Microtubule involvement in embryogenic sunflower protoplasts division.

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Abstract

When cultured in liquid medium, sunflower protoplasts divide symmetrically giving rise to loose microcolonies. When embedded in agarose beads they mainly divide asymmetrically leading to compact structures ressembling embryos and thus called embryoids. The present study focused on the cytoskeleton dynamic of cultured protoplasts in relation to the determination of the division plane. Cortical microtubules appeared to be randomly arranged in freshly isolated protoplasts. During the first days of culture this cortical array gradually showed a parrallel orientation of microtubules whatever the conditions of culture. In agarose beads cultured protoplasts, microtubules form a more or less complete basket around the nucleus whereas, in liquid medium, protoplasts only showed a non fibrillar perinuclear material. This suggest that agarose embedding promote microtubule nucleation by the microtubules organizing centers. How and where microtubules are nucleated in plant cells remain highly debated, but centrosomal-like proteins and gamma-tubulin have been shown to label plant nuclear surface which may function as a microtubule nucleating site. We are now investigating about localization and dynamic of gamma-tubulin in embryogenic and non-embryogenic culture conditions.

Keywords: *Helianthus annuus*; Protoplasts; Microtubules; Cell wall; Cell division;

Introduction

In *Helianthus annuus* protoplasts divide symmetrically and form loose microcolonies when cultured in liquid medium, whereas they divide mostly asymmetrically, presenting a polar organization at the onset of their development and give rise to compact embryo-like structures when they are embedded in agarose beads (Alibert et al. 1994, Petitprez et al. 1995).The agarose matrix surrounding the protoplasts could likely trigger an intracellular polarity, which could induce the asymmetry of the first division.

Previous works have led to the idea, that the cytoskeleton and the cell wall play a major role in the polarization process (Akashi et al. 1994, Kropf 1994, Shibaoka 1992). Therefore, in order to study the role played by microtubules (MT) in the division of sunflower hypocotyl protoplasts, we have

studied the evolution of the MT network during the first days of culture, in liquid and agarose medium.

Material and Methods

Seeds of *Helianthus annuus* genotype Emil (Pioneer Hi-bred International) were cultured as described by Chanabé et al (1989). Hypocotyls of seven day-old plantlets were used for the isolation of protoplasts, which were cultured at a final concentration of 5.10^4/ml in TLD medium, either in the liquid medium or after inclusion in agarose beads (Chanabé et al. 1991). Protoplasts were fixed during 1h in 2% paraformaldehyde containing 0,03% glutaraldehyde in Traas buffer (Traas 1989). When cultured in liquid medium, fixed protoplasts were spread onto poly-L-lysine (Sigma) coated coverslips, and covered with a thin melting layer of 1% Sea Plaque agarose (FMC Bioproducts USA). In the case of agarose-embedded protoplasts, beads containing the fixed protoplasts were molten at 50°C and spread onto poly-L-lysine coated coverslips.

Immunolabeling of microtubules was performed according to De Mey et al (1982). Fixed protoplasts were extracted in Triton X100 (0,25% in Traas buffer) for 30 min. to increase membrane permeability. In order to reduce aldehyde-induced fluorescence, the slides were treated three times with NaBH3CN (0,01M in PBS pH8) for 10min, rinsed twice in TBS-Mg pH 7,6 and treated with 5% Normal Goat serum in TBS-Mg for 30 min. Protoplasts were incubated overnight at room temperature with monoclonal anti-b Tubulin (Amersham UK), diluted 5000-fold in TBS-Mg. They were rinsed twice in TBS-Mg, once in 0,1% BSA dissolved in TBS-Mg, and further incubated with fluorescein isothiocyanate (FITC)-conjugated sheep anti mouse immunoglobulin antibody (Amersham, UK), diluted 1/50 in TBS-BSA. After one wash in the same buffer, nuclei were stained with 0,2 μg/ml Hoechst 33342 (Sigma) and coverslips were mounted in antifading medium Citifluor (Link Analytical, France)

Observations were performed with a Leitz epifluorescence microscope equipped with excitation and barrier filters BP340-380/LP430 for Hoechst, BP450-490/BP520-525 for FITC. Images were recorded with a CCD SIT intensified camera and image processing was performed using the MIMA image analysis software (Biocom, France).

Results

Just after isolation, protoplasts showed cytoplasmic strands characterized by an extensive network of randomly arranged cortical microtubules (Fig.1).radiating from the nuclear area and directing towards the cortical area. When first divisions start, and whatever the culture conditions, 40 to 50% of the protoplasts showed cortical arrays of microtubules organized in parallel bundle sheaths (Fig.2). A non-fibrillar perinuclear fluorescence (PNF), was observed during prophase, microtubular strands radiating from the nuclear envelope towards the cortex, and the cortical network occurred as a circular band known as the preprophasic band (PPB) (Fig.3 and 4). This structure was present in about 50% of the prophasic protoplasts when cultured in liquid medium, but appeared very scarcely when protoplasts were cultured in agarose beads. At metaphase, cortical microtubules disappeared and labeling was only located in the mitotic spindle emerging from broad poles whatever the culture conditions. At telophase the decondensed chromosomes were enclosed in non-fibrillar microtubules, closely associated with the phragmoplast microtubules (Fig.5).

A few hours after agarose embedding, protoplasts showed a perinuclear β-tubulin labeling corresponding to individual MTs forming a loose basket around the nucleus (Fig.7). Protoplasts suspended in liquid medium showed no such structure, up to the third day of culture and β-tubulin exhibited only a diffuse perinuclear fluorescence (Fig.8). Individual MTs only appeared as from day 3 and thereafter concerned an increasing number of protoplasts (Fig.9).

In liquid medium, protoplasts entering early in prophase, showed a PPB arranged in a central position (Fig.3). Later, in telophase the phragmoplast was located in a symmetrical plane (Fig.5), and the division gave rise to identical daughter cells. When cultured in agarose beads, a few protoplasts divided symetrically, but most of them divided in an asymmetrical way : in this case the parallel cortical array shrank during prophase, but a narrow preprophase band was never seen. In telophase the phragmoplast set up in an excentric position (Fig.6), leading to the unequal division of the cell.

Discussion

In sunflower interphasic protoplasts, the cortical network of microtubules gradually organized during the first days of culture by forming a parallel array of MTs bundles. According to Kengen and Derksen (1991), parallel MTs arrays characterize elongating cells, whereas cross-linked network are related to spherical cells and protoplasts. So by 3 or 4 days of culture, sunflower protoplasts were able to form a new cell wall, to polarize, and to divide. When sunflower protoplasts entered into prophase, the MT cortical network was resolved and a preprophasic band (PPB) appeared, as described by Meijer and Simmonds (1988) in Medicago and Nicotiana.

Agarose enbedded protoplasts never formed a narrow PPB. This structure is generally assessed to be a determinant of the division plane : Traas et al. (1995), using *Arabidopsis* mutants lacking PPB, showed that this mutation, even affecting cell elongation and division plane alignment, did not alter differentiation patterns or organ position. Mineyuki and Gunning (1990) when inhibiting PPB narrowing by cytochalasin D induced asymmetrical division of the cell and Murata and Wada (1991) have shown in *Adiantium* protonema cells that disruption of PPB induced oblique cell plates formation. Thus the PPB does not seem to be essential to morphogenesis, but the absence of a PPB, or a broad PPB, as observed in agarose embedded protoplasts, could lead to the asymmetric division. Thus the absence of PPB in dividing protoplasts cultured in agarose matrix could be one determinant of the occuring assymetrical division.

A diffuse perinuclear fluorescence is shown in protoplasts cultured in liquid medium. In contrast agarose embedded protoplasts showed well defined MTs around the nucleus. This suggests that agarose embedding enhances MT nucleation in the vicinity of the nucleus. If in *Haemanthus*, microtubule organizing centers (MTOC) seem to be located around the nucleus (Lambert 1993), it is still unclear whether a unique or multiple MTOC organize the different types of microtubules arrays in plants. In centriolar cells γ-tubulin is a universal component of MTOC (Joshi et al. 1993). In *Allium* and *Arabidopsis* cells, Liu et al.(1993) localized γ-tubulin at the nuclear envelope, the cortex, the PPB and on the phragmoplast suggesting that different nucleating centers could exist in plant cells. We have recently identified the presence of γ-tubulin in protein extracts of sunflower hypocotyls. Experiments are in progress to investigate on the localization and dynamics of the γ-tubulin in order to elucidate its modulation by agarose embedding culture conditions.

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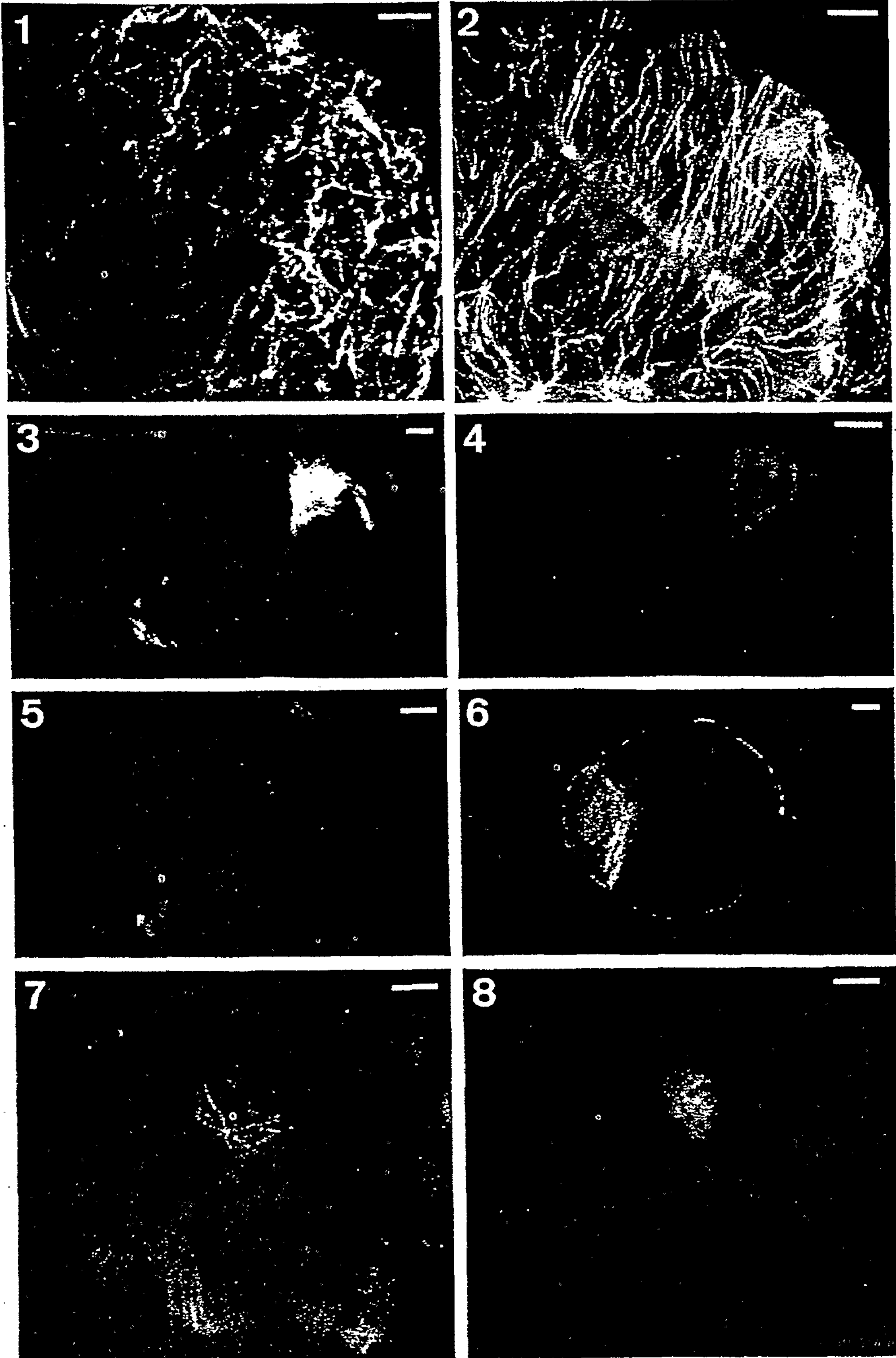
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Fig 1 -8. Microtubule dynamics of protoplasts cultured either in agarose beads (6, 7) or in liquid medium (1-5 and 8):study by immunolabeling of β-tubulin (1, 2, 3, 5, 6, 7, 8) and Hoechst staining of nuclei (4). Bar=5μm

Fig.1 Freshly isolated protoplast showing a dense, randomly oriented cortical microtubule network.

Fig.2. Protoplast cultured for 3 days showing areas of ordered cortical microtubules where long microtubule strands with little branching are present.

Fig.3and 4. Protoplast cultured for 4 days in liquid medium. At this stage nucleus moved to an excentric position and microtubules form around the protoplast a ring (PPB) connected to the PNF (3). Hoechst stained nucleus of the same protoplast: chromatin condensation and loss of nucleolar shape are indicative of early prophase (4).

Fig.5 Four day-old liquid cultured protoplast showing a large phragmoplast in a central position which will lead to a symmetrical division.

Fig.6. Agarose embedded protoplast cultured for 5 days. The phragmoplast is located in an excentric position leading to an unequal division and cortical microtubules reappear at the surface of the two daughter cells.

Fig.7. Agarose embedded protoplast cultured for 2 days showing short well defined microtubules around the nucleus.

Fig.8. Two day-old liquid cultured protoplast showing a diffuse PNF.

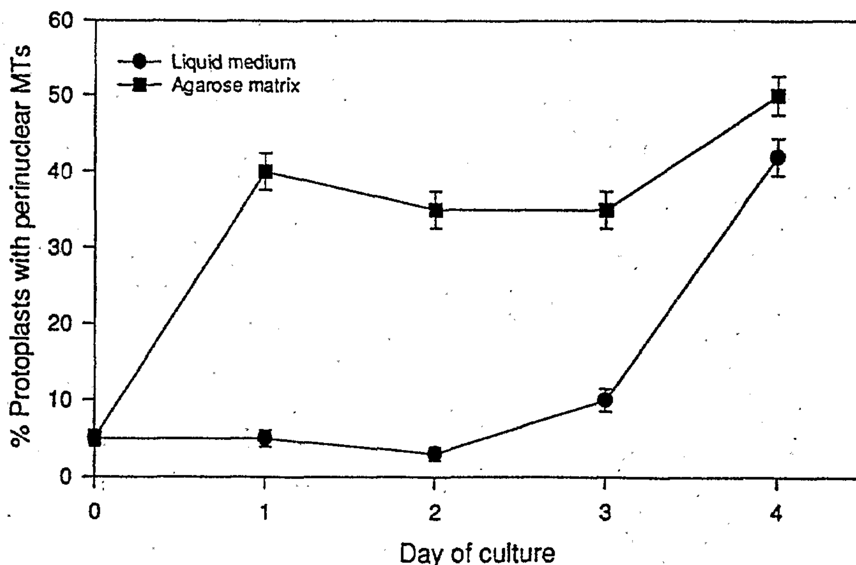

Fig.9. Percentage of protoplasts showing a perinuclear MT network when cultured in liquid medium (●) or agarose matrix (■).