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Effect of anti-microtubular drug amiprophos-methyl on somatic embryogenesis and DNA ploidy levels in alfalfa and carrot cell suspension cultures

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Abstract

A short treatment with the anti-microtubular drug amiprophos-methyl (APM) blocked somatic embryogenesis in alfalfa (*Medicago sativa* L.) and carrot (*Daucus carota* L.). The interruption was temporary and restoration of somatic embryogenesis was observed in long-term cultures. In addition to the effect on somatic embryogenesis, APM treatment induced polyploidization the extent of which was concentration dependent. In long-term alfalfa cultures, APM-induced loss of somatic embryogenesis led to ploidy instability and to a shift to DNA aneuploidy. Critical stages of somatic embryogenesis sensitive to disruption of microtubule-mediated processes were determined in carrot cell cultures. Complete embryogenic arrest occurred when APM was added within the first 5 d of embryogenesis from single cells. The role of the cytoskeleton in the first events of somatic embryogenesis and the relation between totipotency and ploidy stability *in vitro* is discussed.

Introduction

Somatic embryogenesis *in vitro* provides a useful system for the study of differentiation at the cellular level. Recently, the role of cytoskeleton in the initiation of somatic embryogenesis received attention (Cyr *et al.* 1987, Dijak and Simmonds 1988). Differences in cortical microtubule arrays, levels of tubulin proteins, and tubulin mRNAs were observed between embryogenic and non-embryogenic cells (Halperin and Jensen 1967, Borkird and Sung 1985, Cyr *et al.* 1987). The cytoskeleton is known to play an important role in generating and maintaining

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Abbreviations: APM - amiprophos-methyl; DAPI - 4,6-diamidino-2-phenylindole; 2,4-D - 2,4'-dichlorophenoxyacetic acid; KIN - kinetin.

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cell polarity (Schnepf 1986) which is probably important also during somatic embryogenesis (Dijak and Simmonds 1988, Nomura and Komamine 1986). Anti-microtubular drugs may be used to clarify the involvement of cytoskeleton in this process.

In our previous work, we have found that a short treatment of alfalfa embryogenic cell suspension cultures with colchicine induced a loss of totipotency followed by unorganized growth and by the onset of ploidy instability (Doležel and Binarová 1989). Because colchicine must be used in relatively high concentrations and may have various nonspecific side effects (Downes and Marshall, 1983, Hague and Jones 1987) the possibility that interruption of somatic embryogenesis and ploidy instability were caused by such effects cannot be ruled out. Among the compounds which inhibit tubulin polymerization, a phosphoric amide herbicide amiprophos-methyl (APM) is one of the most effective (Morejohn and Fosket 1984) and can be used in much lower concentrations than colchicine (De Laat *et al.* 1987). The purpose of this work was to study the effect of APM on somatic embryogenesis in alfalfa and carrot cell suspension cultures. Embryogenic carrot cultures, which provide a higher efficiency of embryogenesis induction combined with partially synchronized development, were used to determine critical stages of somatic embryogenesis sensitive to disruption of microtubule-mediated processes. Flow cytometry was used to examine changes in DNA ploidy level induced by APM, and to study ploidy level instability in long-term cultures following APM-induced loss of somatic embryogenesis.

Materials and methods

Alfalfa cell suspension culture: Embryogenic cell suspension culture G13/K5 was derived from embryogenic calli initiated from petiole explants of alfalfa (*Medicago sativa* L., 2n = 4x = 32) genotype G13 as described earlier (Binarová and Novák 1985). G13/K5 line maintains high embryogenic capacity and ploidy stability in long-term culture (Binarová and Doležel 1988). The suspensions were maintained in BL medium (Blaydess 1966) supplemented with 5 μM 2,4-D and 1 μM KIN and subcultured at 10 d intervals for 6 months. The cultures were sieved through a 200 μm pore size nylon filter before every subculture.

Carrot cell suspension culture: The embryogenic carrot cell suspension was initiated from hypocotyls of the domestic carrot *Daucus carota* L. (cv. Karotina) seedlings. The stock cell suspension culture was maintained in MS medium (Murashige and Skoog 1962) supplemented with 2 μM 2,4-D and μM KIN for 6 months. Embryogenesis was started by diluting and transferring the cells to an auxin-free MS medium supplemented with 0.5 μM KIN and 20 μM proline.

APM treatment of alfalfa embryogenic cell suspensions: A stock solution of APM (10 g l^{-1}) was prepared in acetone and the treatments at final concentrations ranging from 0.64 to 128 μM were given to actively growing cell suspensions at the 3rd day of

subculture. The cultures were treated for 72 h. Then, APM was removed by rinsing the cells three times in a fresh medium, and the suspensions were maintained under standard conditions. Changes in DNA ploidy levels were analysed during the APM treatments. The number of somatic embryos was determined 21 d after subculture. Somatic embryogenesis and DNA ploidy level stability was further followed in long term culture.

APM treatment of carrot embryogenic cell suspensions: *Treatment of fractionated cultures*: In order to obtain fractions of pro-embryogenic cell clusters differing in size, the stock cell suspension maintained in an auxin medium was filtered through 47, 100 and 230 μm pore size nylon filters before subculture. Individual fractions were transferred into an auxin-free medium to induce somatic embryo development. All fractions were treated with 12.8 μM APM for 72 h, APM was removed by washing the cells three times in fresh auxin-free media. Embryos were scored 21 d later. The efficiency of the APM treatment was monitored using flow cytometry of nuclear DNA content.

Timing of APM treatment: Sieving of the cell cultures and Percol gradient centrifugation procedure according to Nomura and Komamine (1986) were used to induce embryogenesis from single cells. Suspensions with the density of 2×10^4 (cells) cm^{-3} were diluted in a medium with auxin and cultured for 5 d. The cells were then transfered to auxin-free medium. To determine the timing of APM developmental inhibition, APM (12.8 μM) was added over a 48-h period on different days of embryogenesis, starting immediately after the transfer of single cells to an auxin medium (-5 d) and continuing every day untill the 5th day after auxin removal (+5 d). Embryos were scored 21 d after auxin removal.

Flow cytometry: The isolation of cell nuclei and DNA staining with DAPI was performed as described earlier (Doležel *et al.* 1989). At least ten thousand nuclei per sample were analysed with a *Leitz MPV Compact Flow Cytometer* (Ernst Leitz Wetzlar GmbH, Wetzlar, FRG) using UV excitation by a *Leitz Ploemopak* and a *Filterblock A*. Histograms were collected over 512 channels and were analysed with a microcomputer using the *FLOWSTAR* program (Doležel 1989).

To simplify the comparison of the APM-polyploidizing effect in different cultures, we expressed this effect as a difference between the frequency of cells with DNA content equal to 8C or higher in treated and control cultures, respectively. To determine DNA ploidy levels of nuclei isolated from alfalfa somatic embryos and from regenerated plantlets, nuclei isolated from leaves of alfalfa plants (genotype G13), were added to samples prior to staining and used as an internal standard. After simultaneous analysis, the DNA index was calculated as the ratio of the G_1 peak mean of a sample to the G_1 peak mean of the standard. The DNA index expresses the DNA content of the sample in question compared with the normal (in tetraploid alfalfa equal to 4C) DNA content. All experiments consisted of 3 replicates and were repeated at least three times.

Results

Alfalfa embryogenic cell suspension cultures: In untreated alfalfa cultures, the cells were predominantly in G_1 phase of the cell cycle (Fig. 1a). The frequency of cells with DNA content higher than 8C was low, ranging from 3 % to 6 %. APM treatments induced a shift to higher DNA ploidy levels (Fig. 1b).

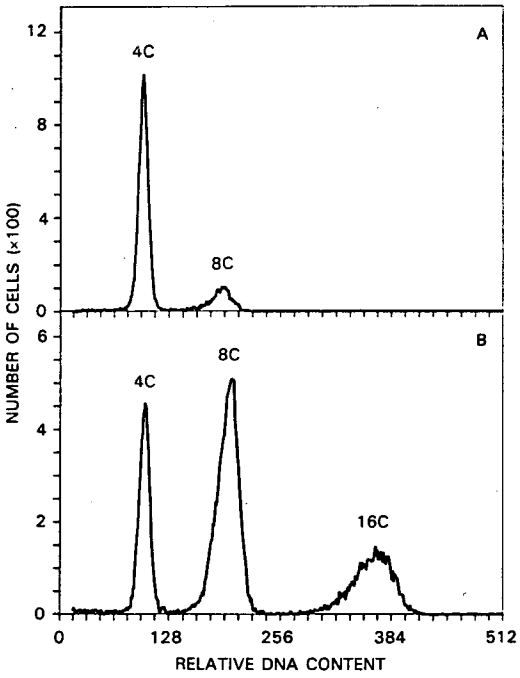

Fig. 1. Frequency distributions of nuclear DNA content in embryogenic alfalfa Gl3/K5 suspension culture. (*a*) the culture in an exponential phase of growth prior to APM treatment. (*b*) the same culture after the treatment with 12.8 μM APM for 72 h.

The extent of the polyploidizing effect was related to the length of the treatment and to APM concentration (Fig.2). The highest increase was induced with APM at concentrations ranging from 1.6 to 12.8 μM. In further experiments, APM was used in concentration 12.8 μM. Even after APM treatments at optimal concentrations for 72 h, an increase in DNA ploidy levels was not observed in all cells. The frequency of non-polyploidized cells with 4C DNA content was 25 %. These cells may represent either non-cycling subpopulation or cells with the cell cycle considerably longer than 72 h.

At the time of APM treatment alfalfa cell suspensions were heterogenous and contained elongated vacuolated cells, small cells with dense cytoplasma, cellular clusters, and small globular embryos. 21 d later, the APM-treated cultures contained unorganized cell clusters, and only 1 - 2 % of torpedo-like embryos, which probably originated from globular embryos treated with APM. No new somatic embryos were

formed, and the cultures continued in unorganized growth. On the other hand, somatic embryos in different stages of development were present in the untreated control culture. Four to five months after APM treatment, partial restoration of somatic embryogenesis was observed.

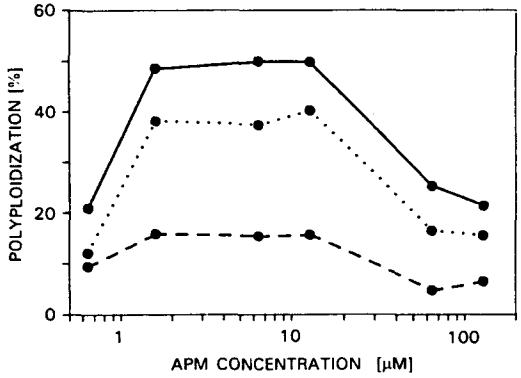

Fig. 2. Relation between the polyploidizing effect and APM concentration in G13/K5 alfalfa cell suspension culture (*dashed line* 24 h, *dotted line* 48 h and *full line* 72 h treatment).

To study the DNA ploidy level stability in long-term alfalfa cultures, more detailed analysis of nuclear DNA content was performed in cell suspensions 5 months after APM treatment at optimal (12.8 μM) and supraoptimal (64 μM) concentrations. The results of the analysis showed that the cultures remained mixoploid and that a shift to DNA aneuploidy occurred. Mean DNA indices were equal to 0.859 and 1.705 for originally tetraploid (4C DNA content) and octoploid (8C DNA content) cells, respectively.

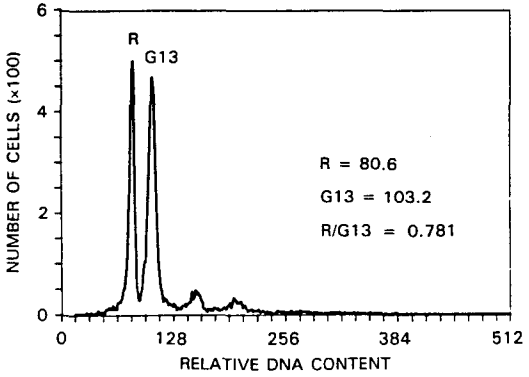

Fig. 3. DNA content distribution obtained after simultaneous analysis of nuclei isolated from a plantlet regenerated from G13/K5 alfalfa suspension culture 5 months after APM treatment (12.8 μM, 72 h) and from a control alfalfa G13 plant. The first and the second peaks from the left correspond to G_1 phase nuclei of the regenerated plantlet (R) and the control plant (G13), respectively. The DNA index of nuclei isolated from the regenerated plantlet is equal to 0.781.

All plantlets differentiated from APM treated suspensions were dwarf, malformed, and did not form roots. Nuclear DNA content was estimated in randomly selected plantlets (Fig. 3, Table 1). None of regenerants showed DNA mixoploidy and aneuploid DNA pattern was found in all of them.

Table 1. DNA indices in plants regenerated from two alfalfa cell suspension cultures five months after 12.8 μM (*A*) and 64 μM (*B*) APM treatment (72 h).

DNA index	Chromosome number*	Frequency of plants**	
		A	B
0.797 - 0.827	26	6.25	9.09
0.828 - 0.858	27	0	9.09
0.859 - 0.890	28	13.50	14.64
1.609 - 1.640	52	6.25	0
1.672 - 1.702	54	19.75	14.64
1.703 - 1.733	55	13.50	18.18
1.734 - 1.765	56	31.25	27.27
1.766 - 1.796	57	0	9.09
1.953 - 1.983	63	6.25	0
2.891 - 2.921	93	6.25	0

* estimated according to the DNA index

**expressed as % of the total number of plantlets analysed (16 in *A* and 22 in *B* variants)

Throughout the whole experiment, control alfalfa cell suspension cultures maintained high embryogenic capacity and a stable nuclear DNA content without any sign of DNA ploidy level instability, regenerated plants were karyologicaly normal.

Carrot embryogenic cell suspension culture: *Fractionated carrot suspension*: In untreated controls observed 14 d after fractionation, the fractions progressed through the individual stages of somatic embryo development (Fig. 4 b,f,j). In APM-treated counterparts, inhibition of somatic embryogenesis was observed and the extent of the embryogenic block depended on the size of pro-embryogenic clusters treated with APM. 21 d after auxin removal, in the 47 μm fraction only dedifferentiated cells were observed, the 100 μm fraction contained 25 % embryos, fraction 230 μm 40 % embryos and 60 % of dedifferentiated cell clusters (Fig. 4 c,g,k). APM treatment induced a shift to higher ploidy levels in all observed fractions. The intensity of polyploidization was highest in the 47 μm fraction and smallest in the 230 μm fraction (Fig. 4 d,h,l). Control fractions maintained a stable DNA ploidy level (Fig. 4 a,e,i).

Timing of APM treatment: During the initial 5 d in an auxin medium the formation of pro-embryogenic clusters was observed but no somatic embryos developed. After transfer to an auxin-free medium early globular stages of somatic embryos occurred within 5 d. Complete embryogenic arrest was observed only when the cultures were

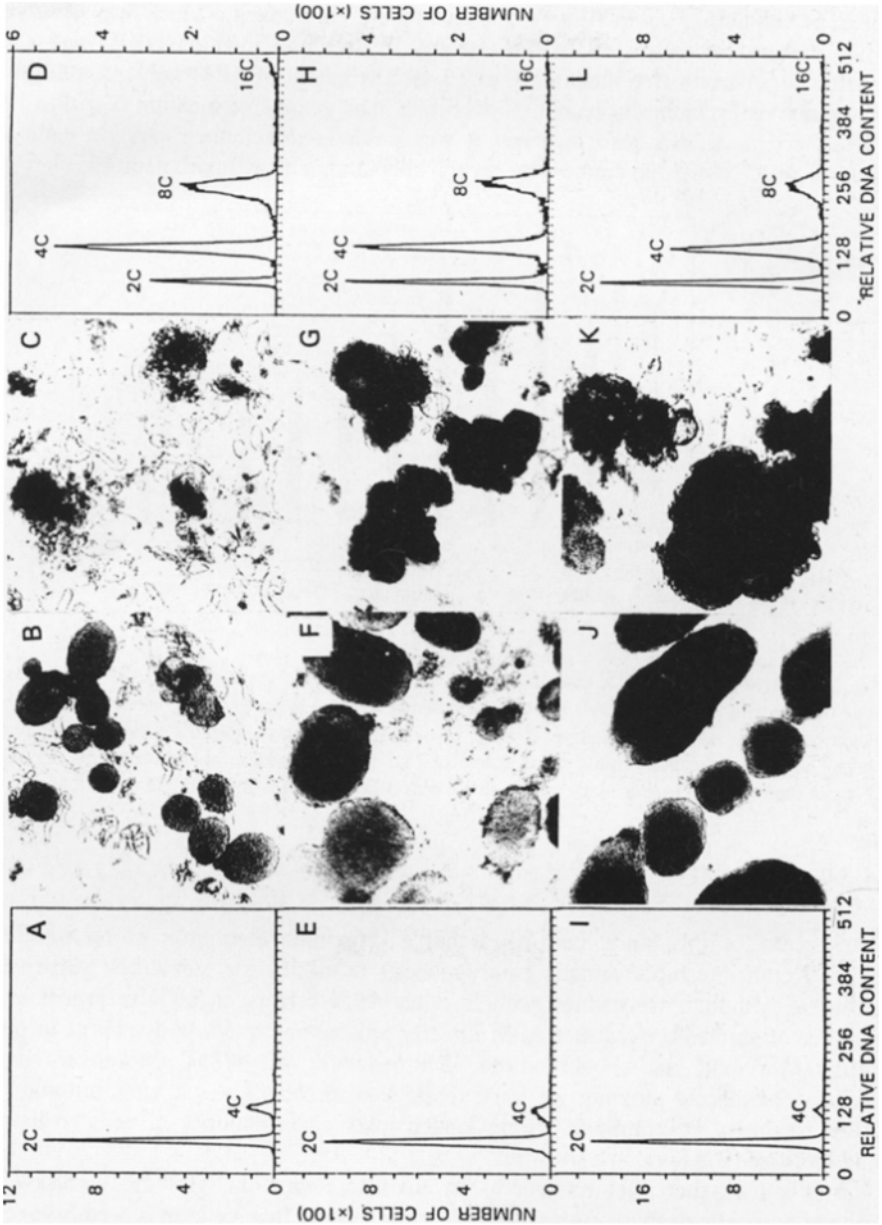

Fig. 4. The effect of APM treatment on somatic embryogenesis and nuclear DNA content distribution observed 14 d after APM treatment in individual fractions of a carrot cell suspension culture (*a, b*- untreated 47 μm fraction, *c, d* - APM treated 47 μm fraction, *e, f* - untreated 100 μm fraction, *g, h* - APM treated 100 μm fraction, *i, j* - untreated 230 μm fraction, *k, l* - APM treated 230 μm fraction.

medium containing auxin (Fig.5). An incomplete embryogenic block was observed when cultures were treated on the last day in an auxin medium (-1 d) and after transfer to an auxin-free medium (0 to +5 d). The extent of APM-induced inhibition of somatic embryogenesis decreased over time in an auxin-free medium (Fig. 5).

Similary to alfalfa, also in carrot it was possible to reinduce somatic embryogenesis in APM-treated cultures by several subcultures in an auxin medium.

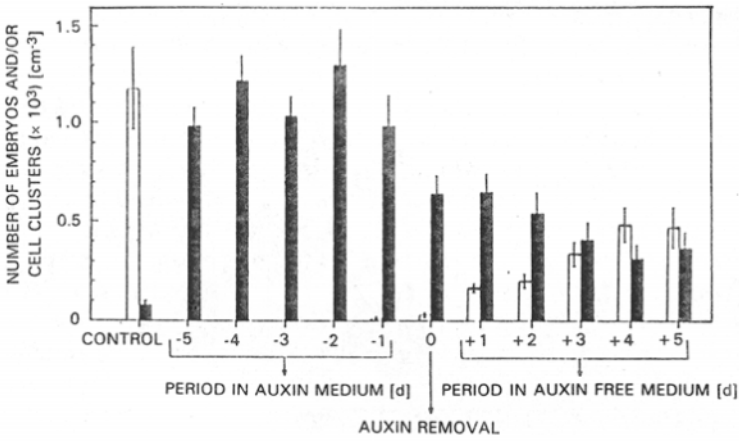

Fig. 5. Determination of APM-sensitive period in carrot somatic embryogenesis. The suspensions were treated with APM (12.8 μM) for 48 h on different days (-5 to +5 d) during embryogenesis from single cells. The number of embryos (*empty columns*) or cell clusters (*full columns*) in untreated control and in APM-treated cultures was estimated 21 d after auxin removal and represents the mean of three separate experiments done in triplicate, *vertical bars* indicate the standard deviations.

Discussion

The results of this study confirmed that a short-time disruption of microtubular cytoskeleton interrupts somatic embryogenesis in alfalfa and carrot cell suspension cultures. Although we cannot exclude other APM effects on cellular processes, a number of studies proved that APM directly poisons microtubule dynamics in plant cells (Morejohn and Fosket 1984, Schroeder *et al.* 1985). Moreover, using immunofluorescent staining we have found that already 1 - 3 h after initiation of APM treatment microtubules are no longer observed in majority of cells (Binarová and Doležel 1991).

It is known that after transfer to an auxin-free medium, globular embryos are formed and cells of these embryos divide more rapidly than cells in pro-embryogenic clusters (Komamine *et al.* 1990). Consistent with this fact, we have found that the highest degree of polyploidization occurred when the rapidly cycling cells of small globular embryos were treated with APM. Interestingly the effect of APM on somatic embryogenesis in these cells was not so strong as compared to APM-treated pro-embryogenic cells and cell clusters before auxin removal. These observations lead to

a conclusion that the block of somatic embryogenesis induced by anti-microtubular drugs is realized in the earliest stages of somatic embryogenesis.

During these developmental stages, increased density of cortical microtubuli was observed (Halperin and Jensen 1967, Dijak and Simmonds 1988). On the other hand, tubulin levels which increase during later stages of somatic embryogenesis, remain unchanged or even decrease during the transition from unorganized growth to embryogenesis (Cyr *et al.* 1987). The treatment with anti-microtubular drugs inhibits synthesis of tubulin in animal and lower plant cells (MacRae and Langdon 1989). Similar effects may be expected also for higher plant cells, however detailed studies are lacking.

The initiation of embryogenesis from single cells is accompanied by unequal cell division creating a developmental polarity which is maintained throughout development (Nomura and Komamine 1986). Microtubuli are not directly involved in the control of polar growth, but their function in nuclear movement has consequences for the realization of polarity (Schnepf and Quader 1987). A short treatment with an anti-microtubular drug was shown to inhibit the movement of the premitotic nucleus and to disturb the normal position of the cross wall in plant cells (Vanverloo and Liebenga 1987). Similarly in developing pollen grains, nuclear movement essential to the unequal division, was inhibited by colchicine treatment (Terasaka and Niitsu 1990).

Disruption of polarity is not the only explanation for the block in embryogenesis induced by anti-microtubular drugs. As it is known from animal systems, information as to the embryogenic pattern and cell proliferation rates are controlled in spatially specific patterns (O'Farell *et al.* 1988). Experiments using colchicine treatment of clam embryos showed that microtubule integrity is necessary for the destruction of cyclin and for the normal progression through the cell cycle (Ruderman 1988), suggesting an important role of cytoskeleton in cell cycle dynamics (Dubel and Little 1988, Verde *et al.* 1990). From this it is implied that mechanisms of regulation of cell cycle are of increasing importance in understanding the process of somatic embryogenesis in higher plants (Komamine *et al.* 1990, Bogre *et al.* 1990).

The loss of totipotency observed in embryogenic cultures is often interpreted as a result of ploidy instability (Coutos-Thevenot *et al.* 1990). Although it is difficult to separate these two phenomena, our previous results (Doležel and Binarová 1989) together with the results of the present study demonstrate that in embryogenic alfalfa cell cultures ploidy instability occurs in long-term culture following the interruption of somatic embryogenesis. This is in agreement with the now widely accepted role of unorganized growth on the destabilization of plant genome *in vitro* (Bayliss 1980, Karp and Bright 1985). Furthermore, the regeneration of aneuploid embryos and plantlets observed in the course of this work and by other authors (Toncelli *et al.* 1985) also does not support the postulate on the primary role of ploidy changes in the loss of embryogenic potential.

It may be concluded that the use of highly efficient anti-microtubular drug enabled us to show that only the first events of somatic embryogenesis are affected when tubulin assembly into microtubuli is inhibited. At the same time, induced block of somatic embryogenesis made it possible to demonstrate that unorganized growth

leads to ploidy instability *in vitro*. Research is in progress to further characterize the role of cytoskeleton in the transition from unorganized to organized growth.

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