Plant Physiol. (1992) 100, 740–748
0032-0889/92/100/0740/09/$01.00/0

Received for publication November 7, 1991
Accepted April 20, 1992

Induction of Cold Stability of Microtubules in Cultured Tobacco Cells

Koichi Mizuno

Department of Biology, Faculty of Science, Osaka University, Machikaneyama 1–1, Toyonaka, Osaka 560, Japan

ABSTRACT

In suspension-cultured tobacco (*Nicotiana tabacum*) cells, we have often encountered cold-stable microtubules (MTs). The cold-stable MTs were found in the pelleted fraction of tobacco cell homogenates. These cold-stable MTs were shown to be accompanied by unidentified filamentous structures that extended along part of their length. However, during the early hours in culture such cold-stable MTs were never observed. They were detectable from 120 h after the beginning of subculture and then their numbers increased gradually. The number of cells with cold-stable MTs eventually accounted for more than 95% of the total population of cells at the stationary phase of culture. The rapid loss of cold stability of MTs occurred when such cells were transferred to fresh medium for subculture. However, if the fresh medium was supplemented with once-used medium, the cold stability of MTs was retained. The active agent in the medium appeared to be of low molecular weight and to be heat resistant. A similar activity was detected in a pectin hydrolyzate. When an inhibitor of protein kinase, either 6-dimethylaminopurine or staurosporin, was added to the cells at an early stage of culture, when cold-stable MTs were normally completely absent, most cells acquired cold-stable MTs. It appears that acquisition or loss of cold stability of MTs in tobacco cells is regulated by the action of a kinase/phosphatase or a phosphorylation/dephosphorylation system on some MT protein(s), such as a cold stabilizer of MTs, some unidentified MT-associated filamentous structure, or even tubulin itself.

MTs[1] are widely regarded as playing a crucial role in cell growth, intracellular transport, and maintenance of cell shape (for reviews, see refs. 9, 16). The diverse functions of MTs in cells often require the precise control of the assembly equilibrium and of polymer stability. These controls are exerted by specialized proteins that selectively associate with MTs for specific purposes (for review, see ref. 7). In mammalian cells, there appear to be different populations of cytoplasmic MTs that can be differentiated on the basis of their resistance to depolymerization at low temperature. Brinkley and Cartwright (5) and Salmon and Begg (26) showed that the kinetochore-to-pole MTs do not depolymerize at low temperature, although the interphase MTs depolymerize. Jones et al. (13), in an electron microscopic study of neurons in the rat brain, found that numerous intact MTs were present after incubation of tissue slices for 1 h at 0°C, and they suggested that resistance to disassembly at low temperature is conferred on MTs by the presence of a nontubulin factor of unknown identity.

Comparing cold-stable and cold-labile populations of MTs, Webb and Wilson (28) found no difference in either the protein composition or the isoelectric properties of tubulin. Margolis and coworkers (11, 12, 17, 23) have also studied similar cold-stable MTs isolated from rat and sheep brain and concluded that the cold stability resides not in the tubulin but in several auxiliary proteins associated with the MTs. However, evidence has been reported that axonal cold-insoluble tubulin is biochemically distinct from tubulin obtained from whole-brain MTs prepared by cold cycling (4, 8). Much of our knowledge about the properties of tubulin and MTs is based on the results of studies of that fraction of MT protein that is solubilized in low-temperature homogenates of cells.

Little is known about the function or properties of either cold-extractable or cold-insoluble MTs in cells of higher plants. It is clear, however, that MTs are not all equivalent. They may differ with respect to ultrastructural details, resistance to destabilizing conditions, cellular distribution, and associated proteins. It is important to determine the mechanism of acquisition and loss of cold stability of MTs if we are to understand MT functions in cells of higher plants.

Depolymerization of MTs has been reported to cause the decrease of chilling resistance (25). Gibberellin was found to decrease cold stability of cortical MTs in epidermal cells of pea internodes (2). However, this result was not consistent with the finding that gibberellin increased cold stability of MTs in onion leaf sheaths (18). Kerr and Carter (14) reported that the different cold stability of MTs in root-tip cells was detected between cold-acclimated and nonacclimated rye, and showed the alteration of tubulin isoforms during cold acclimation (15). However, only small differences in MT stability were detected between cold-acclimated and nonacclimated spinach (*Spinacia oleracea*) mesophyll cells (3).

Suspension-cultured tobacco (*Nicotiana tabacum* Bright Yellow 2) cells are a suitable material for investigations of cold-stable MTs because their MTs lack cold stability completely during the early stages of culture and acquire cold stability gradually but lose it again when transferred to fresh medium for subculture. Therefore, this system appears to be useful for examining the process and mechanism whereby the cold stability of MTs is acquired and lost.

6-DMAP has been shown to block the cell cycle in the bivalve *Spisula* and in the sea urchin without affecting protein synthesis (24), and it was found to affect the activity of protein kinase exclusively both in vivo in starfish oocytes and

[1] Abbreviations: MTs, microtubules; 6-DMAP, 6-dimethylaminipurine; CERF, cytoskeletal element-rich fraction; MTOC, microtubule organizing center; 2-D, two-dimensional.

in vitro in extracts of these oocytes (20). Furthermore, *cdc-2* kinase was shown to be inhibited by 6-DMAP in extracts of *Xenopus* eggs (27). In the present report, 6-DMAP was used effectively to induce cold stability of MTs in tobacco cells, as was staurosporin, which is a well-known inhibitor of protein kinases.

MATERIALS AND METHODS

Plant Material

Tobacco (*Nicotiana tabacum* Bright Yellow 2) cells, provided by Professor T. Nagata of Tokyo University, were cultured in suspension in Linsmaier and Skoog's medium supplemented with 3% (w/v) sucrose and 0.2 mg/L 2,4-D, at pH 5.8, at 27°C in the dark. These cells were subcultured at 7-d intervals in 300-mL Erlenmeyer flasks that contained 100 mL of medium.

For the production of protoplasts, cells were treated with wall-lysis medium that consisted of 1% Cellulase Onozuka RS (Yakult Honsha Co. Ltd., Tokyo, Japan) and 0.1% pectolyase Y23 (Seishin Pharmaceutical Co. Ltd., Chiba, Japan) in 0.4 M mannitol, pH 5.5. After incubation at 27°C for 1.5 h, the suspension of cells was filtered through 50-μm nylon mesh. The protoplasts were then sedimented by centrifugation for 5 min at 500*g* and washed with 0.4 M mannitol.

Immunofluorescence Observations

For cold treatment, intact cells and protoplasts were collected in Eppendorf centrifuge tubes and put under ice water, usually for 1 h. As controls, cells in Eppendorf centrifuge tubes were incubated at 27°C for 1 h. For the immunofluorescence observation, cells were handled according to the similar procedures described by Wick et al. (29). Cells were fixed with 3.7% formaldehyde in PBS, pH 7.4, for 1 h at 27°C. After two washes in PBS for 3 min each, cells were treated with a solution of wall-digesting enzymes (1% Cellulase Onozuka RS, 0.1% pectolyase Y23 in 0.4 M mannitol, pH 5.5) for 3 min at 27°C, then washed twice with PBS that contained 0.05% Tween 20. After a 5-min treatment in permeabilizing solution (0.1% Nonidet P-40 in PBS), the cells or protoplasts were dispersed on glass slides that had previously been coated with 0.1% (w/v) polylysine and allowed to settle on the slides. After a brief wash with PBS, they were treated with monoclonal antibody against mung bean tubulin (19) for 1 h at 36°C. Fluorescein isothiocyanate-labeled rabbit antibodies against mouse immunoglobulin G (ICN, ImmunoBiologicals, Lisle, IL) were applied as second antibody and specimens were incubated for 1 h at 36°C. Finally, single drops of a solution of 5′,6′-diamidino-2-phenylindole at 0.1 μg/mL were applied to stain nuclei for the counting of cell numbers. The preparations were mounted with 50% glycerol in PBS that contained 0.1% *p*-phenylenediamine and the fluorescence was observed with an Olympus BH-2 fluorescence microscope fitted with a DApo 40 UV PL lens. The cells containing more than 10 MTs that stained over more than 10 μm of their length were defined as cells that contained cold-stable MTs.

Preparation of a Cold-Stable MT-Rich Fraction

Cells cultured for 8 d were used as a source of cold-stable MTs. The cells (10 g) were homogenized with a Tomy ultrasonic disruptor, model UD-201 (Tomy Seiko Co. Ltd., Tokyo, Japan) in 50 mL of extraction buffer (100 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), 1 mM $MgSO_4$, 1 mM DTT, 0.2 mM PMSF, 25 μg/mL of leupeptin, 25 μg/mL of pepstatin A, 1 mM GTP, 1 mM EGTA, pH 6.9). The homogenate was centrifuged at 100,000*g* for 1 h at 4°C. The resultant pellet was used as a source of cold-stable MTs. The pellet was resuspended in extraction buffer and dispersed in a Teflon homogenizer, then the suspension was centrifuged at 30,000*g* for 1 h at 4°C. The supernatant was centrifuged again at 100,000*g* for 1 h at 4°C. Because the pellet contained various filamentous structures, including cold-stable MTs, this fraction is referred to as CERF.

Treatment of Tobacco Cells with Inhibitors of Protein Kinase

The preparations of tobacco cells in suspension were supplemented with solution in DMSO of an inhibitor of protein kinase, either 6-DMAP or staurosporin, and incubated at 27°C for an appropriate period. The final concentration of DMSO was adjusted so that it did not exceed 5% (v/v). Then the cells were incubated at 0°C for 1 h to investigate cold-stable MTs.

Preparation of Pectin Hydrolysate

Citrus pectin (10 mg/mL) was hydrolyzed by addition of pectinase from *Aspergillus niger* at a concentration of 10 μL/mL (Sigma). The mixture was incubated for 1 h at 30°C and then the reaction was terminated by heating over boiling water for 1 min. After removal of the precipitate with centrifugation (5000*g* for 10 min at 4°C), the supernatant was added to fresh medium at an appropriate concentration.

2-D PAGE and Immunoblotting

The 2-D PAGE system of O'Farrell (22) was used. The preparations of cells were homogenized in a glass homogenizer in equal volumes of SDS-sample buffer (0.25 M Tris-HCl buffer, pH 6.9, containing 1% SDS, 20% glycerol, 10% 2-mercaptoethanol) over boiling water for 3 min to avoid proteolysis, and centrifuged at 10,000*g* for 5 min at 4°C. Resultant supernatants were used for electrophoresis. To each of the samples, urea, Nonidet P-40, ampholine (1.5% pH 5–7, 0.5% pH 3.5–10; Pharmacia LKB, Uppsala, Sweden) and 2-mercaptoethanol were added to final concentrations of 8.5 M, 2%, 2%, and 5%, respectively, before isoelectric focusing. Isoelectric focusing was performed in gels that contained a mixture of 1.5% pH 5 to 7 and 0.5% pH 3.5 to 10 ampholine. Isoelectric focusing was performed at 300 V for 12 h, then at 700 V for 1 h. After immersion in SDS-sample buffer for 2 h at 36°C, the gels were used for electrophoresis in the second dimension. SDS-10% polyacrylamide gels were used in the second dimension. After 2-D PAGE, proteins were transferred to nitrocellulose membranes using the blotting system of Bio-Rad (Richmond, CA). The membranes were soaked

for 1 h in a 1% (w/v) solution of BSA in PBS; they were then incubated with a monoclonal antibody raised against α- or β-tubulin from chick brain (Amersham Japan, Tokyo) for 1 h at 36°C, washed with PBS that contained 0.05% Tween 20 and incubated for 1 h with peroxidase-conjugated goat antibodies against mouse immunoglobulin G (Bio-Rad). Tubulin on nitrocellulose membranes was visualized by incubation with 0.06% 4-chloro-1-naphtol and 0.03% H_2O_2 in PBS.

EM

For the negative staining of cold-stable MTs, single drops of preparations were applied to Formvar-coated copper grids. The grids were washed with distilled water and stained with a 0.1% aqueous solution of uranyl acetate. The electron micrographs were taken with a JEM 100C (JEOL Ltd., Tokyo, Japan) transmission electron microscope.

Chemicals

6-DMAP was purchased from Fluka Chemie AG (Buchs, Switzerland) and staurosporin was a generous gift from Dr. H. Takizawa (Osaka University). Piperazine-*N*,*N*′-bis(2-ethanesulfuric acid), DMSO, EGTA, and citrus pectin were purchased from Wako Pure Chemicals (Osaka, Japan). GTP was purchased from Yamasa Shoyu, Ltd. (Chiba, Japan). Leupeptin and pepstatin A were obtained from the Peptide Institute, Inc. (Osaka, Japan).

RESULTS

Acquisition of Cold Stability by MTs in Cultured Tobacco Cells

Tobacco cells were transferred every 168 h to a fresh medium for subculture at a cell density of 1.5×10^7 cells/100 mL. No cold-stable MTs were detected at early stages of culture. As shown in Figure 1, for at least 96 h from the beginning of subculture, complete depolymerization of MTs by cold treatment for 1 h was achieved. The first recognizable cold-stable MTs were generally found 120 h after subculture; they were thin and short, their number was very small, and they were seen in only a minor population of cells (less than 1% of the total cells). Then, both the number of cells that contained cold-stable MTs and the numbers of cold-stable MTs in each cell increased gradually with time. After 192 h, the cells containing cold-stable MTs accounted for more than 95% of the total, as shown in Figure 1A. The cold-stable MTs in the cells at a late stage appeared to be much more organized than those in the cells at 120 h. Timing of the first appearance of cold-stable MTs after subculture depended upon the density at which cells were subcultured. When a subculture was started at a high cell density (7.5×10^7 cells/100 mL), the disappearance of cold-stable MTs ordinarily occurred rapidly, but the time lag until reappearance of cold-stable MTs was shortened and cold-stable MTs were detected at 96 h. At lower cell density (3×10^6 cells/100 mL), the appearance of cold-stable MTs was delayed until 168 or 196 h, and sometimes cells failed to acquire cold-stable MTs throughout the culture.

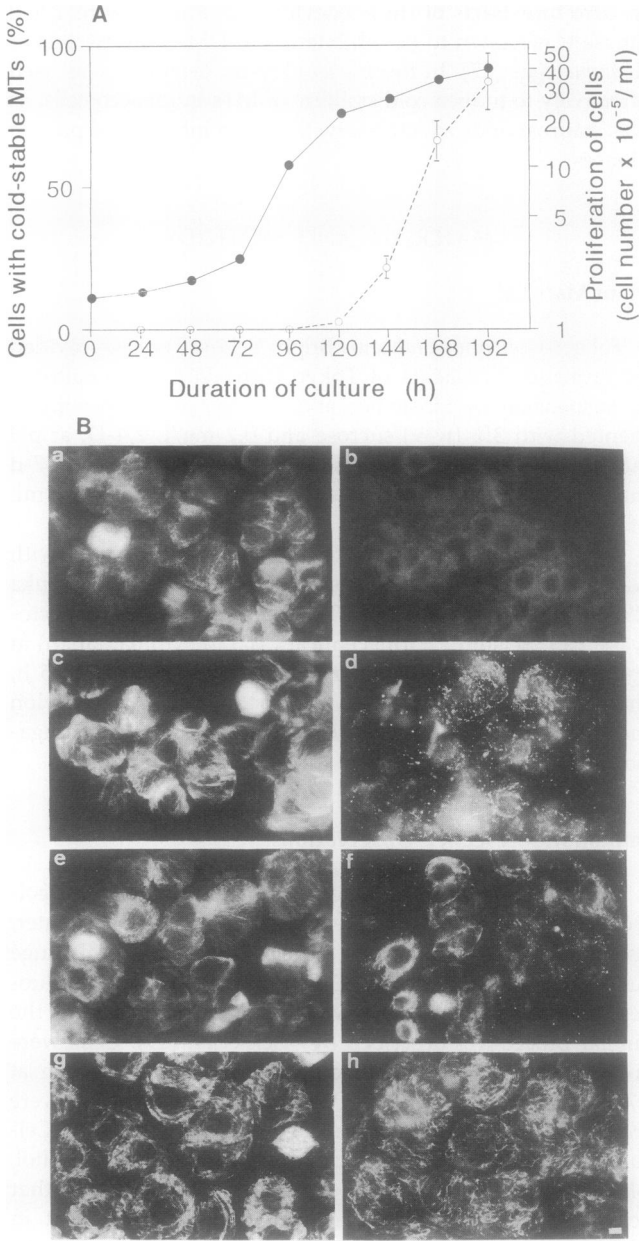

Figure 1. Cold-stable MTs are detected in cultured tobacco cells during culture. A, The numbers of cells having cold-stable MTs are expressed as percentages of the total number of cells (—○—). Vertical bars represent SD of results from three experiments. The proliferation of cells during culture is also shown (—●—). B, MTs in cells incubated for 1 h at 27°C (a, c, e, g) and at 0°C (b, d, f, h) are revealed by indirect immunofluorescence staining with a monoclonal antibody against mung bean tubulin. MTs in cells cultured for 48 (a, b), 96 (c, d), 144 (e, f), and 192 h (g, h) are shown. Bar = 10 μm.

Cold-Labile and Cold-Stable MTs in Tobacco Cells

As described above, tobacco cells at an early stage of culture have no cold-stable MTs. The properties of cold-labile MTs were determined in cells that had been cultured at 27°C for 48 h after transfer to fresh medium. As shown in Figure 2, cold treatment for 10 min induced a marked decrease in the

number of MTs in each cell, but most cells still retained a small number of MTs. Most MTs were disassembled by cold treatment for 20 min, but the remnants of MTs were still observed on occasion, and cold treatment for more than 30 min was required for the complete disappearance of MTs. In the present study, therefore, cold treatment was performed for 1 h. One hour was deemed to be sufficient for the complete disassembly of MTs and, thus, treatment defined such disassembling MTs as cold labile. Those MTs that were not depolymerized by the cold treatment of cells for 1 h were defined as cold-stable MTs. These cold-stable MTs did not depolymerize, for the most part, even as a result of prolonged cold treatment for at least 3 h.

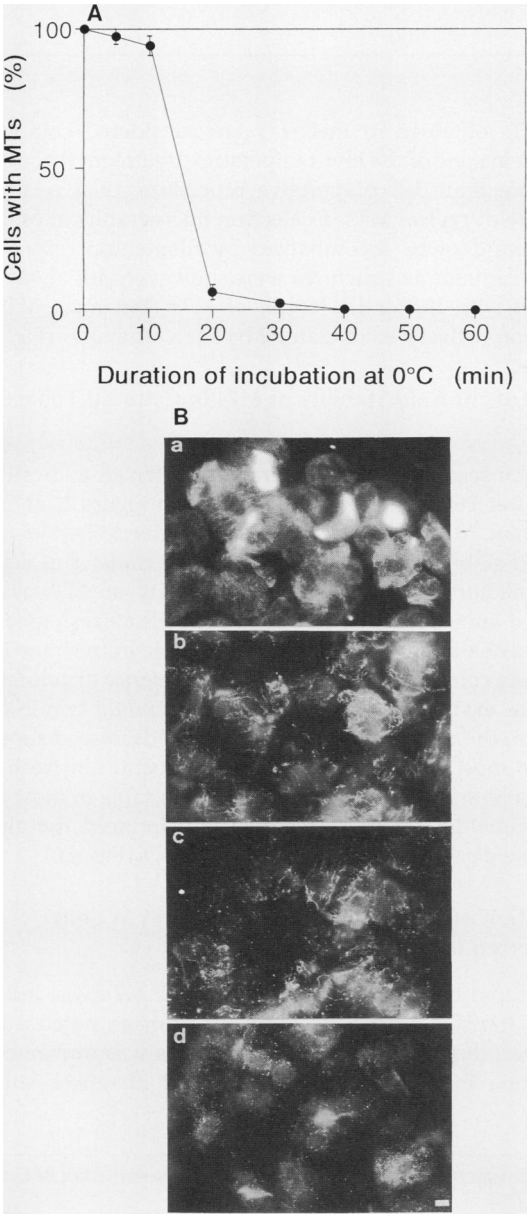

Figure 2. The effects of low temperature on cold-labile MTs as determined with cells cultured for 48 h at 27°C, which do not have any cold-stable MTs. A, The numbers of cells with MTs are expressed as percentages of the total number of cells. Vertical bars represent SD of results from three experiments. B, Cold-labile MTs as observed by indirect immunofluorescence. MTs in the initial population of cells (a), MTs in cells incubated for 10 (b), 20 (c), and 30 min (d) at 0°C. Bar = 10 μm.

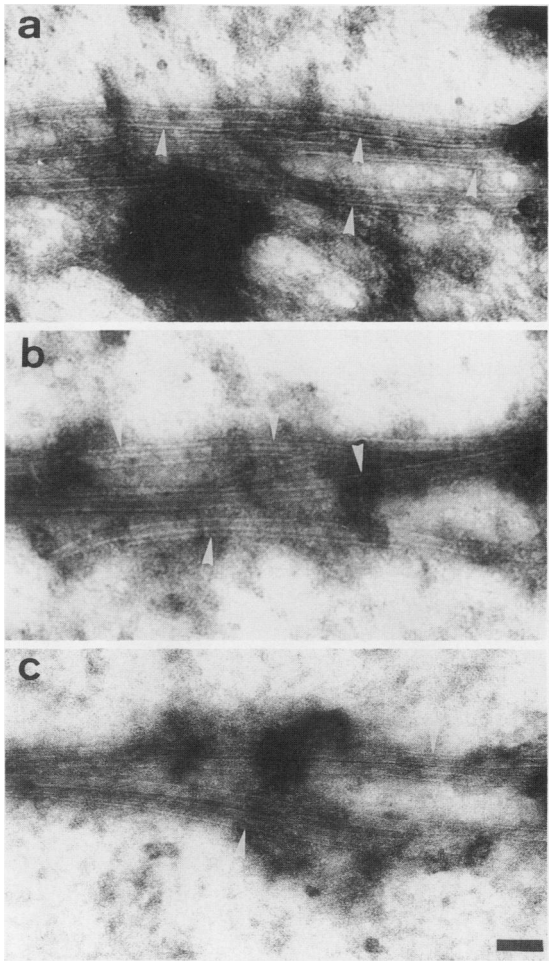

Figure 3. The cold-stable MTs in the CERF from cells cultured for 168 h are shown on electron micrographs. As indicated by arrowheads, most MTs in the CERF are accompanied by filamentous structures. Such filamentous structures do not always extend along the entire length of MTs. Bar = 0.1 μm.

Occurrence of Cold-Stable MTs in the Extracts of Cultured Tobacco Cells

At 168 h from the beginning of subculture, the tobacco cells with cold-stable MTs in about 70% of the total population were homogenized in an extraction buffer with a sonicator. The homogenate was centrifuged for 1 h at 100,000*g* at 4°C. The supernatant solution containing free tubulin was discarded and the pellet was suspended in the extraction buffer and the suspension was then centrifuged at 30,000*g*. The resultant supernatant was centrifuged again at 100,000*g*. In addition to MTs, the resultant pellet contained various filamentous structures identified as components of cytoskeletal elements, such as microfilaments, intermediate filament-like structures, and other unidentified filamentous materials.

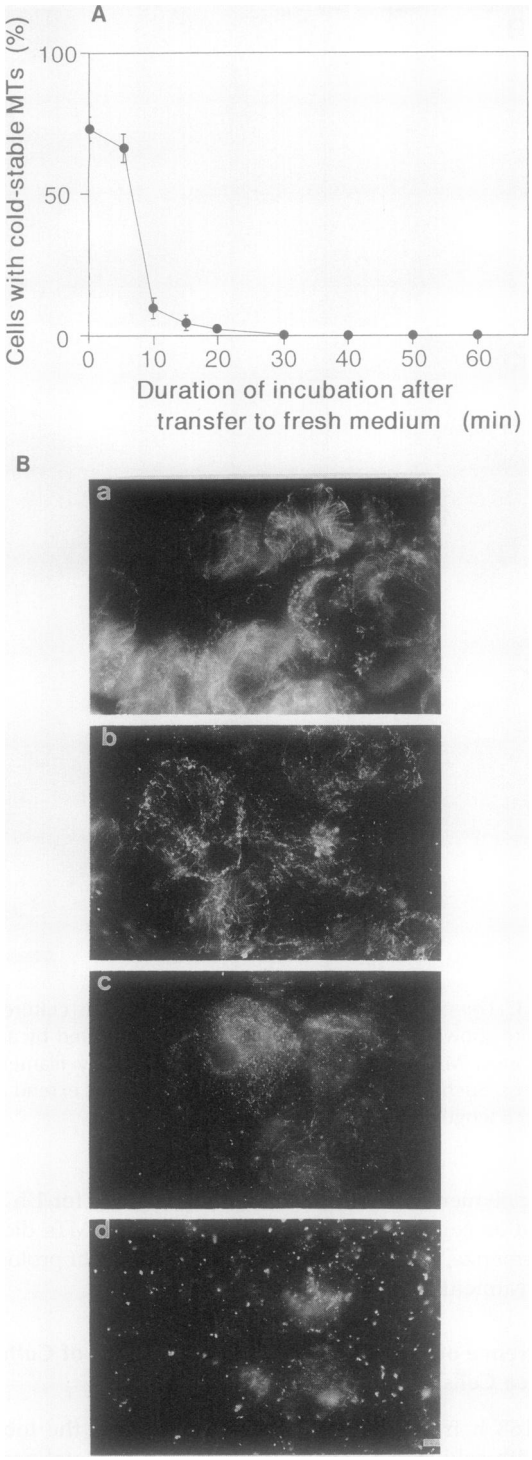

Figure 4. Fresh medium induces rapid loss of cold-stable MTs. A, The number of cells having cold-stable MTs are expressed as percentages of the total number of cells after the transfer to fresh medium. Cells cultured for 168 h lose their cold-stable MTs rapidly upon transfer to fresh medium at 27°C. Vertical bars represent SD of results from four experiments. B, Cold-stable MTs as detected by indirect immunofluorescence staining. The initial population of cells cultured for 168 h contains cold-stable MTs (a). After incubation for 5 (b), 15 (c), and 20 min (d) in fresh medium at 27°C, cold-stable MTs are examined. Bar = 10 μm.

Table I. *Prevention of the Loss of Cold-Stable MTs in Cells Transferred to Fresh Medium*

Amount of Once-Used Medium	Percentage of Cells with Cold-Stable MTs
%, v/v	
0	70
10	0
20	27
40	55
50	57
100	74

The MTs obtained in this way are considered cold stable because in spite of the low-temperature treatment throughout the more than 3-h preparative procedure, they retain the structure of typical MTs. In electron micrographs, these MTs were found to be accompanied by filamentous structures. These filamentous structures were, however, not always associated with the entire length of MTs, but most MTs on photographs were accompanied by such structures (Fig. 3).

Rapid Loss of Cold Stability of MTs in Cultured Tobacco Cells

The tobacco cells were routinely transferred to fresh medium after 168 h in culture. As shown in Figure 1, at 168 h more than 70% of cells acquired cold-stable MTs. However, the cold stability was lost soon after the transfer of cells to fresh medium. This loss of cold stability of MTs always occurred immediately upon transfer of cells to fresh medium. After incubation for various periods of time in fresh medium, cells were cold treated for 1 h and the presence of cold-stable MTs was examined. With incubation for only 5 min in fresh medium, the number of cold-stable MTs decreased substantially in most cells. After a 15-min incubation in fresh medium, cold-stable MTs were scarcely detectable in most cells, and incubation for more than 20 min induced the almost complete disappearance of cold-stable MTs (Fig. 4).

Prevention of the Loss of Cold-Stable MTs in Cells Transferred to Fresh Medium

The rapid disappearance of cold-stable MTs was induced by the transfer of cells to fresh medium as noted above. However, the loss of the cold-stable MTs was prevented by incubating the cells with fresh medium that was supple-

Table II. *Effect of Pectin Hydrolyzate on the Preservation of Cold-Stable MTs*

Amount of Pectin Hydrolyzate Added	Percentage of Cells with Cold-Stable MTs
μg/mL	
0	0
10	5.3
100	52
500	60

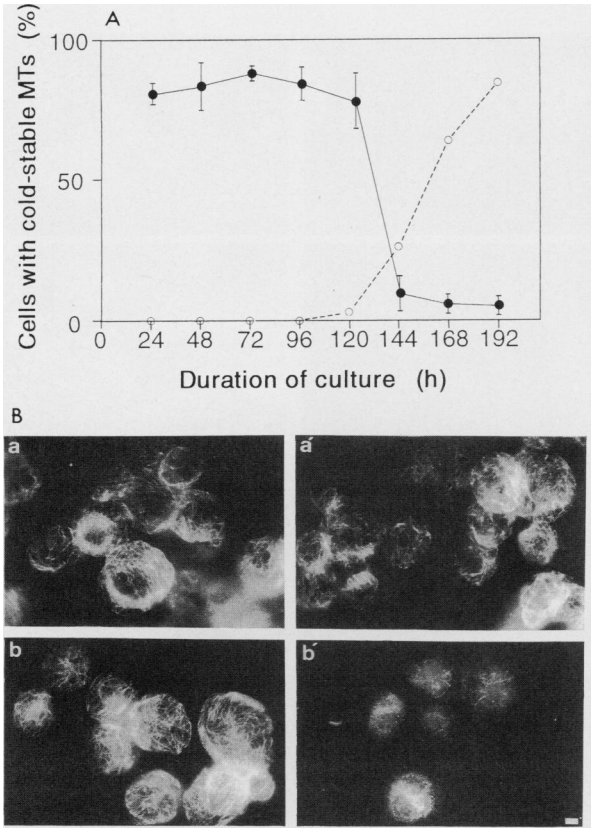

Figure 5. Cold stability of MTs in tobacco protoplasts. A, The number of protoplasts with cold-stable MTs (—●—) are expressed as percentages of the total number of protoplasts. Control of intact cells is also shown (—○—). Vertical bars represent SD of results of three experiments. B, MTs in protoplasts as visualized by indirect immunofluorescence staining. MTs in early-stage protoplasts (prepared from cells cultured for 48 h in fresh medium, a,a'). MTs in late-stage protoplasts (prepared from cells cultured for 168 h, b,b'). Protoplasts were incubated at 27°C (a, b) or at 0°C (a', b') for 1 h prior to fixation. Bar = 10 μm.

mented with once-used medium at 27°C for 2 h. After the cells were incubated at 0°C for 1 h, they were prepared for immunofluorescence microscopy to determine the number of cells having cold-stable MTs. The addition of once-used medium to 20% (v/v) preserved cold-stable MTs in about 27% of cells. The preservation of cold stability increased with increasing amounts of once-used medium. Addition of once-used medium to 40% (v/v) resulted in the preservation of cold-stable MTs in about 55% of cells (Tables I and II). This result indicates that some unidentified factor(s) that causes the preservation of cold stability may be excreted into the medium during culture. Because the activity that preserved cold-stable MTs in tobacco cells was found in the dialyzable fraction of once-used medium and was resistant to heating at 100°C for 1 min, the active substance(s) appeared to have a low mol wt.

A pectin hydrolyzate had a similar effect on the preservation of cold-stable MTs in tobacco cells when added to fresh medium, as shown in Tables I and II. However, the medium factor(s) and the pectin hydrolyzate had no ability to confer cold stability to cold-labile MTs. The cells that lost cold-stable MTs by the incubation for 1 h after the transfer to a fresh medium were supplemented with either once-used medium or pectin hydrolyzate and were then cultured at 27°C for 2, 24, 48, and 72 h, but cold-stable MTs were not detected in any of these cultures.

Cold-Stable MTs in Protoplasts

With protoplasts, the results were different from those obtained with intact cells (Fig. 5). During early stages of culture, more than 80% of protoplasts had cold-stable MTs, whereas intact cells at the same stages had no cold-stable MTs. By contrast, most protoplasts at a late stage lost cold-stable MTs, but intact cells at this stage retained cold-stable MTs at high frequency. Thus, the profile of changes in cold stability of MTs during culture of protoplasts was the mirror image of that in intact cells.

Induction of Cold Stability of MTs by Inhibitors of Protein Kinase Activity

Application of inhibitors of protein kinase to the cells that were cultured for 48 h at 27°C induced cold stabilization of their MTs. Treatment with 6-DMAP at more than 1 mM or staurosporin at 10 μM induced cold stabilization of MTs in nearly 100% of cells (Fig. 6A). The induction of cold stabilization of MTs during culture at 27°C by 1 mM 6-DMAP was investigated. This inhibitor of protein phosphorylation caused marked acquisition of cold-stable MTs in most cells at every stage of culture (Fig. 6B).

The time lag for the induction of the complete establishment of cold stability of MTs in cultured cells depended upon the stage of culture. At 48 h after the transfer to fresh medium, cells treated with an inhibitor of protein kinase at 27°C for 30 min did not show evidence of any significant cold stabilization of MTs, and the treatment for 60 min induced cold stabilization only in MTs of spindles and phragmoplasts, but not in cortical MTs. Treatment for more than 90 min induced maximal cold stabilization of every type of MT in almost 100% of cells (Fig. 6C). The cells cultured for 72 h showed similar responses.

However, cells cultured for more than 168 h, in about 70% of which cold stabilization of MTs had already occurred, had a rapid response to protein kinase inhibitors. Treatment for only 30 min with 6-DMAP or staurosporin at 27°C induced a remarkable stimulation of cold stabilization of cortical MTs. The maximal effect was obtained with treatment for 60 min and, apparently, most MTs in each cell acquired cold stability (Fig. 6C). Thus, inhibitors of protein kinase induced a remarkable cold stabilization of MTs in intact cells at a late stage of culture.

The response of protoplasts to protein kinase inhibitors was very poor. But characteristic cold-stable MTs from MTOC-like structures were detected on nuclei and/or in the periphery of the cytoplasm in protoplasts from the population of actively dividing cells at an early stage of culture, as shown in Figure 7.

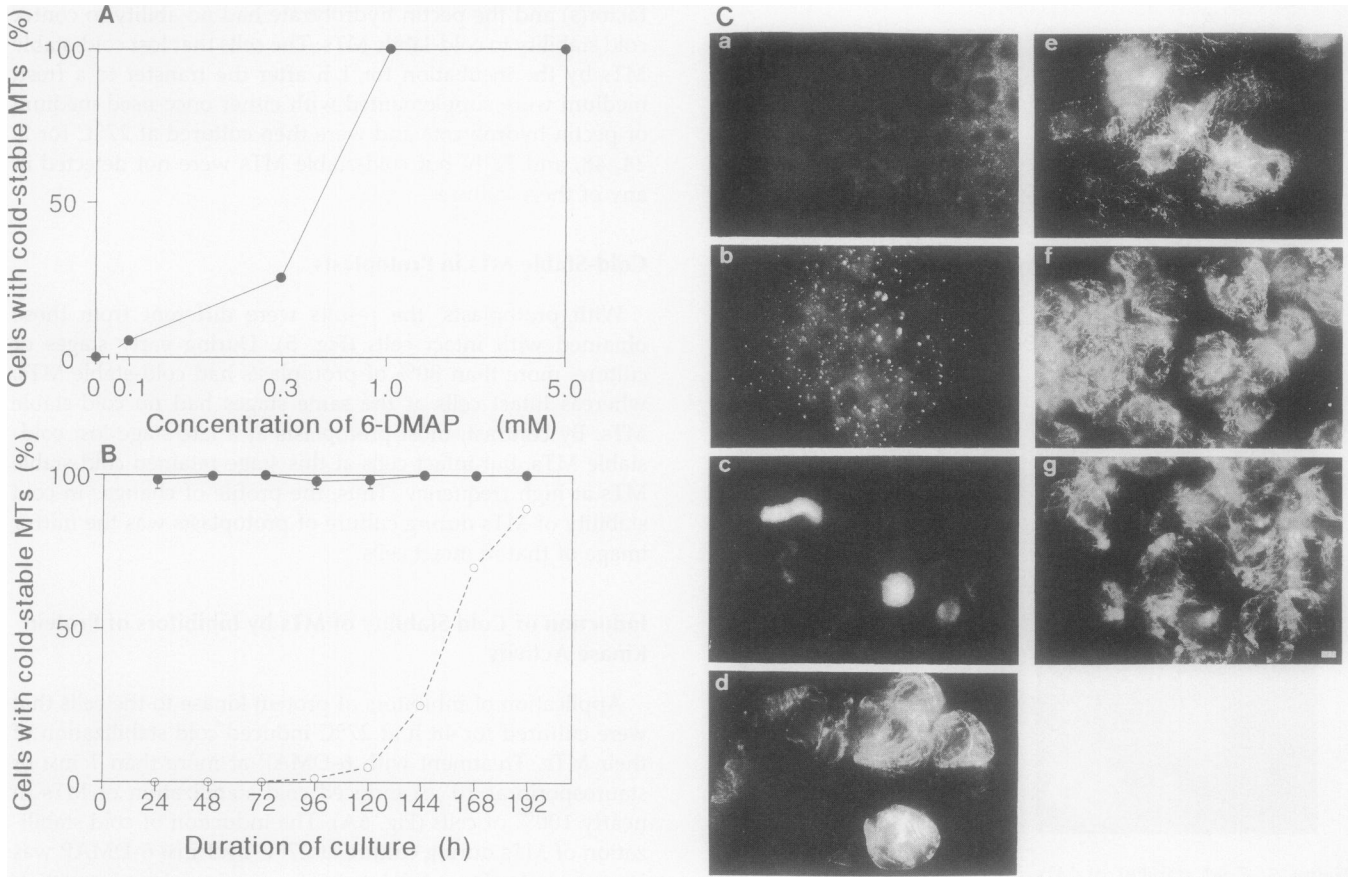

Figure 6. Protein kinase inhibitors confer cold stability on MTs in tobacco cells. A, Effect of various concentrations of 6-DMAP on acquisition of cold stability of cold-labile MTs. Cells cultured for 48 h at 27°C were incubated with 6-DMAP for 2 h at 27°C, then at 0°C for 1 h before fixation. B, The time course of induction of cold stabilization of MTs by 1 mM 6-DMAP (—●—). Control is also shown (—○—). C, Cold-stable MTs in the cells treated with 6-DMAP. Cells cultured for 48 (a, b, c, d) and for 168 h (e, f, g) were used. Initial cells cultured for 48 h (a) and for 168 (e). Cells treated with 6-DMAP for 30 min (b and f). Cells treated with 6-DMAP for 60 min (c and g). Cells treated with 6-DMAP for 90 min (d). Treatment with staurosporin has a similar effect on cold stabilization of MTs in tobacco cells. Bar = 10 μm.

Tubulin Subunits in Tobacco Cells

The modification of tubulin subunits was also expected to be involved in the acquisition of cold-stability of MTs. 2-D PAGE was performed by the method of O'Farrell (22). No significant differences were found in either α- or β-tubulin isoforms between cells cultured for 168 h with cold-stable MTs (Fig. 8, a, and a') and cells transferred to a fresh medium and incubated for 1 h without cold-stable MTs (Fig. 8, b and b').

DISCUSSION

In suspension-cultured tobacco cells, the absence of cold-stable MTs was found at early stages of culture. Cold stability was gradually conferred on MTs and eventually most cells contained almost entirely cold-stable MTs. The cold-stable MTs were detected in the CERF of the extract and were found to be associated with unidentified filamentous structures along their length. Such structures did not, however, always extend along the full length of MTs. In the case of animal nerve cells, the factors that confer cold stability on MTs also appear not to be associated with the entire length of MTs (12). Because tubulins from cold-stable and cold-labile MTs in tobacco cells showed no significant differences after 2-D PAGE, the filamentous structures or other MAPs can be assumed to participate in the cold stabilization of MTs. When microtubules lose their cold stability, the associated filamentous structures may be detached from the surface of MTs. The gradual acquisition of cold stability by MTs during culture might be dependent on the increased synthesis of such a cold stabilizer(s) and the ability of such a factor(s) to associate with the surface of MTs. The rapid loss of the cold stability of MTs, induced by the transfer of cells to fresh medium, also might be caused by the detachment of stabilizer from the surface of MTs. Intracellular interconversion of cold-stable and cold-labile MTs would be governed by the balance between phosphorylation and dephosphorylation of a cold stabilizer, depending on the actions of a protein kinase and a phosphoprotein phosphatase.

The striking ability of protein kinase inhibitors to confer cold stability on cold-labile MTs and to maintain the cold stability of MTs in cells supports the above hypothesis. The

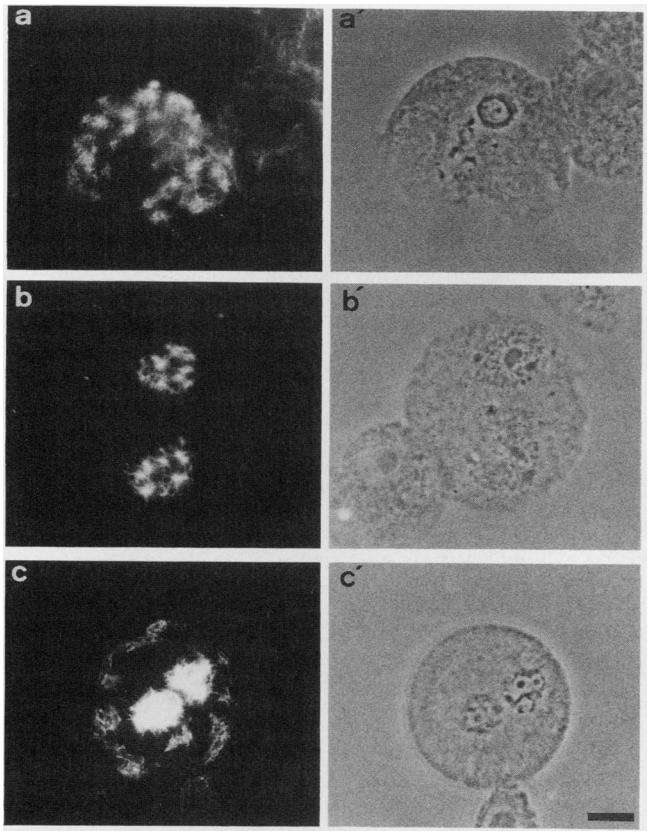

Figure 7. Appearance of MTOC-like structures in protoplasts by treatment with protein kinase inhibitors. MTOC-like structures scattering in the cytoplasm (a). MTOC-like structures associating with the surface of nuclei (b). In some protoplasts, MTOC-like structures are distributed to the surface of nuclei and in the periphery of the cytoplasm (c). Panels a', b', and c' show phase-contrast images that correspond to panels a, b, and c, respectively. Bar = 10 μm.

fact that MTs in spindles and phragmoplasts acquired cold stability more rapidly than cortical MTs upon treatment with inhibitors of protein kinase suggests that the interconversion between phosphorylated and dephosphorylated states of the cold stabilizer might be more rapid in spindles and phragmoplasts than in cortical MTs.

The factor(s) excreted into the medium was dialyzable and resistant to heating, and it prevented the rapid change of cold-stable MTs to cold-labile MTs when cells were transferred to fresh medium. The action of the pectin hydrolyzate may involve a process similar to that mediated by the factor(s) in the once-used medium. However, these substances lacked the ability to confer cold stability on cold-labile MTs in the cells. Therefore, these substances may act on the MTs indirectly.

Fragments of pectin are known to act as an endogenous elicitor and to cause accumulation of phytoalexin in soybean cells (10, 21). Moreover, Yoshikawa et al. (30) demonstrated the presence of a receptor for a fungal elicitor on the membrane of soybean cells. Dietrich et al. (6), using suspension-cultured parsley cells, reported that fungal elicitor triggers rapid, transient, and sequential phosphorylation of proteins, and that this response is rapidly reversed by removal of the elicitor from the medium. The medium factor(s) and pectin hydrolyzate were speculated to act also on the plasma membrane of tobacco cells and to affect the function of protein kinase, although the apparent response of tobacco cells concerning phosphorylation seems to be inconsistent with the above reports.

Akashi et al. (1), using only cells at a late stage of culture, reported that polycationic substances, including extensin, induced cold stabilization of cortical MTs, and they proposed that the cell wall or wall material plays an important role in expression of cold stability of cortical MTs in tobacco protoplasts. In fact, most MTs were cold labile in the protoplasts prepared from the cells at a late stage of culture, even though the MTs in intact cells were cold stable. However, in the protoplasts prepared from cells at an early stage, plenty of cold-stable MTs were retained in spite of the complete absence of cold-stable MTs in intact cells. This result indicates that, at least in cells at an early stage, cell wall or wall material is not necessarily involved in cold stabilization of MTs. The protoplasts at an early stage tend to aggregate and form many clusters in mannitol solution, but protoplasts at a late stage never form such aggregates. This observation suggests the change in the properties of the plasma membrane during culture. The cell wall could function to support the maintenance of normal functions of the plasma membrane.

The treatment of protoplasts with protein kinase inhibitors induced only a faint stimulation of the acquisition of cold stability by MTs. The poor effect of the protein kinase inhibitors might be due to the removal of the cell wall. However, the distinctive cold-stable MTs were often observed to extend from the surface of nuclei and/or MTOC-like organizing centers in protoplasts. This observation suggests that the active interconversion between phosphorylated and dephos-

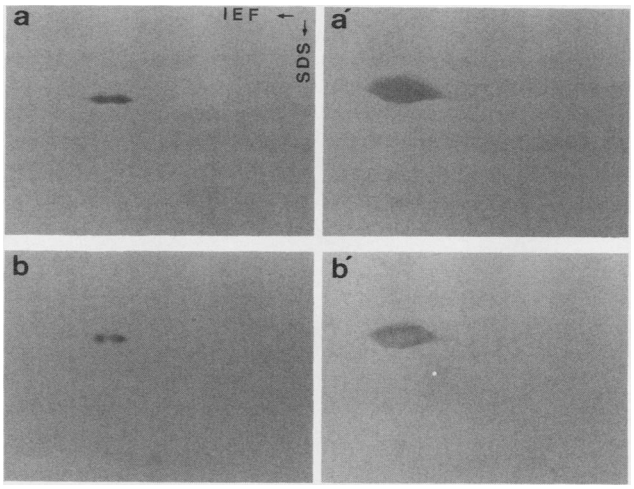

Figure 8. Immunoblot analysis of tubulin isoforms in cold-stable and cold-labile MTs after 2-D PAGE. Tubulin from cells cultured for 168 h with cold-stable MTs (a, a'). Tubulin from cells cultured for 168 h and then transferred to fresh medium and incubated for 1 h completely without cold-stable MTs (b, b'). Blots a and b are probed with monoclonal antibody against α-tubulin. Blots a' and b' are probed with monoclonal antibody against β-tubulin.

phorylated states of MT proteins might take place at the surface of nuclei and on MTOC-like structures.

In intact cells, abundant MTs acquire cold stability upon treatment of cells with protein kinase inhibitors, and profiles of MTs extending from the surface of nuclei and/or MTOC-like structures would be masked by other overlapping MTs. The cold stabilization of MTs in intact tobacco cells seems to occur in an age-dependent manner, being maximal just prior to the stationary phase of cell proliferation. Upon acquisition of cold stability, MTs may be rendered static. Although it is difficult to conclude that the establishment of the stationary phase is somehow related to the formation of static MTs, it is possible that some correlation exists. For the initiation of a new round of cell division, the loss of cold-stabilized MTs or a change from the static form of MTs to the dynamic form may be required.

Determination of the mechanism that controls the formation and disappearance of cold-stable MTs is essential if we are to understand the physiological role of cold-stable MTs in plant cells. The establishment of an in vitro system for reconstruction of cold-stabilized MTs is also necessary, as is the isolation and identification of the cold-stabilizing factor, which is now under investigation.

LITERATURE CITED

1. **Akashi T, Kawasaki S, Shibaoka H** (1990) Stabilization of cortical microtubules by the cell wall in cultured tobacco cells: effects of extensin on the cold-stability of cortical microtubules. Planta **182:** 363–369
2. **Akashi T, Shibaoka H** (1987) Effects of gibberellin on the arrangement and the cold stability of cortical microtubules in epidermal cells of pea internodes. Plant Cell Physiol **28:** 339–348
3. **Bartolo ME, Carter JV** (1991) Microtubules in mesophyll cells of nonacclimated and cold-acclimated spinach. Plant Physiol **97:** 175–181
4. **Brady ST, Tytell M, Lasek RJ** (1984) Axonal tubulin and axonal microtubules: biochemical evidence for cold stability. J Cell Biol **99:** 1716–1724
5. **Brinkley BR, Cartwright J** (1975) Cold-labile and cold-stable microtubules in the mitotic spindle of mammalian cells. Ann NY Acad Sci **253:** 428–439
6. **Dietrich A, Mayer JE, Hahlbrock K** (1990) Fungal elicitor triggers rapid, transient, and specific protein phosphorylation in parsley cell suspension cultures. J Biol Chem **265:** 6360–6368
7. **Dustin P** (1984) Microtubules. Springer, Berlin
8. **Gozes I** (1982) Tubulin in the nervous system. Neurochem Int **4:** 101–120
9. **Gunning BES, Hardham AR** (1982) Microtubules. Annu Rev Plant Physiol **33:** 651–698
10. **Hahn MG, Darvill AG, Albersheim P** (1981) Host-pathogen interactions. XIX. The endogenous elicitor, a fragment of a plant cell wall polysaccharide that elicits phytoalexin accumulation in soybeans. Plant Physiol **68:** 1161–1169
11. **Job D, Fischer EH, Margolis RL** (1981) Rapid disassembly of cold-stable microtubules by calmodulin. Proc Natl Acad Sci USA **78:** 4679–4682
12. **Job D, Margolis RL** (1982) Recycling of cold-stable microtubules: evidence that cold stability is due to substoichiometric polymer blocks. Biochemistry **21:** 509–515
13. **Jones DH, Grey E, Barron J** (1980) Cold-stable microtubules in brain studied in fractions and slices. J Neurocytol **9:** 493–504
14. **Kerr GP, Carter JV** (1990) Relationship between freezing tolerance of root-tip cells and cold stability of microtubules in rye (*Secale cereale* L. cv Puma). Plant Physiol **93:** 77–82
15. **Kerr GP, Carter JV** (1990) Tubulin isotypes in rye roots are altered during cold acclimation. Plant Physiol **93:** 83–88
16. **Lloyd CW** (1987) The plant cytoskeleton: the impact of fluorescence microscopy. Annu Rev Plant Physiol **38:** 119–139
17. **Margolis RL, Rauch CT** (1981) Characterization of rat brain crude extract microtubule assembly: correlation of cold stability with the phosphorylation state of microtubule associated 64 K protein. Biochemistry **20:** 4451–4458
18. **Mita T, Shibaoka H** (1984) Gibberellin stabilizes microtubules in onion leaf sheath cells. Protoplasma **119:** 100–109
19. **Mizuno K, Sek F, Perkin J, Wick S, Duniec J, Gunning B** (1985) Monoclonal antibodies specific to plant tubulin. Protoplasma **129:** 100–108
20. **Neant I, Guerrier P** (1988) 6-Dimethylaminopurine blocks starfish oocyte maturation by inhibiting a relevant protein kinase activity. Exp Cell Res **176:** 68–79
21. **Nothnagel EA, McNeil M, Dell A, Albersheim P** (1983) Host-pathogen interactions. XXII. A galacturonic acid oligosaccharide from plant cell walls elicits phytoalexins. Plant Physiol **71:** 916–926
22. **O'Farrell P** (1975) High-resolution two-dimensional electrophoresis of proteins. J Biol Chem **250:** 4007–4021
23. **Pirollet F, Job D, Fischer EH, Margolis RL** (1983) Purification and characterization of sheep brain cold-stable microtubules. Proc Natl Acad Sci USA **80:** 1560–1564
24. **Rebhun LI, White D, Sander G, Ivy N** (1973) Cleavage inhibition in marine eggs by puromycin and 6-dimethylaminopurine. Exp Cell Res **77:** 312–318
25. **Rikin A, Atsmon D, Gitler C** (1980) Chilling injury in cotton (*Gossypium hirsutum* L.): effects of antimicrotubular drugs. Plant Cell Physiol **21:** 829–837
26. **Salmon ED, Begg DA** (1980) Functional implication of cold-stable microtubules in kinetochore fibers of insect spermatocytes during anaphase. J Cell Biol **85:** 853–865
27. **Verde F, Labbé J, Dorée M, Karsenti E** (1990) Regulation of microtubule dynamics by *cdc2* protein kinase in cell-free extracts of *Xenopus* eggs. Nature **343:** 233–238
28. **Webb BC, Wilson L** (1980) Cold-stable microtubules from brain. Biochemistry **19:** 1993–2001
29. **Wick SM, Seagull RW, Osborn M, Weber K, Gunning BES** (1981) Immunofluorescence microscopy of organized microtubule arrays in structurally-stabilized meristematic plant cells. J Cell Biol **89:** 685–690
30. **Yoshikawa M, Keen NT, Wang M-C** (1983) A receptor on soybean membranes for a fungal elicitor of phytoalexin accumulation. Plant Physiol **73:** 497–506