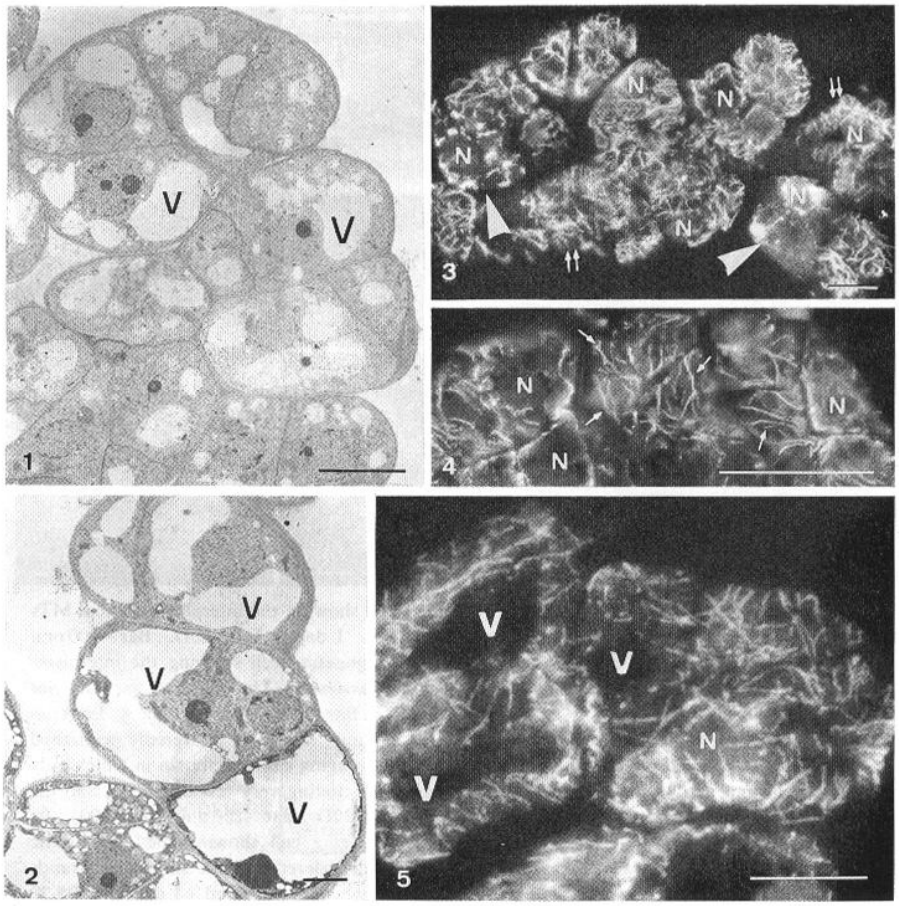
In both elongated cells and enlarged cells, the cortical MTs were found rather in an individual appearance (Fig. 8 with reference to Fig. 6).

Cell aging-related microtubular configurations

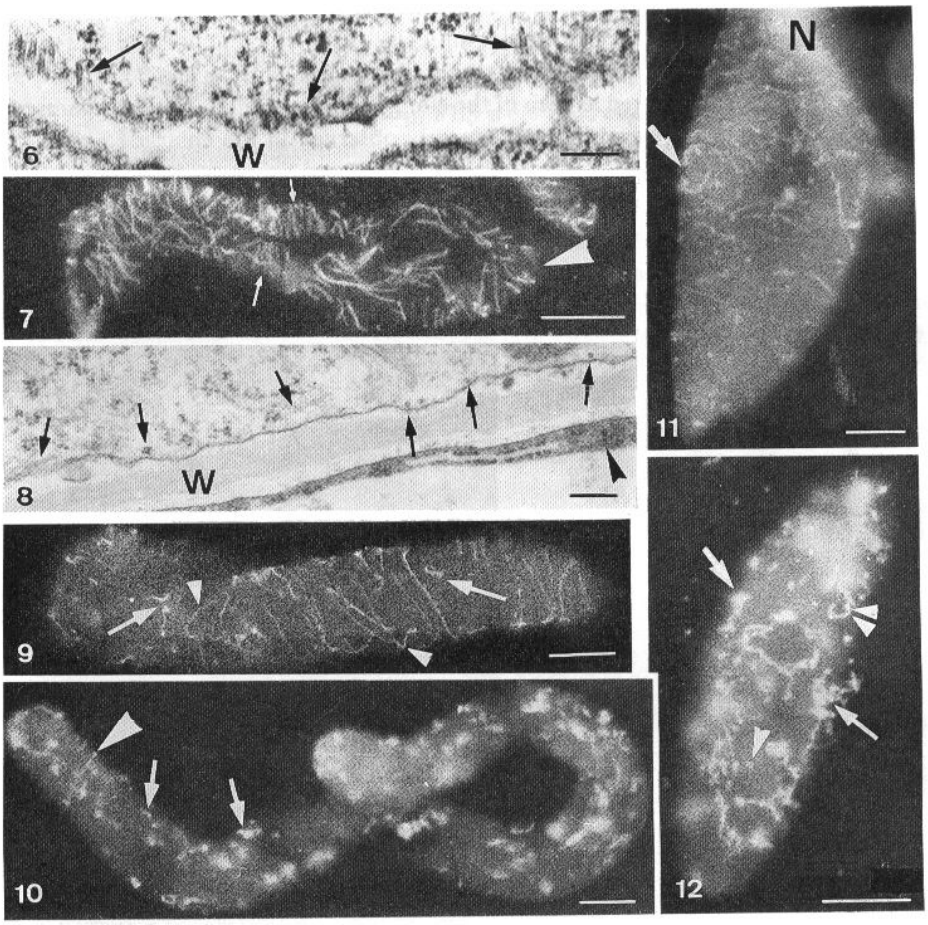
Type III cells finally entered the post-mitotic phase in which these well differentiated cells underwent cell aging prosesses without cell divisions. With electron microscopy, only a very thin layer of condensed cytoplasm was observed in the aging cells (Fig. 8). The cytoplasm lost most organelles. MTs were scarcely observed. With immunocytochemical method, the disorganised MT configurations were revealed. In the elongated aging cells, the cortical MT bundles became zigzag and shortened (Fig. 9). Swollen ends of the MT bundles were

frequently observed. However, the originally ordered parallel MT bundles were still able to see. The MT bundles lost the ordered parallel configuration and further shortened to be "islands" in the aged cells (Fig. 10). In the enlarged aging cells (Fig. 11), the cortical MT arrays lost smoothly-curved configuration and started to be zigzag, and similar shortened and swollen-ended cortical MT arrays occurred as seen in the elongated aging cells. The disorganised zigzag and "islands" configurations were rather frequently observed in the enlarged aged cells (Fig. 12).

MTs were not observed in even aged cells either with electron microscopy or with immunocytochemical method.



Figs. 1–5. 1, 2. Electron micrographs. 1. Pro-embryogenic cell masses derived from type I cells. Note that the vacuoles in the cells were commonly small and the cells were rich in cytoplasm. 3 dac in L.B5-20. V=vacuole. Bar=10μm. (×1170). 2. A part of a colony derived from type II cells. Note that the cells were undergoing vacuolation which represented early stage of non-embryogenic differentiation. 3 dac in L.B5-20. V=vacuole. Bar=10μm. (×1580). 3, 4. Immunocytochemical micrographs. 3. An overview showing MT configurations in type I cells, type II (double arrows) and type III (Arrowheads) cells. 4 dac in L.B5-20D. N=nucleus. Bar=200μm. (×32). 4. An enlargement of the smoothly-curved cortical MT configuration in type I cells. Note the thick MT bundles (arrows) in various but ordered orientations. N=nucleus. 4 dac in L.B5-20D. Bar=200μm. (×80). 5. Type II cells showing that the interphase cortical MT bundles were thin and loosely positioned. The vacuoles (V) were well developed. 4 dac in L.B5-20D. Bar=100μm. (×160).



Figs. 6–12. 6. Electron micrograph of a part of type I cell showing the interphase cortical MTs grouped in bundles (arrows) underneath the cell wall (W). 1 dac in L.B5-20D. Bar = 200 nm. (×38,400). 7. Immunocytochemical micrograph of an elongating cell showing the interphase cortical MTs oriented parallel to the axis of elongation (arrows). The enlarged part did not exhibit parallel arrays (arrowhead). 1 dac in L.B5-20D. Bar = 100 μm. (×107). 8. Electron micrograph of a very vacuolated cell showing that the cortical MTs were scatteredly positioned (arrows). At the other side of the cell wall (W) a part of an aging cell was shown in which only a thin layer of condensed cytoplasm was left (arrowhead) including very few organelles and the MTs were scarcely observed (unshown here). 1 dac in L.B5-20D. Bar = 250 nm. (×24,600). 9–12. Immunocytochemical micrographs. 9. An elongated aging cell showing the post-mitotic phase cortical MTs. The parallel arrays of MTs became disordered and the shortened strands with swollen ends (arrows) as well as zigzag bundles (arrowheads) appeared. 1 dac in L.B5-20 D. Bar = 200 μm. (×45). 10. An elongated aged cell showing the cortical MT arrays had become shortened into scattered islands (arrows). Only very few of the MT bundles could be seen (arrowhead). 1 dac in L.B5-20D. Bar = 200 μm. (×37). 11. An enlarged aging cell showing that the post-mitotic phase cortical MT arrays became thin and zigzag. Swollen ends and points on the MT bundles started to appear. Note the circular configuration of some MT strands (arrow) did not correspond to the position of the nucleus (N). 1 dac in L.B5-20D. Bar = 200 μm. (×39). 12. An enlarged aged cell showing the cortical MT arrays had become shortened into scattered islands (arrowhead) or short arrays with swollen ends (double arrowhead). Only very few MT bundles were present and they were associated with disordered groups of the MT arrays at the ends (arrows). 1 dac in L.B5-20D. Bar = 200 μm. (×60).

Discussion

It has been well documented that somatic embryogenesis in carrot cell suspension is readily elicited when cells cultured in a medium supplemented with 2,4-D are subsequently transferred to an auxin-free medium (Sung *et al.* 1984, Nomura and Komamine 1986). Such culture system has been routinely maintained in our laboratory (Roustan *et al.* 1989, Roustan *et al.* 1990). That allows us by using the model culture system to confine the aging related microtubular configuration.

The concept of cell aging in higher plants was interpreted variously, and seldom is an attempt made to integrate the data using a culture system analysis approach, and even more rarely is aging investigation by the point of view of cytoskeleton (for a recent review of broad interests in plant cell aging see Rodríguez *et al.* 1990). To avoid misunderstanding, in the present paper, cell aging is defined as losing totipotency-depended cell structure and function during cell development.

The previous work conducted using carrot suspension cells were addressed to cell culture but not to cell aging process (Israel and Steward 1966, Halperin and Jensen 1967, Fujimura and Komamine 1980, Lloyd *et al.* 1988, Hawes 1985, Cyr *et al.* 1987, Traas *et al.* 1987). The efforts of those studies, if cytoskeleton was the topic, were mainly paid to the determining morphogenic potential of preprophase band (PPB) and spindle MTs (also see reviews on protoplast, Simmonds 1991, Fowke and Wang 1992). In our research, we associate ultrastructural and immunocytochemical methods, focus on the cortical MT configurations of the interphase or post-mitotic phase which exist much longer in cell cycle than PPB or spindle does, and can be, therefore, rather easy to be used as a marker for the cell development. Additionally, this knowledge enables us to select type I cell dominant population, *i.e.* which include a maximum number of embryogenic cells, to study embryogenesis related cytological events and molecular metabolism.

The present work reveals that the amount of the cortical MTs decrease during cell aging and the cortical MTs show disordered configurations, *i.e.* shortened and zigzag MT strands and swollen ends of MT bundles. As concluded in Fig. 13, the type I cells, *e.g.* embryogenic cells,

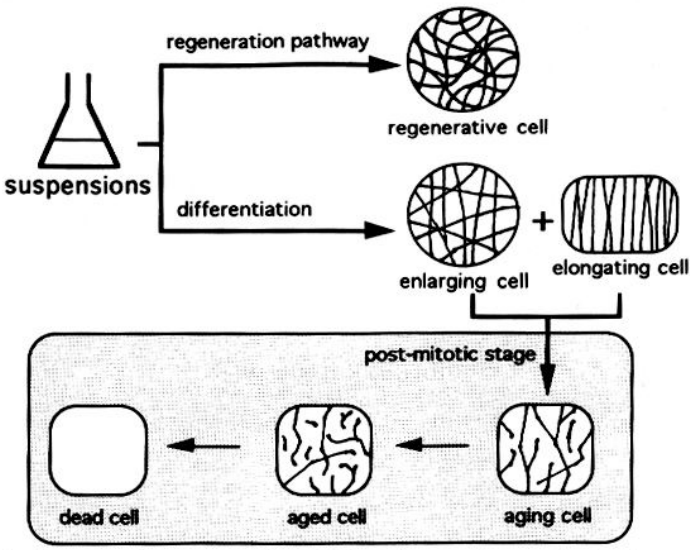


Fig. 13. A diagrammatic representation of interphase and post-mitotic phase cortical MT configurations associated with various suspension cell developments. For details see the text.

commonly display extensive unparallel cortical MT bundles which smoothly curved underneath the cell wall. The non-embryogenic but meristematic isodiametric cells, *i.e.* type II cells, generally show medium density of the MT bundles which are more or less straight and thin. Very vacuolated and enlarged cells exhibit loosely positioned, zigzag and thin cortical MT strands. Parallel arrays of cortical MTs mostly occur in elongating and elongated cells. Similar arrangements of MTs in parallel hoops were previously observed in elongated cells of carrot (Llyod *et al.* 1980), in elongated and protoplast derived cells of tobacco (Hasezawa *et al.* 1988) and in cylindrical cells of *Mougeotia* filaments (Galway and Hardham 1986). Type III cells, *e.g.* aging cells, often associate with disorganised and scattered MT arrays or islands. Condensed and shortened strands, thin and zigzag bundles and swollen strand ends are characteristics of the aging-related configurations in this species.

The result presented here is also suggestive that for a good understanding of the relationship between cytoskeletal function and cytomorphogenesis, the aging related MT configurations should be distinguished and not be intermingled with that in embryogenic/regenerative cell. On the other hand, compared with protoplast, cell suspension averts cell wall removal and regeneration, both of which were found affecting the configurations of cortical MTs (Simmonds 1991). Therefore, modifications might be made before the conclusion from this study being applied to protoplast.

Summary

Configurations of the cortical microtubules (MTs) in carrot (*Daucus carota* L.) suspension cells were investigated by electron microscopy and immunocytochemical method. In the carrot cell suspensions, some cells followed the regeneration pathway and gave rise to pro-embryogenic cell masses. They were observed to have a specific configuration of the cortical microtubules, *i.e.* a dense framework formed by smoothly-curved MT bundles. Other cells were involved in the non-embryogenic differentiation which eventually resulted in cell aging. The non-embryogenic differentiation included cell vacuolation followed by cell enlargement and cell elongation which were associated with the formation of rather loose frameworks of the cortical MTs. The enlarging cell showed more or less straight MT strands and the elongating cell showed parallel-strand-dominated MTs. After several divisions, those differentiated cells entered the post-mitotic phase and the cytoplasm became disorganised. The cortical MTs continued to decrease in number during the cell differentiation and aging processes. Most cortical MT strands were shortened or appeared zigzag in aging cells. In aged cells, the MTs in thin strands or condensed short strands with swollen ends became the majority. Finally, all the MTs disappeared simultaneously with the death of the aged cells. Disorganised random arrays of the cortical MTs including the zigzag strands and shortened strands as well as the swollen ends were concluded to be specific of cell aging. This result showed it possible to use configurations of the cortical MTs as a marker to distinguish the potential of regeneration in cell cultures.

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