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Sites of microtubule reorganization in tobacco BY-2 cells during cell-cycle progression

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Summary. The sites of microtubule (MT) reorganization were examined in synchronized tobacco BY-2 cells. The MTs of these cells were completely destroyed by a combined cold and drug treatment at 0 °C with 100 μM propyzamide for 3 h. After the cells were washed and cultured at 30 °C, the reorganization of MTs was observed in detail. Sites for MT reorganization at each stage of the cell cycle were identified on the cell cortex and nuclei, the mitotic apparatus, the nuclei (or the nuclei and cell cortex), and the cell cortex in the S–G_2 phase, M phase, M/G_1 interface, and G_1 phase, respectively. The polypeptide synthesis elongation factor (EF)-1α is co-localized with these sites of MT reorganization. At some stages, microfilaments (MFs) were found to be involved in the reorganization of MTs. Based on these results, the mode of MT reorganization during cell cycle progression is discussed.

Keywords: Cell cycle; Elongation factor 1α; Microtubule reorganization; Microfilament; Tobacco BY-2 cells.

Abbreviations: EF-1α elongation factor 1α; MAP microtubule-associated protein; MF microfilament; MIs mitotic indices; MT microtubule.

Introduction

In plant cells, five types of microtubule (MT) configuration can be identified at various stages of the cell cycle; namely, cortical arrays, radial cytoplasmic arrays, preprophase bands, mitotic spindles, and phragmoplasts. Sites of MT organization occur at these respective stages and vary between different cell cycle stages. Since higher plant cells do not possess distinct structures for organizing MTs, such as the centrosomes of animal cells, it is intriguing to characterize the sites of MT organization in plant cells in order to understand cellular architecture.

Several studies have suggested that the nuclear surface is one such site of MT organization. Mizuno (1993) reported that MTs were nucleated on isolated nuclei that had been injured to some extent, and also on particles prepared from nuclei of tobacco BY-2 cells, to which tubulins from bovine or tobacco were added. Stoppin et al. (1994) also demonstrated MT nucleation on isolated maize nuclei after supplementation with bovine tubulins. We previously showed by immunofluorescence microscopy that, during the cell-cycle transition from M to G_1 phase, MTs were organized at the perinuclear region at the expense of decomposing phragmoplasts (Hasezawa and Nagata 1991). MT elongation up to the cell cortex was followed by further elongation parallel to the long axis of cells. As soon as the tips of the elongated MTs reached the distal end of the division plane, the transversely oriented MTs were formed proximal to the division plane (Nagata et al. 1994). Although the nuclear surface is the site of MT organization, the organized MTs are not the direct precursors of cortical MTs (Nagata et al. 1994). At different stages of M phase and G_1 phase, the nuclear surface cannot act as a site for organizing MTs, since MTs are predominantly observed on the cell cortex in the G_1 phase and the nuclear envelope is not discernible during M

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phase. Thus, the characterization of MT organization sites at different stages of the cell cycle is an intriguing and fundamental issue that needs to be resolved.

By following the reorganization of MTs after the destruction of preexisting MTs by some process provides a simple means by which the mode of organizing MTs at the respective stages can be better understood. There have been several reports, in which the reorganization of MTs was followed after the destruction of MTs by various treatments, such as cold treatment in *Closterium* (Hogetsu 1986) and drug treatment in *Spirogyra* (Hogetsu 1987) and *Nitella* (Wasteneys and Williamson 1989). A critical point in such studies, however, should be whether MTs are sufficiently depolymerized, and the absence of structurally discernible fibrous components of MTs after immunostaining with anti-tubulin antibody under fluorescence microscopy should be a prerequisite. In this context, as the MTs of tobacco BY-2 cells were reported to show substantial resistance to cold treatment at 0 °C for 3 h (Akashi et al. 1990), a combination of cold treatment and drug treatment could be a plausible means by which MTs in tobacco BY-2 cells could be efficiently destroyed in order to fulfill the above requirement. It should also be noted, however, that treatments used to destroy MTs should have minimal detrimental effects on the treated cells. A preliminary trial demonstrated that the complete destruction of MTs in tobacco BY-2 cells with a combined treatment of cold and propyzamide, a microtubule depolymerizing drug, had no apparent detrimental effects on these cells. In this study, therefore, MT reorganizations during cell cycle progression have been observed using highly synchronized tobacco BY-2 cells after complete destruction of MTs by that combined cold and drug treatment, for examining where and how the five types of MT configuration organized.

In addition, some proteins, such as γ-tubulin, appear to be involved in MT nucleation in plant cells (Liu et al. 1993). The polypeptide synthesis elongation factor (EF)-1α, which is thought to function as MT-bundling, MF-bundling, and calmodulin-binding protein (Numata 1996), may play some role on MT nucleation. We have already shown that EF-1α (a 49 kDa protein) is associated with organizing MTs in tobacco BY-2 cells (Hasezawa and Nagata 1993), and that this association occurs exclusively with organizing MTs on the perinuclear region during the cell-cycle transition from the M to G_1 phase (Kumagai et al. 1995). Thus, an examination of the association of EF-1α and the sites of reorganization of MTs throughout the cell cycle would be expected to provide further insight into the MT organization sites at the respective cell cycle stages.

Material and methods

Synchronization of tobacco BY-2 cells.

Tobacco BY-2 cells, developed from *Nicotiana tabacum* L. cv. Bright Yellow 2, were maintained by dilution with a modified Linsmaier and Skoog (1965) medium at weekly intervals, as described by Nagata et al. (1992). Synchronization was carried out as described by Hasezawa and Nagata (1991). Tobacco BY-2 cells were released from a 24 h treatment with aphidicolin, and the cells were then harvested at 1, 6, and 9 h after the release from aphidicolin to allow examinations of S, G_2, and M phases, respectively. To obtain highly-synchronized cells starting from metaphase, propyzamide (3 μM) was added to the BY-2 cells to induce mitotic arrest 6 h after the release from aphidicolin. The propyzamide was then removed after a 4 h treatment period. The cells were harvested at 2 and 4 h after the release from propyzamide for the examinations of M/G_1 and G_1 phases, respectively.

Destruction and reorganization of MTs

Tobacco BY-2 cells were treated with 100 μM propyzamide at 0 °C for 3 h (Akashi et al. 1988, Murata 1996), washed once with the ice-cold medium and then cultured at 30 °C for 1, 5, 15, 30, and 60 min for the reorganization of MTs. Subsequently, the cells were harvested by centrifugation and fixed in 3.7% (w/v) formaldehyde solution (Hasezawa and Nagata 1991) 1 min after the termination of the culture period.

Immunofluorescence microscopy

The fixed cells were placed onto coverslips coated with poly-L-lysine. To stain MTs and nuclei, the cells were first treated with an enzyme solution containing Cellulase Y-C and Pectolyase Y23 (Seishin Co., Tokyo, Japan), and then with a detergent solution as described by Hasezawa and Nagata (1991). Subsequently, the cells were incubated with antibodies against tubulin (1:1 mixture of mouse anti-α-tubulin and anti-β-tubulin; Amersham International plc., Buckinghamshire, U.K.) and then with a fluorescein (FITC)-conjugated anti-mouse antibody (Sigma Chemical Co., St. Louis, MO, U.S.A.). After staining with a 4′,6-diamidino-2-phenylindole (DAPI) solution, the specimens were finally embedded in a glycerol solution.

For double staining with antibodies against tubulin and the 49 kDa protein (tobacco EF-1α), two sets of primary and secondary antibodies were employed. To stain MTs, YL 1/2 against tubulin (a rat monoclonal antibody; Sera Laboratories, Crawley Down, U.K.) and a FITC-conjugated anti-rat antibody (no cross to mouse) were used. For staining the 49 kDa protein, KU-2 (a polyclonal antibody against the 49 kDa protein raised in mouse) and a rhodamine-conjugated antibody against mouse (Cappel Laboratories, West Chester, PA, U.S.A.) were employed.

The specimens were examined with a fluorescence microscope (Axiophot FL; Carl Zeiss, Oberkochen, Federal Republic of Germany). Photomicrographs were recorded on Super Prest film (Fuji Photo Film Co., Tokyo, Japan)

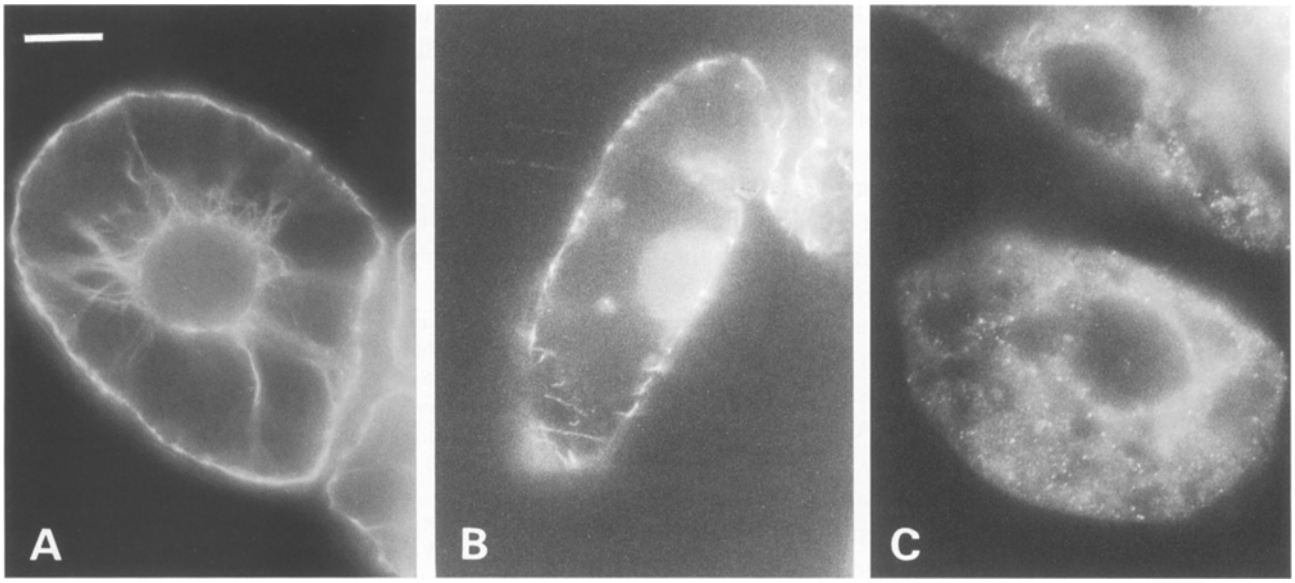


Fig. 1 A–C. Destruction of MTs of BY-2 cells by cold and propyzamide treatments. The cells were stained with anti-tubulin antibody. **A** Cortical and cytoplasmic MTs can be observed in control BY-2 cells. **B** Some cortical MTs remained even though the BY-2 cells were maintained at 0 °C for as long as 3 h. **C** MTs were completely destroyed by treatment of BY-2 cells with 100 μM propyzamide at 0 °C for 3 h. Bar: 10 μm

Results

Destruction and reorganization of MTs during cell-cycle progression

In order to follow the reorganization of MTs during cell-cycle progression, the complete absence of polymerized forms of MTs is an essential prerequisite. It has been reported that the MTs of tobacco BY-2 cells are rather resistant to cold treatment (Akashi et al. 1990), whereas the MTs of animal cells are quite susceptible. As these findings were confirmed in an early part of this study (Fig. 1 B), a combination of cold treatment and 100 μM propyzamide for 3 h was examined for its ability to completely destroy MTs. Indeed, the MTs were found to be completely depolymerized throughout the cell cycle (Fig. 1 C) by this treatment, with no appreciable differences in MT destruction between the four cell cycle stages. Consequently, throughout this study, a combination of cold and propyzamide treatment was used for the destruction of MTs.

Subsequently, we examined whether the destructive treatment of MTs had any appreciable detrimental effects upon the reorganization of MTs in tobacco BY-2 cells. This was achieved by examining whether treatment retarded cell-cycle progression in highly synchronized tobacco BY-2 cells. As shown in Table 1, the mitotic indices (MIs) of tobacco BY-2 cells following combined treatment were 1.5%, 28.4%, and 53.5% at 5, 7, and 9 h, respectively, after the release from aphidicolin treatment. As these MIs did not differ significantly from those of control cells without the cold and drug treatments, it appears that cell-cycle progression was completely halted by the combined treatments. Furthermore, when treated cells were recultured after treatment, their MIs did not differ significantly from MIs of cells with no treatment (Table 1). Thus, based on the progression of mitosis, it appears that treated cells showed no signs of damage resulting from treatment, and that they were able to recover immediately after treatment. The recovery of MT organization as determined by immunofluorescence microscopy was detected when the cells were cultured for 1 min after the combined cold and drug treatment, and was mostly complete within 15 min.

Reorganization of MTs at different cell cycle stages

At the S and G_2 phases, MTs were reorganized in the perinuclear region as well as on the cell cortex (Fig. 2 A). Cytoplasmic MTs appeared to emanate from the perinuclear regions. At late G_2 phase, MTs including preprophase band were reorganized in the same way (Fig. 2 B). At prophase, short MTs first accumulated around the chromosomes (Fig. 2 C, D), at which time the spindle started to be reorganized, although the poles were obscure at this stage. Subsequently, the regenerated spindles began to display their typical

Table 1. Cell-cycle progression of synchronized BY-2 cells after the destruction of microtubules

Time after release from aphidicolin (h)	Mitotic index (%)
Control	
5	1.9
7	29.8
9	51.7
Cold and drug treated	
5 + 3	1.5
7 + 3	28.4
9 + 3	53.5
Re-cultured	
5 + 3 + 2	26.8
5 + 3 + 4	52.9

5, 7, and 9 h after the release from aphidicolin, the microtubules of synchronized BY-2 cells were destroyed by the combined cold and propyzamide treatment. The similarity between the mitotic indices (MIs) of cells after destructive treatment and those of the control cells indicates that cell-cycle progression was completely halted by the treatment. The similarity of the MIs of cells re-cultured after the destructive treatment until 10 and 12 h after release from aphidicolin and the MIs of control cells indicates that the destructive treatment had no detrimental effects, at least in terms of cell-cycle progression. Three more examinations were conducted independently, and the trend was very similar

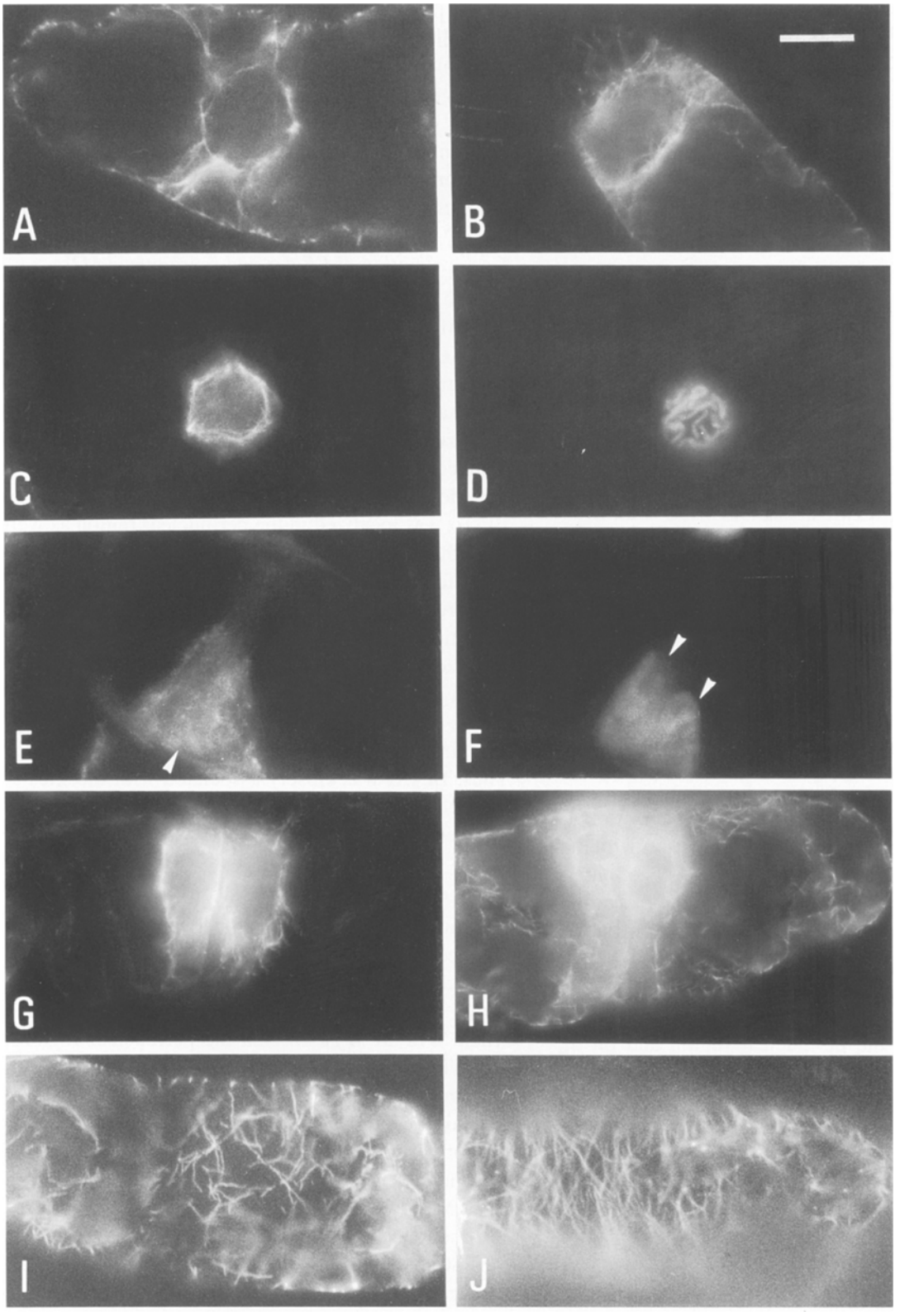
shapes with two distinct poles. Reorganization of spindle MTs was rather rapid in comparison with that of phragmoplast MTs at telophase. The MTs of phragmoplast were slowly reorganized around the daughter nuclei and obscure at first (Fig. 2 E, F). During the cell-cycle transition from M to G_1 phase, MTs were organized solely on the perinuclear regions (Fig. 2 G). At some frequencies, MT organization was also observed both in the perinuclear region and on the cell cortex (Fig. 2 H). These patterns of MT organization correspond to the five types observed during the cell-cycle transition from M to G_1 phases (Nagata et al. 1994); short MTs were first formed in the perinuclear region (I) and elongated to the distal end of the cells (II–IIIa), and then the transverse cortical MTs were formed near the division plane (IIIb–IIIc). In this study, the reorganization of MTs solely on the perinuclear regions reflected earlier types of I to IIIa, and MT reorganization at both the nuclear surface and cell cortex reflected the later types of IIIb to IIIc. This supposition was in fact confirmed by scoring the frequency of the respective types (data not shown). At the G_1 phase, MTs were predominantly reorganized in the cell cortex, and MT organization on the nuclei was seldom observed (Figs. 2 I and 3 E). Although the regenerated cortical MTs showed rather random arrays in direction, shortly after recovery from the combined cold and drug treatment, they showed typical transversely-oriented MTs perpendicular to the long axis at least 15 min after the recovery (Fig. 2 J).

Co-localization of EF-1α and reorganized MTs

As we had shown previously that tobacco EF-1α (identified earlier as a 49 kDa protein) was closely associated with the MT organization sites during cell-cycle transitions (Hasezawa and Nagata 1993, Kumagai et al. 1995), we examined whether EF-1α is co-localized with the reorganized MTs after the release from cold and drug treatment. Staining with the KU-2 polyclonal antibody against EF-1α demonstrated that EF-1α was localized in the perinuclear region as well as in the cell cortex at the S and G_2 phases (Fig. 3 B), at which time MTs were reorganized after the cold and drug treatment (Fig. 3 A). During the cell-cycle transition from the M to the G_1 phase, EF-1α was localized in the perinuclear region (Fig. 3 D), from which MTs were reorganized (Fig. 3 C). At the G_1 phase, EF-1α was localized in the cell cortex (Fig. 3 F), in which MTs were also reorganized (Fig. 3 E). Thus, EF-1α was closely associated with the sites at which MTs were reorganized after release from the MT-destructive treatment.

Effect of cytochalasin D on the reorganization of MTs

Although a close association between MTs and MFs has been reported, it appears that this association is dependent on the stage of the cell cycle (Staiger and Lloyd 1991). In fact, we have shown previously that when MTs are organized in the perinuclear regions, the MTs are not associated with MFs but rather with EF-1α (Kumagai et al. 1995). This association could be classified by examining the effect of the destruction of MFs on the reorganization of MT-destructive treatment. When tobacco BY-2 cells were treated with 100 μM cytochalasin D throughout the destructive treatment and re-culture periods, MTs regenerated at the S–G_2, M, M/G_1, and G_1 phases (Fig. 4 A–D). Only during the S–G_2 phase was the development of radial MTs severely retarded, with only vestigial MTs being formed. It suggests that the MFs may play some role on the reorganization of radial MTs; however, the role of MFs may not be so critical on those of other MTs.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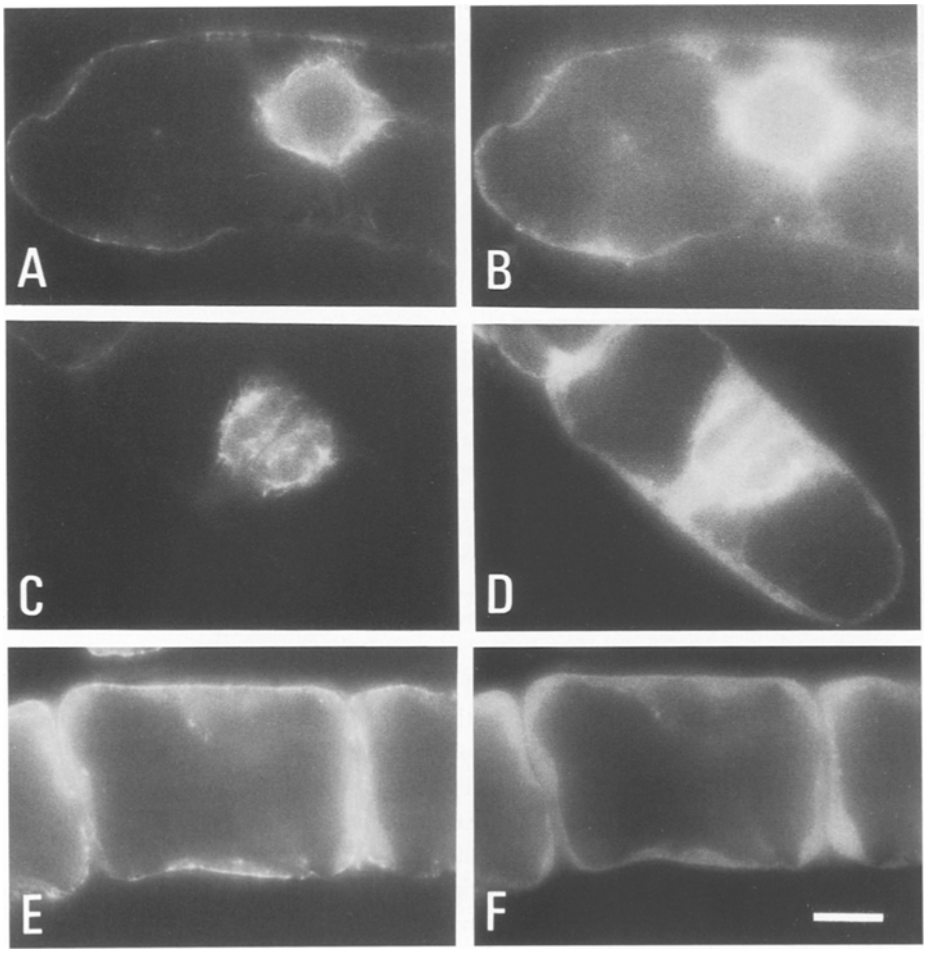

Fig. 3 A–F. Co-localization of EF-1α with the sites of MT reorganization. **A** After the destruction of MTs, BY-2 cells were cultured for 1 min. Regenerated MTs and EF-1α were observed by staining with anti-tubulin and anti-EF-1α antibodies, respectively. **A** and **B** EF-1α (**B**) was localized in the perinuclear region and in the cell cortex where the MTs (**A**) were reorganized at the S and G_2 phases. **C** and **D** EF-1α (**D**) was mainly localized in the perinuclear region where the MTs (**C**) were reorganized at the M/G_1 interface. **E** and **F** EF-1α (**F**) was predominantly localized in the cell cortex where the MTs (**E**) were reorganized at the G_1 phase. Bar: 10 μm

Discussion

In this study, we have established that a combination of cold treatment and 100 μM propyzamide completely destroys the organized MTs in tobacco BY-2 cells throughout the plant cell cycle. When these cells were released from this treatment, MTs were immediately and very rapidly reorganized. Furthermore, it appears that this treatment had no detrimental effects on the biological activity of the treated cells, at least in terms of cell-cycle progression. Although some retardation was observed in phragmoplast reorganization, this may be an intrinsic feature of its structure,

Fig. 2 A–J. Reorganization of MTs at different cell-cycle stages. MTs of BY-2 cells at different stages were destroyed by a combined cold and drug treatment. Subsequently, the cells were washed and cultured in fresh medium at 30 °C for 1 min (**A–I**) or 5 min (**J**), and stained with anti-tubulin antibody and DAPI. **A** MTs were reorganized in the perinuclear region and the cell cortex at S and G_2 phases. **B** MTs including preprophase band were reorganized in the perinuclear region and the cell cortex at late G_2 phase. **C** and **D** Short MTs (**C**) accumulated around the chromosomes (**D**) to reconstruct the spindle at prophase. **E** and **F** MTs of phragmoplast were slowly reorganized around nuclei and obscure at fist (arrowhead in **E** indicates the division plane; arrowheads in **F** indicate the daughter nuclei). **G** and **H** MTs were reorganized solely in the perinuclear region (**G**), or in both the perinuclear region and in the cell cortex (**H**) during the cell-cycle transition from M to G_1 phase. **I** and **J** MTs were predominantly reorganized in the cell cortex at the G_1 phase. The regenerated MTs were initially rather randomly oriented (**I**), but then became arranged into transverse cortical arrays (**J**). Bar: 10 μm

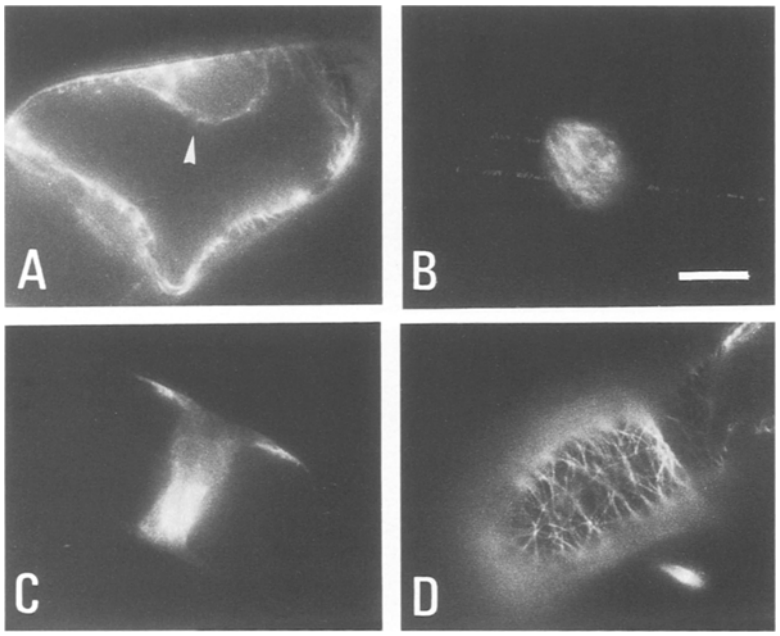

Fig. 4 A–D. Effects of cytochalasin D (CD) on the reorganization of MTs. After the destruction of MTs, BY-2 cells were cultured for 30 min and stained with anti-tubulin antibody. Throughout these procedures, 100 μM CD was included in the culture medium. **A** Cortical MTs were reorganized, whereas radial MTs were not (arrowhead), in response to the effects of CD at the S and G_2 phases. **B–D** MTs were reorganized at M (**B**), M/G_1 (**C**), and G_1 (**D**) phases similar to that observed without CD treatment. Bar: 10 μm

since it exists only temporarily towards the end of the M phase and has a complex structure.

The current study constitutes the first report in which the regeneration of all five types of MTs was followed experimentally. The results can be summarized as follows: After destruction, MTs were reorganized as under natural reorganization conditions. Thus, MT reorganization after MT destruction appears to reflect the natural process of MT organization. The sites of MT reorganization were well preserved in the cells after treatment with cold and propyzamide. Furthermore, EF-1α, previously shown to be closely associated with the organization of MTs when the MTs were organized during the transition from M to G_1 phase (Hasezawa and Nagata 1991, Kumagai et al. 1995). In this study, EF-1α was co-localized with the reorganizing MTs through cell cycle, suggesting some role in MT organization. MFs were not necessarily required for the reorganization of MTs, except for the development of radial MTs.

Therefore, it appears that the reorganization of MTs could be utilized as a model system for the study of the natural organization of MTs. In this context it should be noted, however, that during the reorganization of MTs after MT destruction, the intracellular concentrations of tubulins were likely to have been biased. The question arises therefore whether localized concentrations of tubulins affect the reorganization of MTs. Although this question could not be addressed directly by the results of this study, we consider this possibility unlikely, since even after the destruction of developing MTs the preexisting sites of MT organization were utilized for MT reorganization. From these obervations we conclude therefore that the sites of MT organization were well preserved.

Our study also clearly demonstrates that the nuclear surfaces are the sites of MT reorganization during the cell-cycle transition from M to G_1 phase, and S and G_2 phases. An intriguing question that remains is as to the type of factors responsible for MT organization on the perinuclear regions. Recently, Stoppin et al. (1996) reported that MT nucleation on isolated nuclei from tobacco BY-2 cells is dependent on microtubule-associated proteins (MAPs) from some specific stages of the cell cycle. MAPs from interphase of tobacco BY-2 cells could induce MT organization in isolated nuclei, whereas those from M phase could not. Thus, MAPs are potential candidates of factors involved in the organization of MTs in isolated nuclei. However, we also unambiguously demonstrate that the cell cortex is a site of MT organization, although, shortly after the release from MT destruction, the reorganized MTs were rather random in orientation. The regenerated MTs were V- or Y-shaped, as described in *Nitella* cells (Wasteneys and Williamson 1989). Subsequently, they became parallel in orientation, and perpendicular to the long axis of the cells. It thus appears that these two steps are involved in the organization of MTs in the cell cortex; first the formation of V- or Y-shaped MTs, and subsequently the orientation of MTs. These steps are most certainly regulated by some specific structures, but, although MFs have

been proposed to fulfill this purpose, the destruction of MFs by cytochalasin D did not significantly affect this organization, indicating that some other factor(s) are involved. To address the question of the regulation of MT organization, further study, for example an examination of the actual reorganization of MTs on protoplast ghosts (Sonobe 1990), may reveal more details of the components that seem to be involved in MT reorganization.

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