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Microtubules in Mesophyll Cells of Nonacclimated and Cold-Acclimated Spinach[1]

Visualization and Responses to Freezing, Low Temperature, and Dehydration

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ABSTRACT

Responses of cortical microtubules in spinach (*Spinacia oleracea* L. cv Bloomsdale) mesophyll cells to freezing, thawing, supercooling, and dehydration were assessed. Microtubules were visualized using a modified procedure for indirect immunofluorescence microscopy. Leaf sections of nonacclimated and cold-acclimated spinach were slowly frozen to various temperatures, fixed while frozen, and microtubules immunolabelled. Both nonacclimated and cold-acclimated cells exhibited nearly complete microtubule depolymerization after ice formation. After 1 hour thawing at 23°C, microtubules in both nonacclimated and cold-acclimated cells repolymerized. With time, however, microtubules in nonacclimated cells again depolymerized. Since microtubules in cells of leaf tissue frozen slowly are subjected to dehydration as well as subzero temperatures, these stresses were applied separately and their effects on microtubules noted. Supercooling induced microtubule depolymerization in both nonacclimated and cold-acclimated cells, but to a smaller extent than did freezing. Exposing leaf sections to solutions of sorbitol (a cell wall-penetrating osmoticum) or polyethylene glycol 10,000 (a nonpenetrating osmoticum) at room temperature caused microtubule depolymerization. The effects of low temperature and dehydration are roughly additive in producing the observed microtubule responses during freezing. Only small differences in microtubule stability were resolved between nonacclimated and cold-acclimated cells.

Microtubules are a major component of the cytoskeleton of all eukaryotic cells. They are composed of a conserved heterodimeric protein called tubulin. Individual subunits, α and β, join to form the wall of the hollow microtubule filament (18). Microtubules play essential roles in mitosis, cell division, cell wall formation, and a variety of other cellular functions (4). Microtubules are dynamic by nature, with an equilibrium existing between soluble subunits and the polymerized filament. Low temperature generally shifts this equilibrium toward depolymerization (6). In plants exposed to low temperature, therefore, microtubule polymerization status could influence normal cellular functions.

When a plant freezes, ice typically forms on the outer surfaces and spreads through the extracellular spaces within the plant. During extracellular ice formation, a cell can be injured due to low temperature, to the dehydration that results from movement of cellular water to extracellular ice (12), or to a combination of these stresses. When a plant undergoes cold acclimation it becomes capable of tolerating being frozen to lower temperatures. During this process, many constituents of the plant cell change in structure or composition to protect the cell as a whole from the different facets of freezing stress. Included in this response can be changes in stability and composition of microtubules. Kerr and Carter (9) noted that microtubules in root-tip cells of cold-acclimated rye, although stable at 4°C, depolymerize when cooled to 0°C to a greater degree than do microtubules in root-tip cells of nonacclimated rye. Also, Western blot analysis of root-tip extracts revealed that growth at low temperature induces changes in tubulin isotypes (10).

Plant microtubule responses to low temperature and freezing have received little attention. Because of the difficulties involved in visualizing microtubules in cells from other tissues, most of the studies that have been done have been limited to cultured cells, hypocotyls, and root-tips. Under natural conditions, however, these latter tissues are seldom exposed to the same thermal fluctuations as are shoots. The objective of this study was to characterize microtubule responses to low temperature-related stress in a species and tissue that is normally exposed to temperature changes. Responses of microtubules in spinach mesophyll cells to freezing and thawing were monitored using IIF[3] microscopy. In addition, microtubule responses to separate components of an extracellular freeze, low temperature and dehydration, were characterized.

MATERIALS AND METHODS

Plant Material

Spinach plants (*Spinacia oleracea* L. cv Bloomsdale) were grown from seed in a controlled environment growth chamber

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[3] Abbreviations: IIF, indirect immunofluorescence; LT_{50}, lethal temperature at which 50% of the tissue is killed.

in a soil, sand, and peat mixture (3:2:2) in 15 cm pots. The temperature at plant height was 23°C in the light and 20°C in the dark. Illumination (12 h/day) was provided by cool white fluorescent tubes supplemented with incandescent lighting. After 5 weeks, half the plants were transferred to a second chamber for cold acclimation. Plants were cold acclimated for 2 weeks at 5°C with an 8 h photoperiod. During the cold acclimation period plants underwent very little growth. Five-week-old plants were used as the nonacclimated controls. Plants were watered daily and quarter-strength Hoagland's solution was provided every other day. Plants that underwent even the slightest water stress (wilting) were discarded. Leaves measuring 5 to 7 cm in length from base to tip were used for all experiments.

Freezing

The bottom epidermis of leaves was manually removed and 4 mm^2 sections excised from the peeled area and placed peeled-side-down on moist filter paper in 10 ml flat-bottomed vials. The leaf sections were held at 2°C for 1 h, and then cooled 1°C every 30 min, a rate slow enough to preclude intracellular freezing. Samples were nucleated with ice at 0°C. At each test temperature, the samples were held an additional 30 min during which time one sample was fixed (as described below). In parallel experiments, electrolyte leakage was used to determine the LT_{50} of leaf sections as described in Sukumaran and Weiser (17).

Thawing and Repolymerization

Leaf pieces were prepared and frozen as above. Some leaf sections were fixed at low temperature while others were warmed to room temperature and fixed at various time intervals up to 20 h after the freezing treatment.

Supercooling

Leaf pieces were prepared as in freezing treatments but were placed on droplets of water rather than moist filter paper and were cooled to subzero temperatures without ice nucleation. The water in which the leaf pieces were cooled was visually inspected prior to the fixation step to verify that it remained liquid.

Dehydration

Peeled leaf pieces were incubated on solutions of sorbitol and PEG 10,000 for 0.5 to 3 h at 22°C. The concentration of the solutions was gradually increased to simulate the freezing rate (2°C/h). Osmotic concentration, based on the vapor pressure of ice at each test temperature, simulated the freeze-induced dehydration at various temperatures (19). Three concentrations of osmotica were examined: 1.12 osmolal (simulating dehydration at −2°C), 2.15 osmolal (simulating dehydration at −4°C), and 3.22 osmolal (simulating dehydration at −6°C). Osmotic concentrations were verified with a vapor pressure osmometer (model 5100C, Wescor, Logan, UT). After incubation, samples were fixed for 1 h, with 5 min of vacuum infiltration.

IIF Microscopy

The polymerization status of microtubules was assessed via IIF microscopy with antitubulin antibodies, based on a procedure by Wick and Duniec (20). The following protocol was used.

Fixation

After freezing, supercooling, or dehydration treatments, leaf pieces were fixed for 1 h with 3.7% paraformaldehyde in buffer (50 mM PIPES, 5 mM EGTA, 0.5 mM $MgCl_2$, 5 μM leupeptin hemisulfate, 2.5 μM pepstatin A, 5 μM PMSF [pH 6.8]) and 0.1% Triton X-100. Control tissue was fixed at 23°C, whereas treated tissues were fixed at their respective test temperatures for 0.5 h and an additional 0.5 h at 23°C. Despite lacking an osmoticum in the fixative, there was no detectable rehydration in tissues that had been dehydrated or frozen. The fixative was removed by three to four rinses with distilled water or buffer.

Digestion with Pectinase

The leaf pieces were treated for 20 min with 1% Macerase (Calbiochem) in buffer containing fresh protease inhibitors (5 μM leupeptin hemisulfate, 2.5 μM pepstatin A, 5 μM PMSF) and then rinsed several times with buffer and distilled water and stored overnight in buffer containing 0.5 M sorbitol at 5°C. After the buffer was removed, the leaf pieces were rinsed three to four times with double distilled water. With fine forceps, moist leaf pieces were lightly blotted onto a clean multi-welled microscope slide. Individual cells were dislodged by this procedure and cell separation was verified by light microscopy. After blotting, the cells were air-dried onto the slide for 10 to 15 min.

Cellulase Treatment

After drying, a solution of 5% Cellulysin and 1% Macerase (Calbiochem), in buffer containing freshly prepared protease inhibitors (5 μM leupeptin hemisulfate, 2.5 μM pepstatin A, 5 μM PMSF), was gently placed on individual wells. The microscope slide with solution was vacuum infiltrated 5 min and then incubated for 2.5 h at 23°C in a moist chamber. After the enzyme treatment, the slide was immersed in PBS for several minutes followed by a final rinse in distilled water. After rinsing, the slide with its affixed cells was air-dried at room temperature for at least 1 h.

Application of Antibodies

After drying, monoclonal anti α-tubulin (B-5-1-2; generous gift of G. Piperno) was placed on each well. The slide was placed in a moist chamber and incubated at 37°C for 1 h. After incubation, the slide was rinsed several times with PBS and then with distilled water. While the wells were still moist the secondary antibody, fluorescein isothiocyanate anti-mouse immunoglobulin G, was applied to the cells. After incubating 1 h at 37°C, the slide was rinsed in distilled water and PBS (5–10 min), then allowed to air dry briefly before mounting. Microtubules were visualized with a Zeiss photo-

microscope equipped with epifluorescence optics. A barrier filter (KP 580 or 600) was used to block interfering Chl fluorescence.

Determining the Index of Polymerization

The index of polymerization refers to the amount of microtubule polymerization in a given treatment. The procedure for determining the index of polymerization is similar to that described by Kerr and Carter (9), and is outlined below. Within each treatment, at least 100 cells, chosen at random, were observed. The fraction of the cells containing full arrays, partial arrays, or no microtubules, was determined. Cells with full arrays were counted as "1," with partial arrays as "0.5," and cells with no microtubules were scored as "0." These numbers were added together and divided by the total number of cells counted. The resulting percentage was the initial index of polymerization.

Cells having partial arrays varied greatly in their content of intact microtubules. In many treatments there were cells in which partial depolymerization had occurred leaving either a reduced number of long microtubules or short microtubules. To account for this variability, an additional means of evaluating treatment differences was added. The treatments were ranked according to the overall degree of depolymerization using a series of pairwise comparisons. The fraction of cells containing partial microtubule arrays were observed in two treatments, and then, by repeated comparisons, the treatment that had the greater amount of microtubules remaining in those cells was determined. Then each of these treatments was compared with a third treatment in the same way and a ranking of the three was obtained. This was repeated until each treatment within a single experiment had been ranked relative to all other treatments in the same experiment. The final ranking and relative differences between adjacent members in the rank was used to adjust the initial index of polymerization obtained from the three-class system. These adjustments were usually no more than 5%. The identity of the treatments was not known by the microscopist until data on all treatments within a single experiment had been recorded. Each experiment was repeated four or more times except where noted, and identical ranking of treatments was obtained in each repetition.

RESULTS

Indirect Immunofluorescence Microscopy

To visualize microtubules in mesophyll cells from expanded leaves of spinach, several additional processing steps were needed in comparison to the procedure commonly used for root-tip cells (20). First, the epidermis was removed which enhanced fixation, digestion, and hence, overall labeling. After fixation, tissue was digested with a pectinase. This treatment allowed the separation of individual cells when tissue pieces were later "blotted" onto a microscope slide. Finally, to remove enough of the cell wall to allow antibody penetration, 5% Cellulysin (Calbiochem) and 1% Macerase (Calbiochem) was applied to the cells. During both enzymatic treatments, it was necessary to include several protease inhibitors in the enzyme solutions due to the presence of proteases in commercial wall-digesting enzymes (13). The use of protease inhibitors, especially leupeptin and PMSF, was essential with higher concentrations of wall-digesting enzymes and long digestion times. Without this precaution only a small percentage of the cells contained any intact microtubules. Using this protocol, full microtubule arrays could be visualized in 90 to 98% of the cells in control tissue.

Freezing

The LT_{50} of spinach leaf tissue was −2°C when plants were nonacclimated and −6°C when plants were cold-acclimated for 2 weeks, as measured by electrolyte leakage (data not shown; see the accompanying paper). During freezing, the majority of microtubules depolymerized between 0 and −2°C (Fig. 1) in both nonacclimated and cold-acclimated cells. No intact microtubules were seen at −4 and −6°C. When frozen at slower freezing rates (1°C/h) and sampled at −1, −2, and −3°C, microtubule depolymerization increased rapidly at about −1°C, the onset of ice formation (data not shown). During the initial stages of freezing, between −1 and −2°C, cells contained partial microtubule arrays in which the microtubule length and number varied. In addition, many cells contained no microtubules at all. The cell in Figure 2A is an example of a full array, Figures 2, B and C, partial arrays, and Figure 2D is an example of a cell with complete microtubule depolymerization.

Thawing and Repolymerization

When samples were frozen to −2°C and then thawed at room temperature, microtubules in both nonacclimated and cold-acclimated tissue repolymerized in approximately 1 h. In nonacclimated cells frozen to −2°C and thawed, total microtubule length decreased by over 80% after 20 h (Fig. 3). In addition, the nonacclimated tissue was flaccid and water

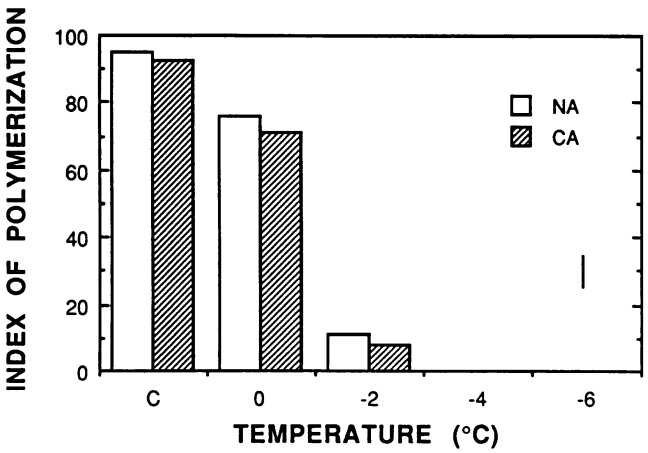

Figure 1. The "index of polymerization" in nonacclimated and cold-acclimated mesophyll cells of spinach that have been cooled to 0°C and frozen to −2, −4, and −6°C. The bars denoted as C represent the index of polymerization in an uncooled control. The height of the bars was determined by averaging data from four separate experiments with similar staining quality. The small solid bar in the lower right indicates the maximum range observed.

soaked. In cold-acclimated cells, repolymerized microtubules remained intact over time. The amount of microtubules 20 h after a treatment closely corresponds to the amount of electrolyte leakage and, hence, to tissue viability.

Low Temperature

In both nonacclimated and cold-acclimated cells, supercooling induced less microtubule depolymerization than did freezing at temperatures from −2 to −6°C (Fig. 4). In cells that had been supercooled to −4 and −6°C many microtubules persisted. In supercooled cells, microtubule length varied greatly. In several instances fully intact cortical arrays were seen in cells supercooled to −6°C, but never in cells frozen to −6°C.

Dehydration

The dehydration that occurs during extracellular freezing was simulated by incubating leaf pieces in an osmoticum (sorbitol or PEG 10,000) at concentrations that provided water potentials equivalent to that of extracellular ice. Dehydration alone induced less microtubule depolymerization than an equivalent extracellular freeze in nonacclimated and cold-acclimated cells, particularly at −4 and −6°C simulations (compare Fig. 5 with Fig. 4). At the various concentrations of osmoticum, microtubules in nonacclimated and cold-acclimated tissue responded similarly to dehydration. At lower concentrations of sorbitol simulating freezing down to approximately −1°C (O.25–1.0 M; data not shown), there were no significant differences between microtubules in nonacclimated and cold-acclimated cells as they underwent dehydration-induced depolymerization. Sorbitol consistently induced less microtubule depolymerization than did PEG 10,000. In more than one experiment, however, this difference did not exceed the range of experimental error.

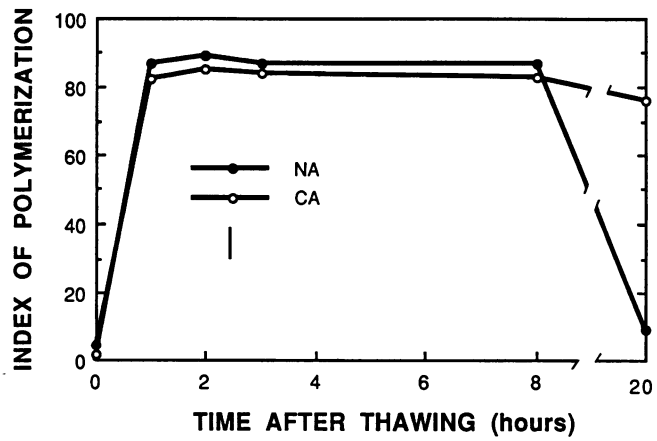

Figure 3. The index of polymerization in nonacclimated and cold-acclimated cells that have been frozen to −2°C, warmed to room temperature (23°C), and held there for up to 20 h. The points represent averages of two separate experiments. The maximum range of any two observations is denoted by the bar in the lower left.

DISCUSSION

Microtubule Arrays Visualized in Mesophyll Cells

By modifying existing immunofluorescence techniques we were able to visualize full microtubule arrays in 90 to 98% of the control cells. As a result, the responses of microtubules to low temperature, dehydration, or freezing treatments could be ascribed to the treatment and not to the vagaries of the visualization method.

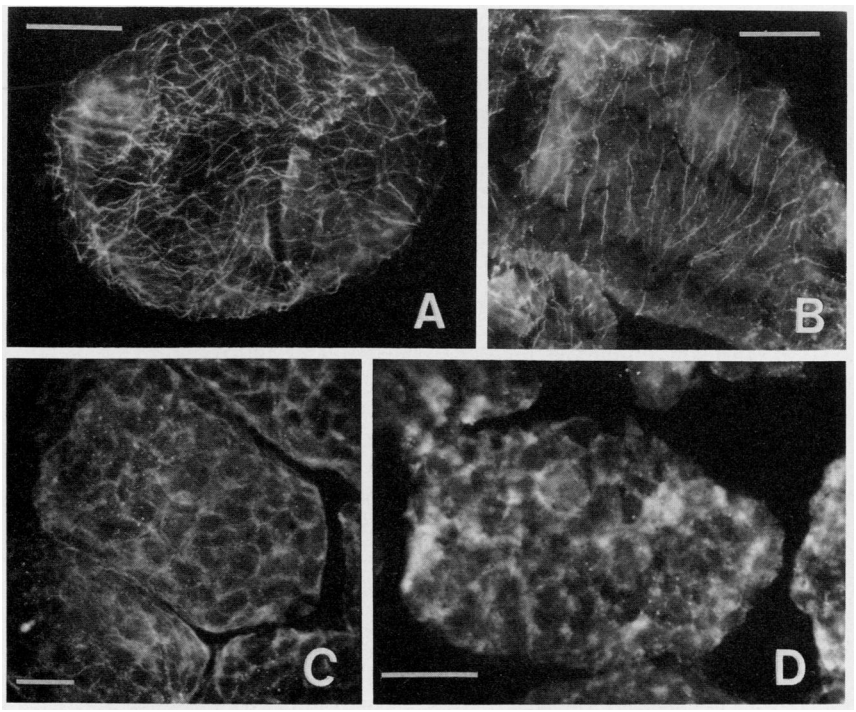

Figure 2. A mesophyll cell of spinach with a fully intact cortical array (A). A population of cells with fully intact cortical arrays would have an index of polymerization of 100. (B) A partial array with a reduced number of long microtubules. A population of cells with this amount of microtubules would have an index of polymerization of about 70. (C) Partial arrays with very short microtubules. A population of cells with this amount of microtubules would have an index of polymerization of about 25. (D) A cell with completely depolymerized microtubules. A population of cells with this amount of microtubules would have an index of polymerization of 0 (bars = 10 μm).

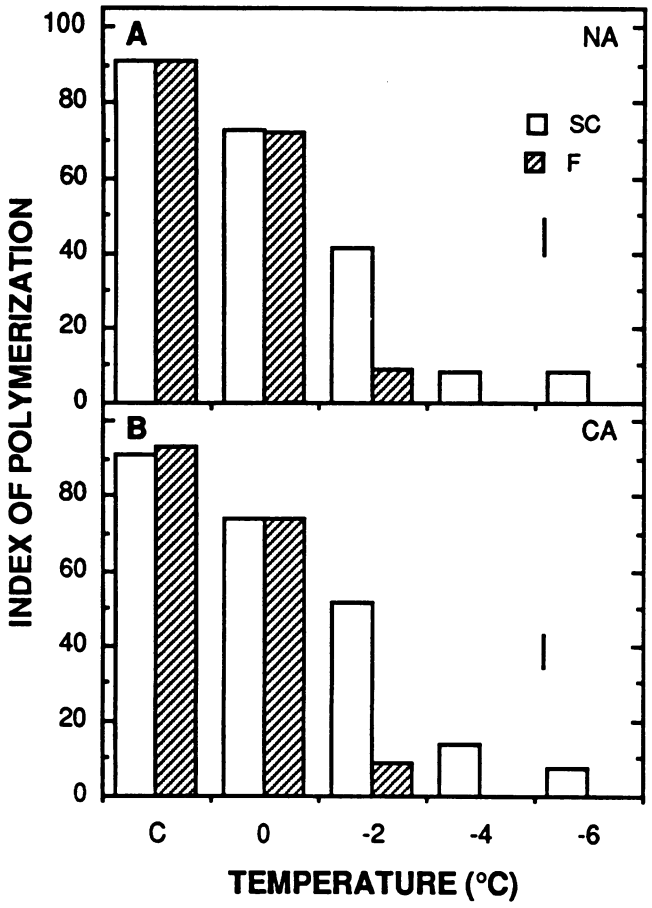

Figure 4. The index of polymerization in nonacclimated (A) and cold-acclimated (B) cells that have been either supercooled (SC) or frozen (F). The bars denoted as C represent the index of polymerization in an uncooled control. The height of the bars was determined by averaging data from four separate experiments with similar control staining quality. The small solid bar in the lower right is the maximum range observed.

To determine the effect of a given treatment we developed a procedure to quantitate microtubule polymerization status. In this procedure, based on a previously described method (9), the fraction of cells that show substantial, but not complete, microtubule depolymerization was accounted for by determining the fraction of cells with normal, partial, and no microtubule arrays and adjusting those values by repeated comparisons between treatments within a single experiment. Individual experiments were repeated several times and comparative index of polymerization values varied by less than 10%.

Freezing Promotes Microtubule Depolymerization

Freezing caused microtubule depolymerization in mesophyll cells of spinach as in root-tip cells of onion (3) and rye (9). In leaf sections frozen to −2°C, nearly all the microtubules in both nonacclimated and cold-acclimated cells depolymerized. They then repolymerized within 1 h after thawing (Fig. 3). The microtubules in cold-acclimated cells recovered fully after the initial freeze-induced depolymerization. In contrast, microtubules were not present in nonacclimated tissue fixed and immunostained 20 h after the initial freeze. This loss of organized structure within the cytoplasm after thawing may be another manifestation of freezing injury. Indeed, the integrity of microtubules 20 h after a freezing treatment correlates sufficiently well with the amount of injury determined by electrolyte leakage that microtubule polymerization status could be used as an independent assay for freezing injury.

The initial repolymerization of microtubules in nonacclimated cells frozen to −2°C and then thawed demonstrates that the components necessary for microtubule assembly are not damaged directly by a damaging freeze. The subsequent disappearance of microtubule arrays in these cells is evidently a part of a gradual onset of freezing damage.

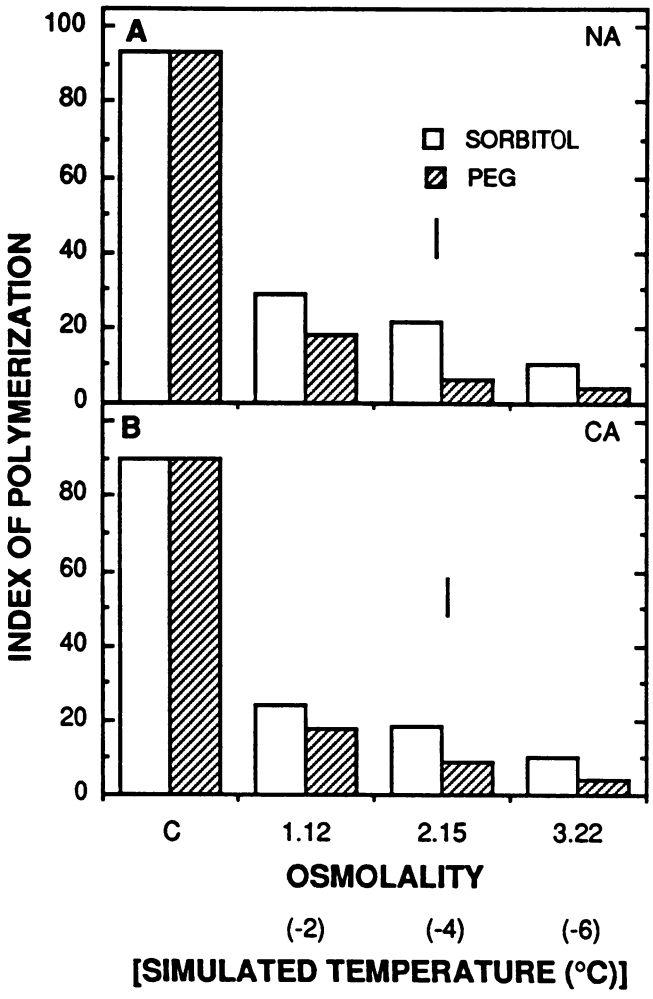

Figure 5. The index of polymerization in nonacclimated (A) and cold-acclimated (B) cells that have been dehydrated with sorbitol and PEG 10,000. Different concentrations of each osmoticum were used to simulate the freeze-induced dehydration that occurs at −2, −4, and −6°C. The bars denoted as C, represent the index of polymerization in an nontreated control. The height of the bars was determined by averaging data from four separate experiments with similar control staining quality. The small solid bar in the lower right indicates the maximum range observed.

Low Temperature and Dehydration Induce Depolymerization

In our system, supercooling caused substantial microtubule depolymerization but not as much as did freezing to the same temperature. In addition, the rate of microtubule depolymerization is slower in response to supercooling than in response to freezing. When supercooled, microtubules in some spinach cells persisted to −6°C. Because the cooling rate was 2°C/h and the tissue was held at 0°C for 0.5 h, these microtubules remained intact for 3.5 h at 0°C or lower. They may represent a subpopulation of cold-stable microtubules.

Those microtubules that did depolymerize in the cold could have done so because of a direct destabilizing effect of low temperature. Low temperature alone is well known to cause depolymerization of certain microtubules *in vitro* (4).

Microtubules have been isolated from certain organisms, however, that do not depolymerize in the cold, even *in vitro* (5). Thus, although low temperature does affect the microtubule-constituent protein equilibrium, this effect is not always sufficient to bring about microtubule depolymerization. Low temperature can cause other cytoplasmic changes that can destabilize microtubules, however. Cytoplasmic calcium ion concentration appears to rise in chilling-sensitive plants when they are cooled (21), and most microtubules are sensitive to micromolar concentrations of calcium ion (4). Thus a rise in calcium ion concentration in spinach mesophyll cells when they are cooled could be responsible for the observed depolymerization of microtubules in supercooled spinach leaf tissue.

Little is known about the response of microtubules to dehydration *in vivo*. Dehydration, like low temperature, can have both direct and indirect effects on the stability of microtubules *in vivo*. As far as direct effects are concerned, slight osmotic perturbations have been reported to change the orientation of microtubules in elongating plant cells (15), and microtubules assembled *in vitro* from calf brain tubulin undergo changes in their x-ray diffraction pattern as a result of reduced hydration of constituent proteins (2). But it seems clear that the most important direct effect on microtubules of dehydration must be a stabilization, since some of the water molecules bound to individual constituent proteins are released when these proteins are incorporated into a microtubule (11). Thus, the dramatic effect of dehydration on microtubules in spinach mesophyll cells is most likely an indirect effect. Cytoplasmic calcium ion concentration, normally low enough not to interfere with microtubule status, may be elevated by dehydration to a level that destabilizes microtubules. Alternatively, dehydration may cause active release into the cytoplasm of calcium ion from the endoplasmic reticulum or vacuole, as is known to occur in response to chilling (21) and salt stress (14). There may be, of course, microtubule destabilizers other than calcium ion the cytoplasmic concentrations of which are actively or passively increased by dehydration.

Differential Stability between Microtubules in Nonacclimated and Cold-Acclimated Cells

Cold acclimation had little or no effect on the lability of microtubules in spinach mesophyll cells. That is, microtubules in cold-acclimated tissue depolymerized at the same temperatures and osmotic potentials as their nonacclimated counterparts. Kerr and Carter (9) found that microtubules in cold-acclimated root-tip cells of rye were more sensitive to low temperature-induced depolymerization than were microtubules in nonacclimated cells. The differences in our findings may be related to the species or tissue we examined since microtubule sensitivity to low temperature can vary widely in different plants and plant parts. For example, in *Haemanthus katherinae*, a tropical species, 1 to 3 h at 0°C caused microtubule depolymerization in its wall-less endosperm cells (1). Similarly, 1 h at 0°C caused a disruption of approximately 70% of the microtubules in epicotyl cells of *Pisum sativum* L. cv Little Marvel (16). In root-tip cells of onion, Carter and Wick (3) observed that 3.7 h at 4°C did not cause microtubule depolymerization. Six hours at 0°C, however, caused complete loss of tubulin immunofluorescence. In the grass species *Lepidium sativum*, microtubule formation was unaffected, but microtubule number was reduced at 3 to 4°C. In another grass, *Lolium temulentum*, microtubules in stem fibers with primary walls were depolymerized by 2 h at 4°C but fiber cells undergoing secondary wall formation were unaffected (8). Finally, in mesophyll cells of tomato cotyledons, microtubules depolymerized when subjected to 5°C for between 4 and 8 h (7). With such diversity it is not surprising that cold acclimation alters microtubule responses to low temperature in rye and not in spinach.

We have observed some of the responses of plant microtubules to freezing and its component stresses. We found plant microtubules *in vivo* are destabilized by both low temperature and dehydration. As components of a freezing stress, the effects of low temperature and dehydration on microtubule status are roughly additive. Since dehydration would be predicted to promote microtubule polymerization (11), the rapid depolymerization of microtubules in response to osmotic stress suggests that freeze-induced microtubule depolymerization may be an indirect result of the effect of freeze-induced dehydration on other cellular responses. The responses may include the concentration of cell solutes or the release of sequestered calcium. Other mechanisms by which dehydration leads to microtubule depolymerization are under investigation.

The finding that freeze-induced microtubule depolymerization in partially cold-acclimated spinach mesophyll cells is complete several degrees above the LT_{50} of the tissue is in accord with similar results reported for microtubules in root-tip cells of winter rye (9). These results suggest that microtubule depolymerization is not a primary cause of freezing injury.

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