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Relationship between Freezing Tolerance of Root-Tip Cells and Cold Stability of Microtubules in Rye (*Secale cereale* L. cv Puma)[1]

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ABSTRACT

The response of cortical microtubules to low temperature and freezing was assessed for root tips of cold-acclimated and nonacclimated winter rye (*Secale cereale* L. cv Puma) seedlings using indirect immunofluorescence microscopy with antitubulin antibodies. Roots cooled to 0 or −3°C were fixed for immunofluorescence microscopy at these temperatures or after an additional hour at 4°C. Typical arrays of cortical microtubules were present in root-tip cells of seedlings exposed to the cold-acclimation treatment of 4°C for 2 days. Microtubules in these cold-acclimated cells were more easily depolymerized by a 0°C treatment than microtubules in root-tip cells of nonacclimated, 22°C-grown seedlings. Microtubules were still present in some cells of both nonacclimated and cold-acclimated roots at 0 and −3°C; however, the number of microtubules in these cells was lower than in controls. Microtubules remaining during the −3°C freeze were shorter than microtubules in unfrozen control cells. Repolymerization of microtubules after both the 0 and −3°C treatments occurred within 1 h. Root tips of nonacclimated seedlings had an LT-50 of −9°C. Cold acclimation lowered this value to −14°C. Treatment of 22°C-grown seedlings for 24 h with the microtubule-stabilizing drug taxol caused a decrease in the freezing tolerance of root tips, indicated by a LT-50 of −3°C. Treatment with D-secotaxol, an analog of taxol that was less effective in stabilizing microtubules, did not alter the freezing tolerance. We interpret these data to indicate that a degree of depolymerization of microtubules is necessary for realization of maximum freezing tolerance in root-tip cells of rye.

Microtubules are dynamic, proteinaceous filaments that have critical roles in morphogenesis, chromosome movement, and organelle transport (for reviews, see refs. 8, 20). Microtubules in plants appear in a number of distinct arrays during the cell cycle, including the interphase cortical array, the preprophase band, the mitotic and meiotic spindles, and the phragmoplast.

Microtubules are of special interest in low-temperature research due to the cold lability of certain microtubule populations; low temperature can cause microtubules to depolymerize into their protein subunits. This characteristic of microtubules suggests the need to determine whether microtubules might be involved in cold acclimation and other responses of plants to low temperature.

Among species, microtubules vary in sensitivity to depolymerization by cold. This is dramatically illustrated with data from algal species. All of the microtubules in the green alga *Closterium ehrenbergii* depolymerize after only 5 min at 0°C (13), while several algal species from antarctic hypersaline lakes maintain polymerized flagellar or cytoplasmic microtubules at −14°C (3). The variability of cold stability of microtubules even extends to microtubules within a single cell, since not all of the microtubules in a cell depolymerize in response to a given cold treatment (2, 9–11).

The above data indicate that at least some microtubule populations in plants are cold stable. In the only published work on the effect of freezing on plant microtubules, Carter and Wick (4) studied microtubules in root-tip cells of onion after a freeze. However, onion roots are not freezing tolerant, and the presence or absence of microtubules in these cells reflected the survival or death of the root after the freeze.

In this study we observed the state of microtubules both during and after low-temperature (0°C) and nonlethal-freezing (−3°C) treatments. Also examined were changes in cold stability of microtubules during cold acclimation and the influence of the degree of microtubule polymerization on freezing tolerance. The drug taxol, which stabilizes microtubules against cold-induced depolymerization (14), was used to artificially manipulate cold stability of microtubules and determine whether cold-induced depolymerization of microtubules is beneficial or detrimental to freezing tolerance of plant cells. Winter rye (*Secale cereale* L. cv Puma) was used in these studies because of its freezing tolerance and ability to cold acclimate, and because microtubules are easily visualized in the roots by immunocytochemical techniques.

MATERIALS AND METHODS

Plant Material

Winter rye (*Secale cereale* L. cv Puma) seeds were treated with 2.5% NaOCl for 2 min, rinsed, then germinated in the dark on moist filter paper at 22°C. Seedlings were used after 2 d growth at 22°C in the dark (nonacclimated), or after an additional 2 d at 4°C (cold-acclimated). Growth of roots during the 2 d at 4°C was negligible. Although the cold-acclimated seedlings were older than the nonacclimated con-

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trols, there was no obvious developmental change during the acclimation period.

Drug Treatments

The microtubule-stabilizing drug taxol (obtained from the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute) and D-secotaxol, a taxol analog (obtained from Dr. D. Kingston, Virginia Polytechnic Institute and State University), were dissolved in DMSO at a concentration of 10 mM and stored in aliquots at −80°C. D-Secotaxol is inactive in mammalian systems (18).

After germinating seedlings for 24 h at 22°C in the dark, they were transferred to a Petri dish containing 10 μM taxol, 10 μM D-secotaxol, or 14 mM DMSO (0.1% v/v) for 24 h. The DMSO control was carried out because of this compound's ability to induce microtubule polymerization at high concentrations (12) and because of its use in cryopreservation. However, the concentration of DMSO used was 50- to 100-fold lower than that used in microtubule stabilization or in cryoprotection. DMSO had no effect in any of our experiments.

Low Temperature Treatments

After holding seedlings at 0°C for 1 h, the temperature was lowered 1°C every 30 min. Ice formation was initiated at −1°C by the addition of ice crystals. Samples were removed at selected temperatures and placed at 4°C for 1 h to thaw.

Although the individual effects of time and temperature cannot be separated by this protocol, the gradual temperature decrease enhances survival of plant tissues and more closely approximates a natural freezing event than an immediate exposure to freezing conditions. As a control for the −3°C treatment in the immunocytochemical experiments, seedlings were kept at 0°C for 2.5 h, the total time required for the −3°C treatment.

Assay for Freezing Injury

After freezing and thawing using the protocol above, seedlings were placed at 22°C for 20 to 24 h, and tested for injury with triphenyl tetrazolium chloride, as described by Towill and Mazur (27). The apical 2 mm of 15 roots were used per treatment. LT-50[2] was taken as the point at which reduction of triphenyl tetrazolium chloride was 50% of the control.

Immunofluorescence Microscopy

After the low-temperature treatments, the roots were fixed either at that temperature or after a 1 h 4°C thaw. Root tips (apical 2 mm) were processed for immunofluorescence microscopy using a procedure based on Wick and Duniec (28). Roots were fixed in buffer (50 mM Pipes, 5 mM EGTA, 0.5 mM $MgCl_2$, 0.4 M sorbitol, 5 μM leupeptin hemisulfate, 2.5 μM pepstatin A [pH 6.8]) with 3.7% (w/v) paraformaldehyde for 1 h. For 0 and −3°C fixation, roots were immersed in fixative at the treatment temperature for 30 min, then transferred to 4°C for 30 min. (Fixative does not freeze at −3°C.) After fixation, roots were rinsed with buffer, digested with 1% w/v Cellulysin (Calbiochem, LaJolla, CA) in buffer for 30 min, rinsed, squashed, and allowed to air-dry. Anti-tubulin (monoclonal anti-α-tubulin B-5-1-2 ([23] or anti-β-tubulin [Amersham, Arlington Heights, IL]) antibodies were applied followed by a fluorescein-labeled second antibody. Cells were then stained for DNA with 1 mg/mL Hoechst 33258 and mounted. Mounting medium contained 1% (w/v) *n*-propyl gallate to reduce fading. Cells were observed with a Zeiss Photomicroscope equipped with epifluorescence optics and photographed with Kodak Technical Pan Film 2415.

Data on the fraction of cells with cortical microtubules were obtained by counting the number of cells with and without any microtubules detectable by immunofluorescence microscopy. Only interphase cells, as determined by DNA staining, were counted to eliminate possible complications arising from including other microtubule arrays that may differ in their cold stability.

RESULTS

Response of Microtubules to Low Temperature and Freezing in Nonacclimated Cells

Typical arrays of cortical microtubule were observed in root-tip cells of 22°C-grown rye seedlings prior to low-temperature treatments (Fig. 1A); 95% of interphase cells had microtubules detectable by immunofluorescence microscopy (Table I). The fraction of cells containing microtubules decreased only to 93% after 1 h at 0°C, but decreased to 54% at −3°C (Table I). Cells without microtubules had a diffuse staining (Fig. 1B). Exposing seedlings to 0°C for 2.5 h did not alter the proportion of cells with microtubules compared to 1 h at 0°C (93% ± 5 and 93% ± 4, respectively), indicating that the difference between the 0 and −3°C treatments was not due to time of exposure to low temperature.

Effect of Cold Acclimation on Microtubules

Microtubules were present in root-tip cells of rye after the cold-acclimation treatment of 2 d at 4°C (Fig. 1D; Table I). Treating these seedlings for 1 h at 0°C caused microtubule depolymerization, with decreases in both the relative number of microtubules per cell and the percentage of cells with microtubules. The 0°C treatment resulted in a 28% decrease in the fraction of cells with microtubules in cold-acclimated roots, compared to only a 2% drop for nonacclimated roots (Table I). This low-temperature treatment is only a decrease of 4°C for the cold-acclimated roots, but a decrease of 22°C for the nonacclimated roots. Microtubules of root-tip cells were more cold stable in nonacclimated, 22°C-grown seedlings than in cold-acclimated, 4°C-grown seedlings exposed to 0°C. At −3°C, microtubules were depolymerized to a similar extent in both nonacclimated and cold-acclimated roots (54 ± 12 and 63 ± 12%, respectively).

Not only did the fraction of cells containing microtubules decrease with the 0 and −3°C treatments, the relative number of microtubules in these cells also diminished compared to controls. Depolymerization caused by the 0°C treatment

[2] Abbreviations: LT-50, the temperature at which 50% of the population is killed.

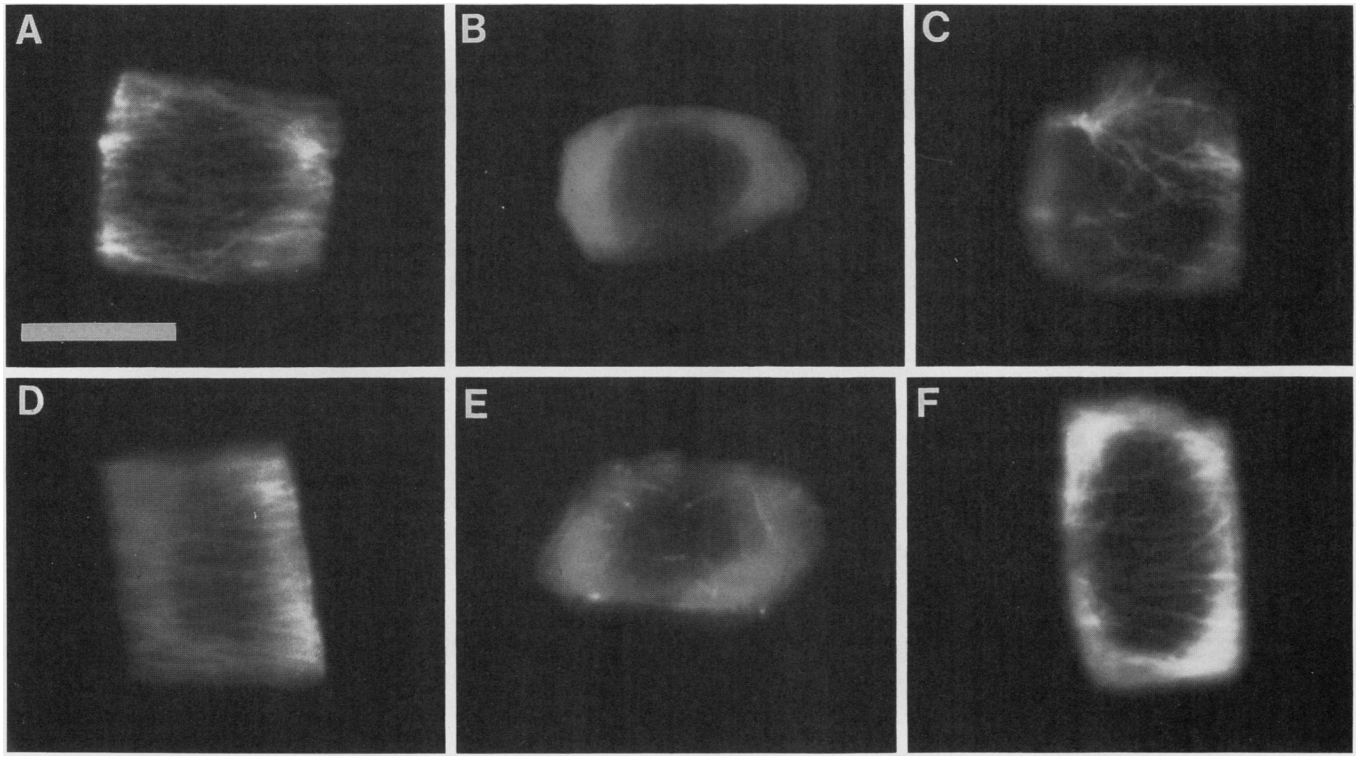

Figure 1. Microtubules of root-tip cells of rye visualized by immunofluorescence microscopy with antitubulin antibodies. A, Nonacclimated control cell with extensive microtubule arrays prior to low temperature treatment (bar = 10 μm); B, nonacclimated cell without microtubules after exposure to −3°C; C, cold-acclimated cell after 2.5 h at 0°C. Fewer microtubules than in controls. D, Cold-acclimated cell with full microtubule array at 4°C, prior to low-temperature treatment; E and F, nonacclimated cells exposed to −3°C followed by 1 h at 4°C. Variation existed in the degree of microtubule repolymerization after the −3°C treatment.

Table I. *Effects of Low Temperature Treatments on Cortical Microtubules*

Cold-acclimated (CA), nonacclimated (NA), or drug-treated rye seedlings were subjected to 0 or −3°C as described in "Materials and Methods," and then either fixed for immunofluorescence microscopy at that temperature or after 1 h recovery at 4°C. Data presented are the percentage of interphase root cells with microtubules ± binomial CI (95%).

Temperature Treatment	Fixed Immediately		Fixed after 1 h Recovery at 4°C	
°C	*% cells with microtubules ± CI*			
	NA	*CA*	*NA*	*CA*
Control[a]	95 ± 4	97 ± 5		
0	93 ± 4	69 ± 6	100	100
−3	54 ± 12	63 ± 12	100	100
	Taxol	*D-secotaxol*		
22	99 ± 2	100		
−3	98 ± 4	73 ± 11		

[a] Controls were fixed at growth temperature: 22°C for nonacclimated and 4°C for cold-acclimated seedlings.

tended to cause a subset of microtubules in both the nonacclimated and cold-acclimated cells to depolymerize completely, resulting in a lower density of microtubules in these cells, as shown in Figure 1C for cold-acclimated cells exposed to 0°C for 2.5 h. The −3°C (freeze) treatment also resulted in fewer microtubules per cell in both nonacclimated and cold-acclimated cells, but the remaining microtubules were of shorter length than in controls. These shorter microtubules were evident even after 1 h recovery at 4°C in some cells (Fig. 1E).

Microtubule Repolymerization after 0°C and −3°C Treatments

Rapid repolymerization of microtubules occurred after both the 0 and −3°C treatments, regardless of acclimation (Table I). The proportion of cells having microtubules rose from 54 to 100% for nonacclimated roots and from 63 to 100% for cold-acclimated roots after a 1 h, 4°C recovery from a −3°C freeze. However, the extent of microtubule repolymerization in the individual cells varied. Some cells had extensive arrays present after 1 h recovery (Fig. 1F), whereas others still exhibited only a few, short microtubules (Fig. 1E). Punctuate fluorescence was also observed in these cells (Fig. 1E).

Effect of Taxol and D-Secotaxol on Cold Stability of Microtubules

Taxol-treated cells had the characteristic microtubule arrays caused by this drug: increased microtubule polymerization

and bundling, steeply pitched helical arrays, and abnormal mitotic figures (Fig. 2, A and B). Taxol caused a decrease in root elongation and an increase in root width. Roots resumed normal growth after removal from taxol. The analog, D-secotaxol, did not cause any change in root growth or mitotic spindles (Fig. 2D); however, some interphase cells did show increased microtubule bundling (Fig. 2C).

Taxol increased the cold stability of microtubules as evidenced by the presence of microtubules in cells during a −3°C freeze (Fig. 2E). The −3°C freeze did not decrease the proportion of cells with cortical microtubules (Table I). The taxol analog did not stabilize microtubules to cold to the same extent as did taxol (Table I). This was especially evident when the relative length and density of the microtubules (in those cells with microtubules) is taken into account. The cells treated with D-secotaxol had microtubules that decreased in length and number as a result of the −3°C treatment, indicating substantial depolymerization. Microtubules in the taxol-treated cells remained long and densely packed.

Freezing Tolerance of Root Tips

Freezing tolerance of rye root tips was influenced by growth temperature and taxol treatment (Table II). Root-tip cells of nonacclimated, 22°C-grown seedlings had an LT-50 of −9°C. Cold acclimation resulted in an increase in freezing tolerance, as indicated by an LT-50 of −14°C. Conversely, taxol decreased the freezing tolerance, with the LT-50 rising from −9°C for D-secotaxol and DMSO controls, to −3°C for the taxol-treated roots.

DISCUSSION

Cold Stability of Microtubules in Root-Tip Cells of Rye is Decreased During Cold Acclimation

Microtubules of nonacclimated and cold-acclimated root tips responded differently to the 1 h, 0°C treatment imposed in this study. Microtubules were more cold stable in nonacclimated, 22°C-grown seedlings than in cold-acclimated, 4°C-grown seedlings (Table I). There were 26% more cells with microtubules after the 0°C treatment in nonacclimated roots than in cold-acclimated roots. At least two means of directly modifying cold stability of microtubules may exist for rye root cells, based on data from other systems. Microtubule-associated proteins can affect cold stability (for example, ref. 16). Cyr and Palevitz (6) recently reported the isolation of a microtubule-bundling protein from carrot which confers cold stability to microtubules *in vitro*. Changes in such a protein as a result of low-temperature growth may be one mechanism

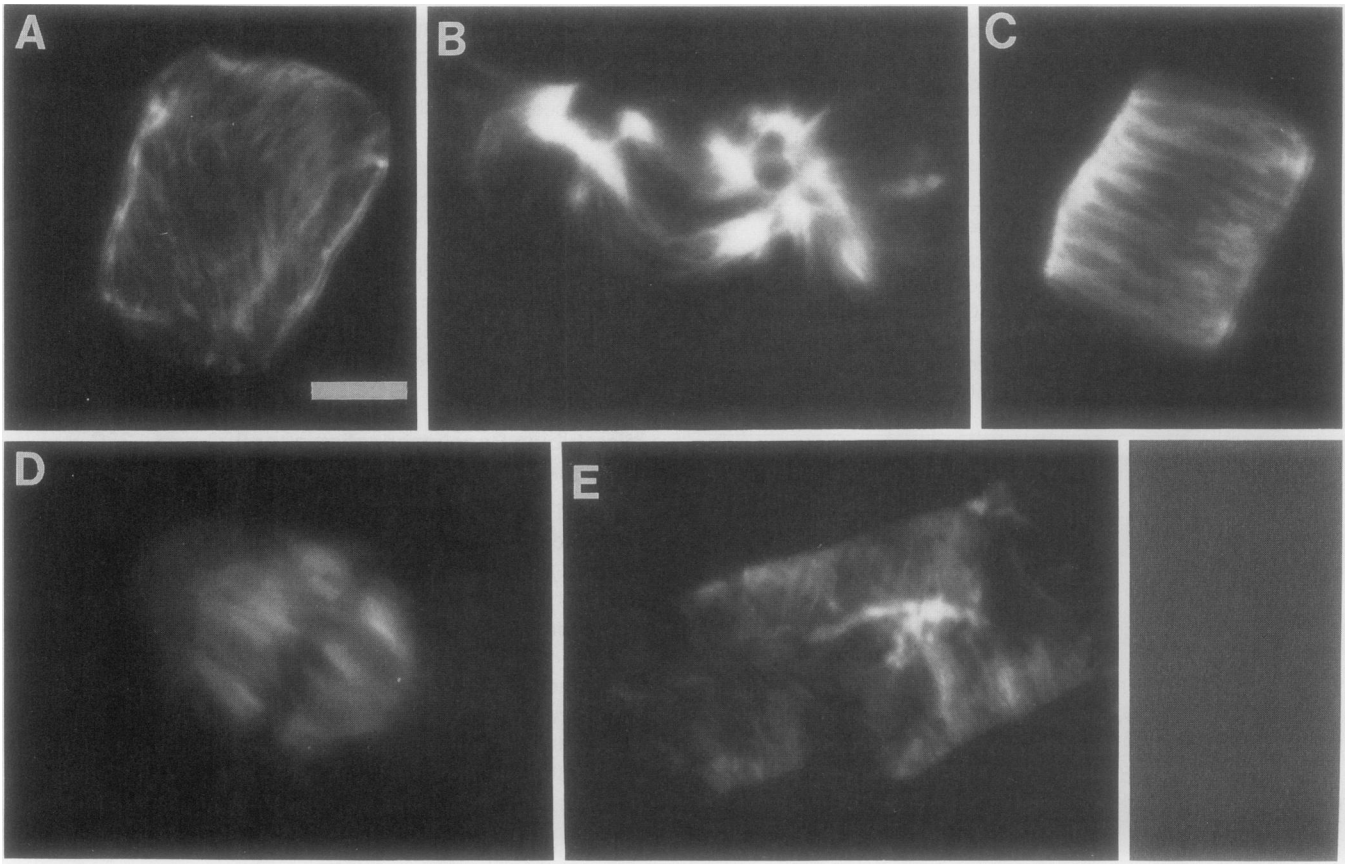

Figure 2. Microtubules in taxol- (A, B, E) or D-secotaxol-treated (C, D) cells of rye. A, Helical microtubule array in taxol-treated cell during interphase (bar = 10 μm); B, abnormal mitotic spindle of taxol-treated cell; C, interphase microtubule array in analog-treated cell; D, normal mitotic spindle in analog-treated cell; E, cold-stable microtubules in taxol-treated cell frozen at −3°C.

Table II. *Influence of Cold Acclimation and Drug Treatments on Freezing Tolerance of Root Tips of Rye*

LT-50 of root tips of rye (*S. cereale* L. cv Puma), treated as described in "Materials and Methods," were determined by a triphenyl tetrazolium chloride reduction assay (27).

Treatment	LT-50
	°C
Nonacclimated	−9
Cold-acclimated	−14
Taxol (10 μM)	−3
D-secotaxol (10 μM)	−9
DMSO (14 mM)	−9

for the change in cold stability of microtubules seen here. Alternately, cold stability of microtubules generated *in vitro* from brain tubulin from antarctic fish has been attributed to changes in α-tubulin (7). The 2 d at 4°C may cause different tubulin isotypes to be present in rye root cells, thereby altering microtubule cold stability directly, or by altering tubulin's interactions with microtubule-associated proteins (5). This hypothesis of altered tubulin is explored further in the accompanying paper (17).

Taxol Decreases Freezing Tolerance

Taxol stabilized the microtubules of root-tip cells of rye to cold (Table I), a characteristic effect of this drug (14). These microtubules were stable even during a −3°C freeze. This stabilization of microtubules may be responsible for the large difference in LT-50 between taxol-treated seedlings and controls (Table II). The possibility that the increased freezing sensitivity of the taxol-treated seedlings is due to an effect of taxol on another cellular system was tested. The taxol analog D-secotaxol, which has a chemical structure very similar to taxol but does not bind to mammalian microtubules (18), was employed. Even though D-secotaxol is similar to taxol, significant conformational changes in the taxane ring system result from opening the oxetane ring of taxol to form D-secotaxol (18), perhaps influencing other properties of this compound. However, no better agent was available to act as a control. Some D-secotaxol-treated cells exhibited microtubule bundling, but this drug did not increase microtubule cold stability to the same extent as taxol (Table I). Although the number of cells with microtubules after a −3°C treatment is higher in D-secotaxol-treated cells (73%) than in controls (54%), the cold-stable microtubules were shorter and fewer in number than in comparable taxol-treated cells, further indicating that more cold-induced depolymerization had occurred in the D-secotaxol-treated cells than in taxol-treated cells. However, the interaction between D-secotaxol and microtubules may be species-specific. When applied to spinach mesophyll cells, D-secotaxol is only slightly less effective than taxol in altering microtubule structure and stability, and in decreasing freezing tolerance (ME Bartolo, JV Carter, unpublished data).

Freezing Tolerance Inversely Related to Cold Stability of Microtubules

Freezing tolerance in rye roots was inversely related to the cold stability of microtubules. Two d at 4°C increased freezing tolerance but decreased microtubule cold stability. Similarly, taxol decreased the freezing tolerance of the roots but increased the cold stability of microtubules. The taxol analog D-secotaxol, which was not as effective as taxol in stabilizing microtubules, did not decrease the freezing tolerance of roots. Thus, it appears to be necessary for microtubules to depolymerize to a threshold level during freezing for root-tip cells of rye to realize their maximal freezing tolerance. The depolymerization experienced in the D-secotaxol-treated cells was apparently above that threshold.

In contrast to our data from these cold-acclimation and taxol experiments, Rikin and coworkers (24, 26) found that chilling injury in cotton was increased by treatment with drugs that depolymerize microtubules, although tubulin depolymerization was not directly observed. The difference between these studies may be due to cotton being a chilling-sensitive plant that does not acclimate appreciably; different mechanisms may exist in cotton cotyledons and winter rye roots for responding to low-temperature stress.

The cause of the relationship we observed between freezing tolerance and microtubule cold stability is unknown, but since one factor in the survival of an organism at low temperature may be the maintenance of fluid cell membranes (19), the depolymerization of microtubules might be a mechanism to maintain a more fluid plasma membrane during a sudden shift to lower temperature. This hypothesis is supported by the work of Aszalos, *et al.* (1), who demonstrated that microtubule depolymerization resulted in increased plasma membrane fluidity in a mammalian system. Presumably, microtubules cross-linked to plasma-membrane components can stabilize plasma membranes. Electron microscopic studies of plant cells have repeatedly shown bridges between cortical microtubules and the plasma membrane (8). Experiments are in progress to address the question of microtubule involvement in freezing tolerance.

Cold Stability of Microtubules

The cold stability of microtubules varies with species and tissue. The high percentage of cells from nonacclimated roots having microtubules after 1 or 2.5 h at 0°C is quite different from results of studies using *Azolla* root tips, in which microtubule depolymerization is observed within 15 min at 0°C (9). Cells of chilling-sensitive tomato cotyledons (15) have microtubules that are depolymerized by 8 h at 5°C. Data of Rikin *et al.* (25) indirectly suggest that microtubules of cotton cotyledons are depolymerized after 3 d at 4°C. In contrast, root-tip cells of rye had complete microtubule arrays after 2 d at 4°C (Table I). We do not know whether the microtubules in rye roots depolymerized at some point during their exposure to cold-acclimating temperatures and then repolymerized by the end of the 2 d at 4°C.

Microtubules with differing cold sensitivity can exist within the same cell (2, 9–11). This is also evident from our observation that in those cells with microtubules, fewer microtubules were present after a 0 or −3°C treatment. (*cf.* Fig. 1, A and D to Fig. 1, C and E). The microtubules that remained were a more cold-stable population.

Microtubules rapidly repolymerize after cold-induced disassembly; repolymerization beginning within 15 min of re-

warming (2, 9, 10). Repolymerization is observed within only 5 min of rewarming in the alga *Closterium* and after 10 min many microtubules are visible (13). Hardham and Gunning (10) calculated that microtubules in an endodermal cell of *Azolla* can polymerize at a total combined rate of 0.6 μm per min after cold-induced depolymerization. Microtubule repolymerization in rye roots after cold-induced disassembly occurred within 1 h of rewarming (Table I). Shorter time intervals were not used because a slow thaw is necessary after a freeze to enhance survival of plant tissues.

The differences in cold stability among the various microtubules within the same cell could be interpreted as indicating that more than one mechanism of depolymerization is occurring in these different microtubule populations. For example, the low-temperature treatments resulted in fewer microtubules in those cells with microtubules (*cf.* Fig. 1, C and D). This suggests that, for a subset of microtubules, a catastrophic depolymerization event occurs of the type described by Mitchison and Kirschner (22) in their dynamic instability model. Freezing to −3°C also resulted in fewer microtubules per cell, but the cold-stable microtubules that withstood this treatment tended to be short (Fig. 1E), perhaps indicating other modes of depolymerization. Low temperature can directly cause microtubule depolymerization. It could also cause microtubule depolymerization indirectly, through such processes as increased cytoplasmic calcium concentration (21). Further work is needed to distinguish between these alternatives.

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