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# Is Microtubule Disassembly a Trigger for Cold Acclimation?

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**Cold acclimation was followed in three cultivars of winter wheat (*Triticum aestivum* L.) that differ in freezing tolerance, using root growth as the indicator. During acclimation (followed through 7 d at 4°C), growth rate progressively recovered. The recovery was fast in the tolerant, slow in the sensitive cultivars. The development of freezing tolerance was followed by a challenging cold shock administered after various time intervals of acclimation. Acclimation proceeded faster in the tolerant cultivars. Microtubules were monitored during the acclimation period. A rapid, but transient partial disassembly in the tolerant cultivars preceded the formation of cold-stable microtubules and the recovery of growth rate. In contrast, this transient disassembly was absent in the sensitive cultivar. When a transient disassembly was artificially generated by a pulse-treatment with the antimicrotubular herbicide pronamide, this could induce freezing tolerance. The appearance of cold-stable microtubules was accompanied by a reduced abundance of type TUA1/2 α-tubulin isotypes. These findings are discussed with respect to a role of microtubule disassembly in the sensing of low-temperature stress.**

**Keywords**: Cold acclimation — Microtubules — Pronamide — Winter wheat (*Triticum aestivum* L.).

Abbreviations: EGTA, ethylene glycol bis(2-aminoethyl)-tetraacetic acid; MAP, microtubule-associated proteins; MSB, microtubule-stabilizing buffer; PIPES, 1,4-piperazine-diethanesulfonic acid; TBS, Tris-buffered saline.

## Introduction

In temperate climates, crop yield is limited by low temperatures. The limiting factor is not photosynthesis, but the high cold sensitivity of growth. As a consequence, the productivity during the spring season is constrained by the retarded unfolding of leaves (Watson 1952). The development of freezing-tolerant wheat cultivars that can be planted in autumn and are able to survive the winter, such that leaf development is advanced at the onset of the spring season, has been a major breakthrough in the food security of the temperate regions during the first half of the last century.

Tolerance to low temperature comprises two phenomena that are mediated by different mechanisms: Resistance to cool, but non-freezing temperatures (below 10°C) is termed chilling tolerance and has to be clearly distinguished from freezing tolerance. Irreversible damage caused by temperatures above the freezing point is quite common in crops that originate from tropical or subtropical regions such as cucumbers, melons, tobacco, maize, rice or cotton. This was investigated as so called *Erkältung* (chilling damage) as long ago as the late 19th century (Molisch 1897). The mechanisms responsible for chilling damage are still far from clear, but the cytoskeleton seems to be one of the primary targets: Cytoplasmic streaming, carried by actomyosin, is blocked within a few minutes when temperature falls to 10°C in cucumber or tomato (Sachs 1865, Woods et al. 1984, Tucker and Allen 1986), whereas it can proceed down to 0°C in chilling-resistant species (Lyons 1973). The sensitivity of chilling is closely correlated to the critical temperature that can induce microtubule disassembly (Jian et al. 1989).

Freezing tolerance, on the other hand, depends on targets and mechanisms that are not related to the cytoskeleton. The major problem during freezing is ice crystals that will disrupt internal and external membranes leading to immediate cell death (Burke et al. 1976) or block water access leading to drought stress (Mazur 1963). Numerous studies have shown that this can be prevented by antifreezing proteins that prevent deleterious phase transitions of membranes (for review, see Guy 1999). For instance, the protein COR15a has been shown to stabilize the lamellar phase of plastids against freezing-induced membrane dehydration (Steponkus et al. 1998).

Freezing tolerance contains a strong genetic component with distinct differences between species and even different cultivars of the same crop. But even between individuals of the same cultivar, freezing tolerance can vary considerably, depending on the developmental status of the plant. Freezing tolerance can be acquired during chilling prior to freezing, a process termed cold acclimation. Thus, the progressive drop of temperature during the autumn season will induce acquired freezing tolerance in winter crops. Cold acclimation has been linked traditionally to increases in the content of abscisic acid (Chen et al. 1983), because cold and drought stress induce similar changes in gene expression (for a recent review, see Thomashow 2001). However, numerous reports have shown that the full expression of freezing tolerance will require cold acclimation (for instance, Mohapatra et al. 1988, Yu and Griffith 2001), and some of the genes that are induced during

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cold acclimation cannot be triggered by abscisic acid (Mäntylä et al. 1995, Medina et al. 1999). This finding supports a model where, in addition to abscisic acid, a second pathway that is independent of abscisic acid contributes to acquired freezing tolerance. Mutant studies in *Arabidopsis* have stimulated a model (for review, see Thomashow 2001) where cold-induced calcium influx will trigger a kinase cascade, inhibiting transcriptional repressors such as HOS1, such that constitutively expressed transcription factors such as ICE can activate a transcriptional cascade (initiated by the transcription factor CBF) that will culminate in the expression of freezing protectants such as the COR proteins. It is far from clear where this pathway merges with abscisic-acid induced signaling, but it might well be relatively downstream of this cascade, for instance at the target promoters of CBF.

Since microtubules disassemble in response to low temperature, they are primary candidates for the pronounced cold sensitivity of cell growth. As expected, microtubules are extremely cold-sensitive in chilling sensitive species, whereas they persist at low temperatures in chilling-tolerant species (Jian et al. 1989). In cotton, a chilling-sensitive species, antimicrotubular drugs significantly accelerate and enhance chilling injury, whereas a pretreatment with abscisic acid prevents chilling injury and counteracts the sensitizing effect of antimicrotubular drugs. Chilling injury is accompanied by a destruction of the microtubular network, and abscisic acid prevents this microtubular destruction (Rikin et al. 1980, Rikin et al. 1983).

The role of microtubules in freezing tolerance, however, seems to be different: When microtubule disassembly is suppressed by taxol, this impairs cold acclimation (Kerr and Carter 1990, Bartolo and Carter 1991). On the other hand, upon completion of cold acclimation, the disassembly of microtubules in response to a freezing shock is prevented (Pihakaski-Maunsbach and Puhakainen 1995, Wang and Nick 2001), indicating that disassembly is a transient event that might be related to signal sensing. In fact, the calcium-influx that seems to trigger cold acclimation (Monroy et al. 1993) is conspicuously enhanced by blockers of microtubule assembly (Mazars et al. 1997). This would support a role of microtubules as "thermometers" that modulate the induction of cold acclimation.

In a previous publication (Wang and Nick 2001), we have shown for a Chinese winter wheat cultivar that microtubules acquire cold stability in response to cold acclimation, demonstrating a function of microtubules as effectors of cold acclimation. In the present work we ventured to ask, whether microtubules fulfill an additional function in the sensing process that triggers cold acclimation. We therefore followed microtubule-responses during different phases of cold acclimation using three cultivars of winter wheat that differ in freezing tolerance (Khokhlova et al. 1997): Albidum 114 is characterized by extreme freezing tolerance, Mironovskaya 808 exhibits moderate tolerance, whereas Bezostaya 1 is only weakly freezing tolerant. We observed (1) that cold acclimation proceeds faster in the freezing tolerant cultivars, (2) that early, transient microtubule disassembly is sufficient for an efficient induction of acclimation, and (3) that α-tubulins of the TUA1/2 type are down-regulated in the freezing-resistant cultivars during cold acclimation.

## Results

*Cold acclimation proceeds faster in freezing-tolerant cultivars*

Root growth was followed over time either under different temperature regimes in the cultivars Albidum 114 (high freezing tolerance; Fig. 1A), Mironovskaya 808 (intermediate freezing-tolerance; Fig. 1B) or Bezostaya 1 (low freezing tolerance; Fig. 1C). Under control conditions (25°C, closed squares in Fig. 1A–C), the growth rate decreased progressively with increasing time of cultivation in all three cultivars from a maximum of about 20 mm $d^{-1}$ around day 2 to 5–10 mm·$d^{-1}$ at day 7. The decrease was most dramatic for Albidum 114 (where the maximum growth rate was reached later than in the other cultivars), somewhat slower for Mironovskaya 808 and even less pronounced for Bezostaya 1. When the roots were challenged by a freezing cold shock (–7°C for 2 h), they were killed, such that growth was completely halted (data not shown). When the roots were cultivated at 4°C (open squares in Fig. 1A–C), growth was initially inhibited, but recovered after a delay of several days. This delay was shorter for Albidum 114 and Mironovskaya 808 (about 3 d), longer for Bezostaya 1 (about 4 d). To test whether this recovery of growth rate was related to the appearance of freezing tolerance, the roots were challenged by a cold shock during different times of the acclimation period and the growth rate followed during the day following the shock treatment (arrows and closed triangles in Fig. 1A–C). When the cold shock was administered prior to the recovery of growth, the roots were killed and did not grow, indicating that they had not acquired freezing tolerance. When the cold shock was administered in the freezing-tolerant cultivars during the phase when growth rate was restored, the roots survived and grew even faster during the first day after the shock compared with the acclimated samples without cold shock (compare closed triangles with the open squares in Fig. 1A, B). Again, this differed for the freezing-sensitive cultivar Bezostaya 1 (Fig. 1C): Here, the freezing tolerance lagged behind the recovery of growth rate. When the cold shock was administered late after the recovery of growth, growth rate did not change compared with the unchallenged, acclimated roots. This indicated that the roots had acquired complete freezing tolerance. This situation was observed shortly after day 4 of the acclimation treatment for the freezing-tolerant cultivars Albidum 114 and Mironovskaya 808, whereas it was found to be reached only after day 6 in the freezing-sensitive cultivar Bezostaya 1. This physiological analysis demonstrates that cold acclimation proceeds faster in the freezing-tolerant cultivars.

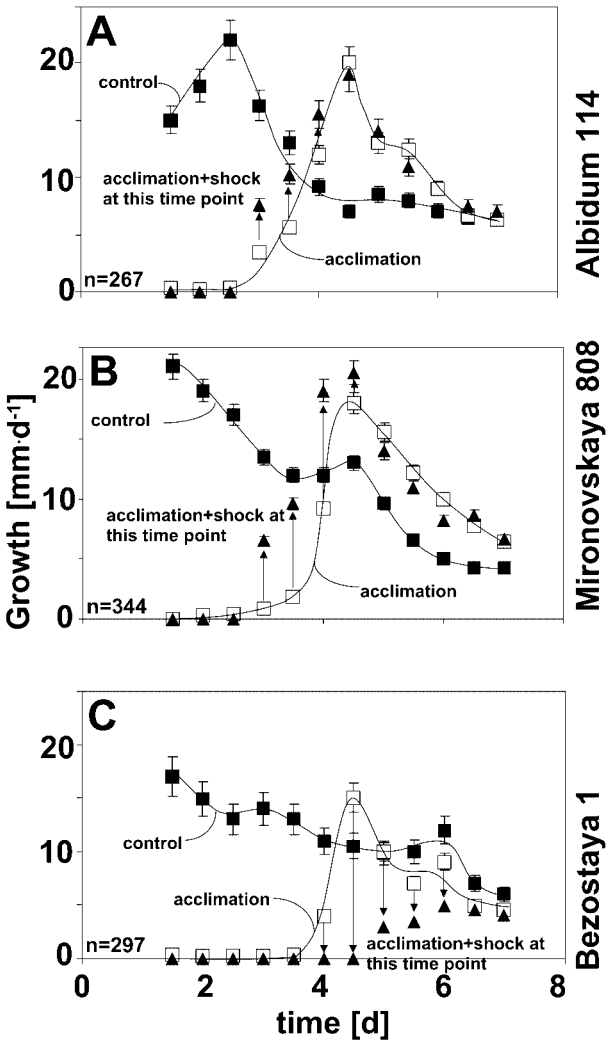


**Fig. 1** Time course of cold acclimation for the response of root growth in the winter-wheat cultivars Albidum 114 (A), Mironovskaya 808 (B) and Bezostaya 1 (C). Growth rates were followed from day 1 after sowing for cultivation at 25°C (closed squares) and for cold acclimation at 4°C from day 1 (open squares). In a parallel set of experiments a challenging freeze shock (–7°C for 2 h) was administered at the times indicated at the abscissae to roots that had been subjected to the acclimation treatment, and the growth rate was followed during the subsequent day (closed triangles and arrows). In non-acclimated roots this treatment caused a complete and irreversible block of growth (data not shown). n indicates the total number of individuals used for each curve, about 20–50 individual roots from two to four independent experimental series were used per data point. Error bars represent the standard deviation of the mean.

*Microtubules acquire cold stability during acclimation in the freezing-tolerant cultivars*

Cortical microtubules were observed throughout the acclimation period in the apical zone of the root for all three cultivars and for all root tissues. However, the cells of the outer cortex were found to be most responsive and were therefore placed in the focus of the present work. Although the behavior of microtubules was followed through meristem, elongation and differentiation zone of the root, the present study is confined to the proximal elongation zone, where the microtubule response was most pronounced. During preparatory studies involving labeling of different root regions, the relative zonation had been found to be similar between the three cultivars. The total length between root tip and the initiation of root hairs was the same for the cultivars Albidum 114 and Mironovskaya 808. It was somewhat reduced (by about 10–15%) in the cultivar Bezostaya 1. To test the cold tolerance of microtubules, they were challenged by the freezing cold shock (–7°C for 2 h) already used to test the freezing tolerance of growth and compared with the situation prior to this challenging shock.

In the cultivar Albidum 114 (characterized by high freezing resistance), microtubules were disassembled into short fragments in response to the challenging freeze shock (compare A and D, Fig. 2). After 0.5 d of acclimation, they showed significant indications of partial disassembly (Fig. 2A) and this deteriorated even more in response to the freeze shock (Fig. 2D).

Interestingly, from 1 d after acclimation the fluorescence intensity increased (Fig. 2G, H), leading to the impression of increased microtubule bundling. In parallel, the effect of a freeze shock disappeared with the time of acclimation. The orientation of microtubule arrays differed between neighboring cells. Oblique microtubules prevailed throughout the acclimation period. The pattern observed for the cultivar Mironovskaya 808 (characterized by intermediate freezing resistance) was very similar. Differences to the cultivar Albidum 114 included a less pronounced initial partial disassembly (after 0.5 d of acclimation) as well as in a less pronounced subsequent increase in fluorescence intensity (Fig. 2B, E).

In contrast, the response of the cultivar Bezostaya 1 (characterized by low freezing resistance), was characteristically different (Fig. 2C, F). In this cultivar, during the early period of acclimation, microtubules did not disassemble in response to the freeze shock, nor did they show the transient partial disassembly after 0.5 d of acclimation. In contrast to the early phase of acclimation, microtubules disassembled when the freeze shock was administered during the late phase of acclimation. When frequency distributions over microtubule orientation were constructed over the whole cell population (data not shown), a progressive increase of transverse microtubule arrays became evident during the later phase of acclimation.

Since the degree of freezing-induced microtubule disassembly varied over the cell population, and the cultivars differed in the density of their microtubular network (compare, for instance, A, B and C, Fig. 2), the integrity of the microtubular network was followed by quantitative image analysis using an algorithm described in Wang and Nick (2001): A grid of probing lines of given length is laid over the cell image such that the lines are oriented perpendicular to the microtubule array (Fig. 3D). The images were processed (see Material and Methods for details) so that the integrated density along this line is proportional to the number of intersected microtubule

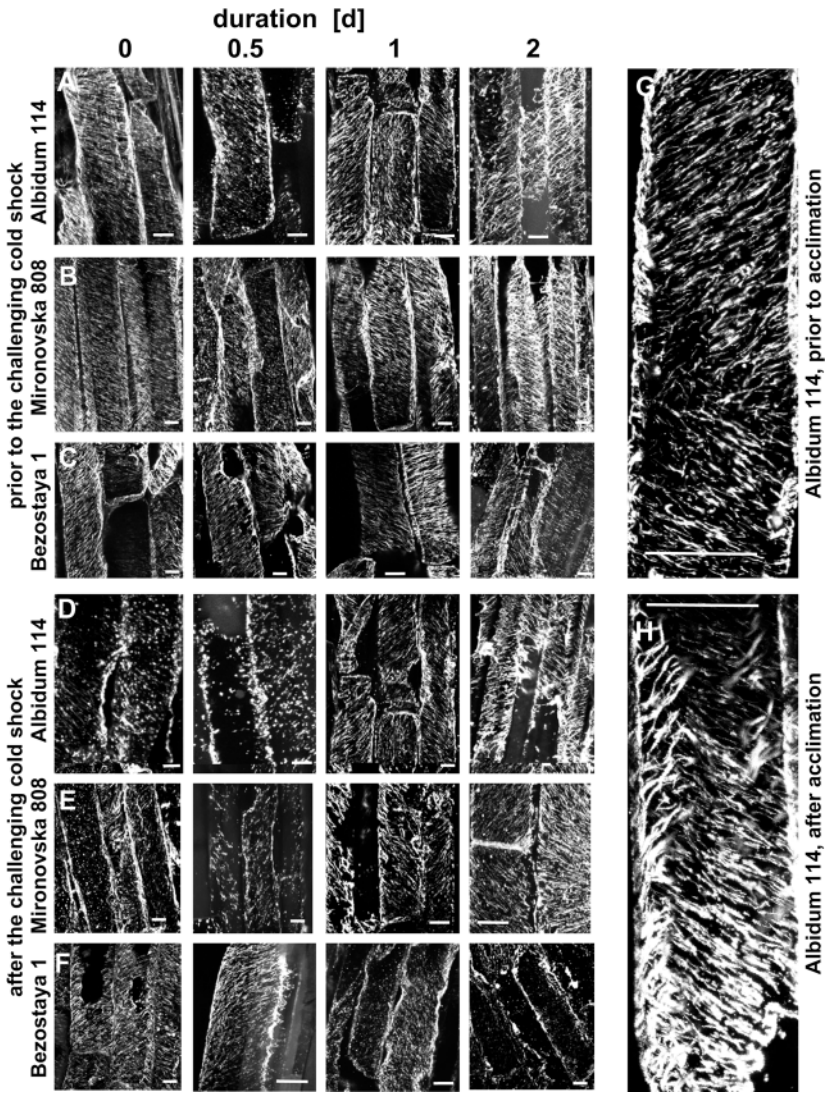


**Fig. 2** Response of cortical microtubules to cold acclimation and freezing in the winter-wheat cultivars Albidum 114 (A, D), Mironovskaya 808 (B, E), and Bezostaya 1 (C, F). Roots were subjected to cold acclimation at 4°C from 1 d after sowing. At the indicated times they were challenged by a freeze shock (–7°C for 2 h). Representative cells from the outer cortex are shown prior (A–C) and after (D–F) this freeze shock. (G, H) Close-up of two cortical cells from Albidum 114 prior to (G) and subsequent to (H) 2 d of acclimation. Scale bars 25 μm throughout.

bundles, irrespective of apparent bundle thickness or apparent fluorescence intensity. For a microtubule array that is intact, this number of intersected microtubules (termed "microtubule frequency") will be high. For a microtubule array that is partially disassembled, the likelihood that the probing line will hit a microtubule is reduced as illustrated by the example shown in Fig. 3D. Thus, microtubule frequency can be used as a measure of microtubular integrity and plotted against the period of acclimation prior to and after the challenging freeze shock (Fig. 3A–C). This reveals a clear difference in the response patterns of the three cultivars. In the cultivars Albidum 114 and Mironovskaya 808 (Fig. 3A, B), microtubule frequency drops transiently after 0.5 d of acclimation, but later recovers to the initial level. A challenging freeze shock reduces this frequency dramatically in the earlier phases of acclimation, whereas this reduction vanishes progressively when the freeze shock is administered in the later phases of acclimation. Both changes are more pronounced in the cultivar Albidum 114, but are clearly present in the cultivar Mironovskaya 808 as well. In contrast, in the cultivar Bezostaya 1 (Fig. 3C), neither a transient decrease nor a subsequent recovery of microtubule frequency is observed. In this cultivar, microtubule frequency decreases continuously during the acclimation period. The freeze shock causes a reduction in microtubule frequency, similar to the other cultivars. However, in contrast to the other cultivars, this reduction is not as pronounced in the beginning, but remains more or less constant even in the later phases of acclimation.

Thus, the response of microtubules during cold acclimation and in response to a challenging freeze shock can be summarized as follows. Microtubules are more susceptible to cold-induced disassembly during the early phase of acclimation in the two cultivars where the recovery of growth rate under acclimation proceeds rapidly (Fig. 1A, B). They are less susceptible in the cultivar Bezostaya 1, where the recovery of growth rate under acclimation is delayed (Fig. 1C). Microtubules

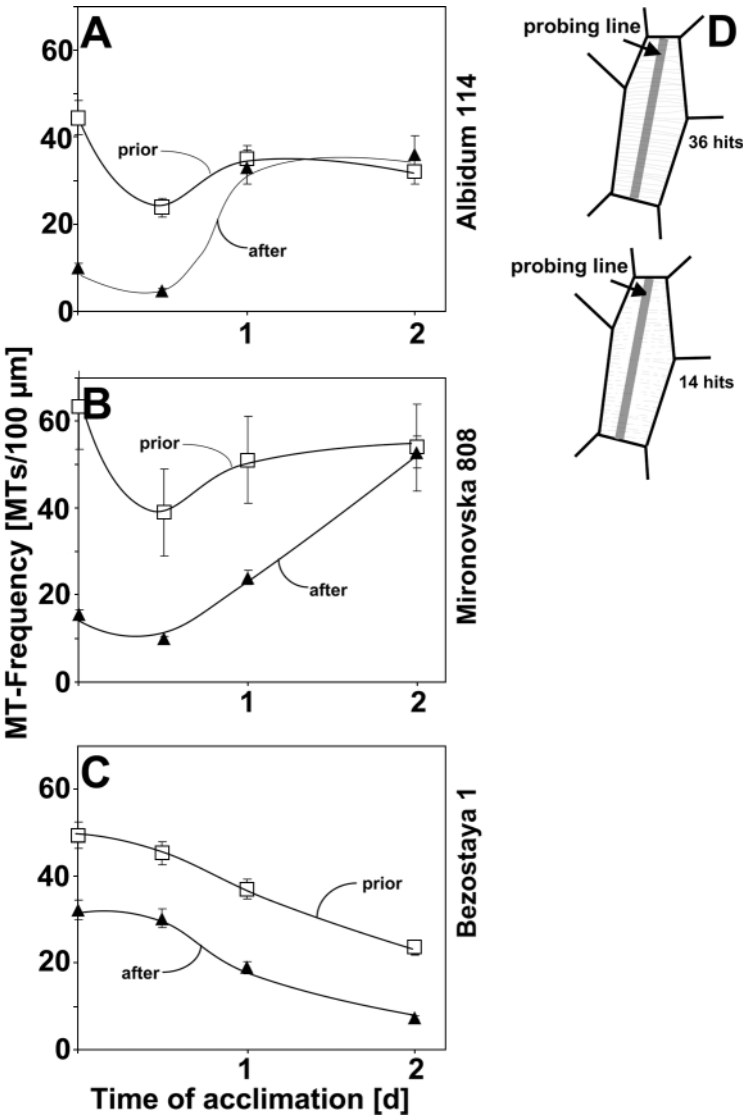


**Fig. 3** Quantitative analysis of the microtubule response to cold acclimation and freezing in the winter-wheat cultivars Albidum 114 (A), Mironovskaya 808 (B), and Bezostaya 1 (C). Microtubule frequency along an intersecting probing line, 100 µm long, as a measure of microtubule integrity (D) is plotted against the time of acclimation. The principle of this measure is illustrated using two arbitrary cells differing in microtubule integrity. The frequency prior to (open squares) and after (closed triangles) a challenging freeze shock (–7°C for 2 h) is shown. Each value for microtubule frequency represents the average of 200 individual cells from 20–30 individual roots.

become increasingly resistant to a challenging freeze shock in the cultivars Albidum 114 and Mironovskaya 808, but not in the cultivar Bezostaya 1. In the cultivars Albidum 114 and Mironovskaya 808, microtubules acquire resistance to a challenging freeze shock before a recovery of growth rate becomes manifest (compare Fig. 1A, B and Fig. 3A, B). This suggests that the microtubular resistance cannot be a consequence of restored growth, and that the resistance of microtubules is not sufficient to mediate the recovery of growth rate.

*Cold acclimation can be induced by pronamide*

The freezing-resistant cultivars where found to show a transient and partial disassembly of microtubules during the early phase of cold acclimation, whereas the sensitive cultivar was observed to lack this disassembly.

We therefore ventured to ask whether freezing resistance could be triggered in Bezostaya 1 by an artificial, reversible disassembly of microtubules induced by incubation with 50 µM of pronamide from 2 h prior to the onset of the experiment (Nick et al. 1991). The pronamide was washed out 12 h after the onset of the experiment (i.e. after an incubation time of 14 h). This treatment left growth essentially unaffected for both cultivation at 25°C, and for cultivation at 4°C (Fig. 4A–C, +pron column). However, it did improve the performance of growth after a challenging freeze shock, even in the sensitive cultivar Bezostaya 1. Interestingly, in this cultivar, a freeze shock, administered after pronamide treatment and subsequent cultivation at 4°C, produced a length increment equalling that of untreated control roots cultivated at 25°C. This means that in this sensitive cultivar, where microtubules fail to disassemble during the early period of acclimation (Fig. 2C, F, 3C), a high degree of freezing resistance can be induced by artificially inducing a transient disassembly of microtubules (Fig. 4C).

To test whether the acquired cold resistance of growth was

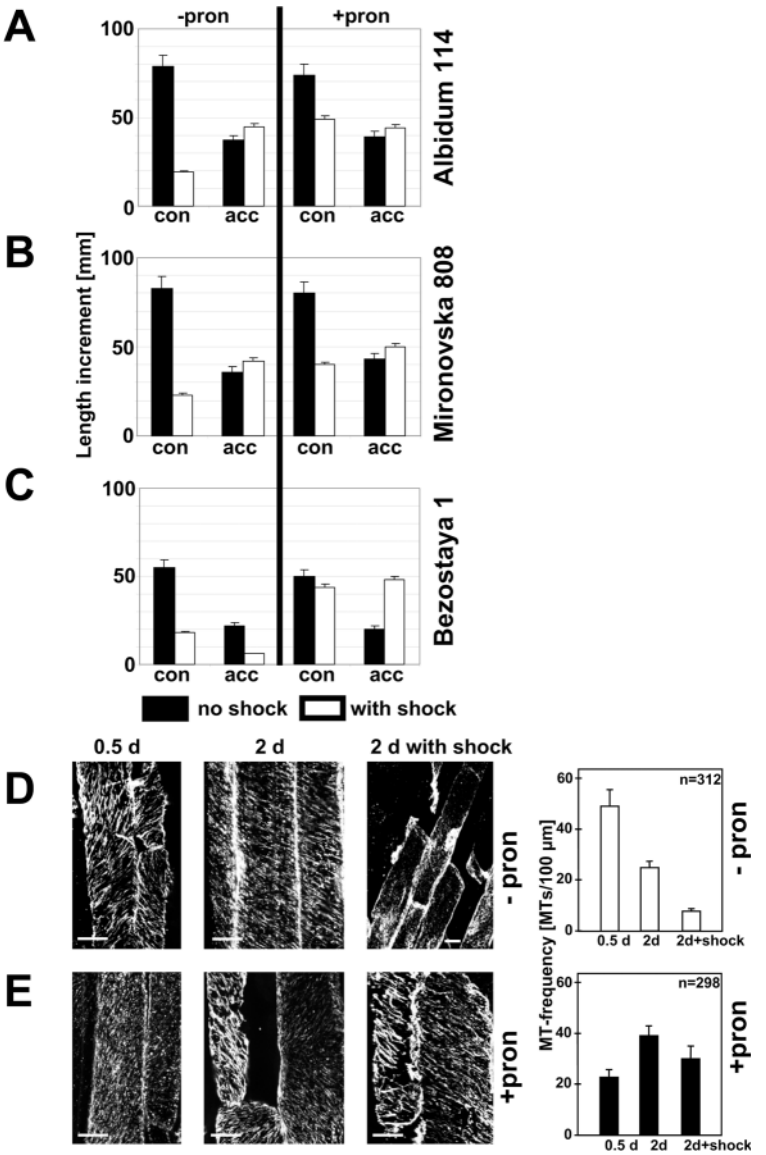


**Fig. 4** Partial microtubule disassembly by pronamide can trigger freezing tolerance in a sensitive cultivar. The response of root growth to freezing is shown for the winter-wheat cultivars Albidum 114 (A), Mironovskaya 808 (B) and Bezostaya 1 (C). Root growth between days 2 and 6 is plotted. Black bars show the increment without the challenging freeze shock (–7°C for 2 h), the white bars show the increment after a freeze shock administered at day 4. Abbreviations: con, cultivation at 25°C; acc, cultivation at 4°C; −pron cultivation without pronamide; +pron, 50 µM of pronamide was added from 2 h prior to the onset of the experiment and washed out 12 h after the onset of the experiment. Each value represents the mean of 20–40 individual roots. Microtubules in cortical cells from Bezostaya 1 roots that were grown for the indicated time intervals at 4°C and either cultivated without pronamide (D) or with pronamide (E). "2d + shock" indicates roots that had been acclimated for 2 d and then challenged by the freeze shock. The bar diagrams in (D) and (E) show the changes of microtubule frequency over the whole cell population.

accompanied by a resistance of microtubules towards the challenging freeze shock, microtubules were visualized at the time of the pronamide treatment and at day 2 of acclimation prior and after the challenging freeze shock (Fig. 4D, E). In contrast to the untreated control under acclimation (Fig. 4D), pronamide-treated cells were characterized by fragmented, partially disassembled microtubules (Fig. 4E). After 2 d of acclimation, the untreated controls displayed clear indications of partial microtubule disassembly, whereas microtubules in the pronamide-treated sample were found to have recovered compared with the situation at the time of the pronamide treatment. However, the decisive difference was observed when these cells were challenged by the freeze shock. Whereas microtubules disassembled almost completely in the untreated control (Fig. 4D), they maintained complete integrity in the pronamide-treated sample (Fig. 4E).

These data show that in the freezing-sensitive cultivar a transient, reversible disassembly of microtubules induced by pronamide can mimic all aspects cold acclimation observed in the freezing-tolerant cultivars. This includes cold resistance of growth as well as cold resistance of microtubules.

*Cold acclimation is related to changes in the pattern of tubulin subpopulations*

Antibodies were raised against carboxy-terminal peptides (Fig. 5A) from the three known rice α-tubulins (Qin et al. 1997) and used to follow the expression of different α-tubulin populations during cold acclimation in roots of the three wheat cultivars.

When cold acclimation was followed by Western-blotting analysis in total root extracts, the total level of α-tubulins

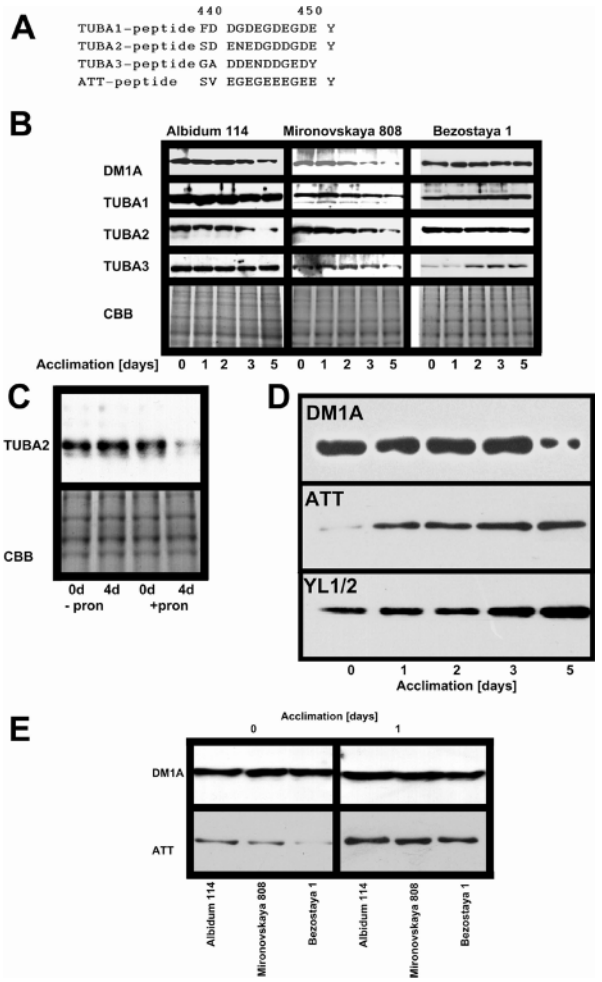


**Fig. 5** Changes in the expression of α-tubulin during cold acclimation. (A) Carboxy-terminal peptides from the three known rice α-tubulins used to raise the antisera and the ATT antibody. (B) Expression of different populations of wheat α-tubulin during cold acclimation as followed using the antisera to rice TUBA1, TUBA2 and TUBA3 in Albidum 114, Mironovskaya 808 and Bezostaya 1. The *CBB* reference gel was loaded in the same way and stained with Coomassie Brilliant Blue. (C) Abundance of isotypes of the TUBA2 group in roots of the freezing-sensitive cultivar Bezostaya 1 prior to and subsequent to 4 d of cultivation at 4°C under incubation without (–pron) or with (+pron) pronamide. For details refer to the legend of Fig. 4. The *CBB* reference gel was loaded in the same way and stained by Coomassie Brilliant Blue. (D) Increase in the abundance of the signal recognized by the DM1A antibody (total α-tubulin) compared with that recognized by the ATT and YL1/2 antibodies (both directed against tyrosinylated α-tubulin) during cold acclimation in roots of the cultivar Albidum 114. (E) Differences in the abundance of tyrosinylated α-tubulin (ATT) compared with total α-tubulin between the three cultivars, at the onset of acclimation, and drastic increase of tubulin tyrosinylation during the first day of acclimation.

(probed by the DM1A-antibody) was found to decrease during acclimation in the freezing-tolerant cultivars Albidum 114 and Mironovskaya 808, whereas it remained constant in the freezing-sensitive cultivar Bezostaya 1 (Fig. 5B). The signal obtained with the TUBA1 antibody showed a similar pattern, although the decrease observed in the tolerant cultivars was less pronounced. In contrast, the signal with the TUBA2 antibody decreased conspicuously in the tolerant cultivars, but remained constant in Bezostaya 1. The TUBA3 antibody revealed a constant signal in Albidum 114, a slight decrease over time in Mironovskaya 808 and a clear increase in Bezostaya 1. These changes of tubulin subpopulations are detectable from about day 2 after the onset of acclimation (Fig. 5B), i.e. later than the partial disassembly of microtubules observed in the freezing-tolerant cultivars during the early phase of acclimation (Fig. 2A, B, 3A, B).

We asked whether a treatment with pronamide that was observed to induce freezing tolerance in Bezostaya (Fig. 4C–E) could induce a decrease of the TUBA2 signal in Bezostaya 1. When the TUBA2 signal in this cultivar at the onset of acclimation was compared with that after 4 d of acclimation, it was found to be suppressed in roots that had been transiently treated with pronamide (Fig. 5C). In contrast, it remained constant in acclimated roots that had not experienced the pronamide treatment. This means that the changes in the pattern of α-tubulin populations, typical for the acclimation of the two freezing-resistant cultivars can also be mimicked by the pronamide treatment.

When the abundance of tyrosinylated α-tubulin as assessed by the ATT and the YL1/2 antibodies (Wiesler et al. 2002) is followed over time, it is observed to change very rapidly (Fig. 5D) in all three cultivars (Fig. 5E). In contrast, the abundance of total α-tubulin as assessed by the DM1A antibody remains constant over the first 3 d of acclimation. The abundance of the epitope detected by ATT and YL1/2 (presumably representing tyrosinylated α-tubulin) increases during the first day of acclimation, i.e. concomitantly with the partial microtubule disassembly in the freezing-tolerant cultivars. Interestingly, prior to acclimation, the ATT signal is most abundant in Albidum 114, and almost undetectable in Bezostaya 1 (Fig. 5E). Again, the signal for DM1A as a measure of total α-tubulin does not differ between the cultivars. This indicates that microtubules are more dynamic in Albidum 114 and Mironovskaya 808 compared with Bezostaya 1.

Thus, the response of tubulins to cold acclimation differs between the freezing-tolerant cultivars Albidum 114 and Mironovskaya 808 compared with the freezing-sensitive cultivar Bezostaya 1. Epitopes recognized by the TUBA2 antibody are down-regulated in the freezing-tolerant cultivars, but not in the freezing-sensitive cultivar. Epitopes recognized by the TUBA3 antibody remain constant in Albidum 114, are slightly decreased in Mironovskaya 808 and are increased in Bezostaya 1. The down-regulation of the TUBA2 signal can be triggered in the freezing-sensitive cultivar by a treatment with pronamide. The ATT and YL1/2 signals (probably measuring the proportion of tyrosinylated α-tubulin) increase during the first day of cold acclimation in all cultivars, but tyrosinylated α-tubulin is already a priori more abundant in the freezing-

tolerant cultivars, indicating that microtubule are a priori more dynamic than in the cultivar Bezostaya 1.

## Discussion

*Freezing tolerance is related to the efficiency of cold acclimation*

Cold acclimation was followed in roots from three winter-wheat cultivars that differ in their degree of freezing resistance (Khokhlova et al. 1997). Root growth was blocked initially but recovered after a lag of several days at 4°C. This recovery was more rapid in the freezing tolerant cultivars Albidum 114 and Mironovskaya 808 compared with the freezing sensitive cultivar Bezostaya 1 (Fig. 1). Concomitantly with this recovery of root growth the tissue acquired increasing tolerance against a challenging freeze shock that caused irreversible damages in unacclimated controls. Again, this tolerance was developed faster in the two freezing-tolerant cultivars, whereas it lagged behind by almost 2 d in Bezostaya 1. In Bezostaya 1, the tolerance lagged behind by almost 2 d and became manifest even later than the recovery of growth. The relative zonation as well as the distance between root tip and root-hair initiation was comparable between the cultivars (the differences were in the range of 10–15% as found during preparatory studies). Moreover, it is not the amplitude of growth rate (which is generally lower by about one-fifth in Bezostaya 1), where the cultivars differ, but a qualitative difference in the onset of recovery and appearance of freezing tolerance. These time-course studies demonstrate that the two freezing-tolerant cultivars are fast in the recovery of growth and fast in the development of resistance to a challenging freeze shock.

*Initial microtubule disassembly is a marker for efficient cold acclimation*

Microtubules were observed to disassemble partially and transiently during the early phase of cold acclimation in the cultivars Albidum 114 and Mironovskaya 808 (Fig. 2A, B, 3A, B), whereas they remained unaffected in the freezing-sensitive cultivar Bezostaya 1 (Fig. 2C, 3C). This finding, at the first sight, is surprising since one would expect that freezing tolerance is mirrored by a cold resistance of microtubules. The situation is different when the final stages of the acclimation process are considered: Whereas in Albidum 114 and Mironovskaya 808, microtubules tolerate a challenging freeze shock (Fig. 2A, B, D, E, 3A, B), they are almost completely disassembled in Bezostaya 1 (Fig. 2C, F, 3C). This is consistent with previous findings in rye (Kerr and Carter 1990, Pihakaski-Maunsbach and Puhakainen 1995) and a Chinese winter-wheat cultivar (Wang and Nick 2001), where microtubules were found to acquire cold stability in response to cold acclimation. However, these studies were investigating the final situation. To our knowledge the present work is the first study in which the early phases of cold acclimation have been investigated with respect to microtubules. Thus, efficient cold acclimation is accompanied by microtubules that initially are easily disassembled in response to chilling but later reorganize into a completely cold-tolerant array. Interestingly, microtubules in unacclimated roots of the same three wheat cultivars differ in their susceptibility to oryzalin (Olinevich et al. 2002): The microtubule damage caused by oryzalin was most pronounced in Albidum 114, followed by Mironovskaya 808 and almost absent in Bezostaya 1. This suggests that initial liability of microtubules to depolymerising factors (chilling in our study or oryzalin) is a marker for efficient cold acclimation.

*Initial microtubule disassembly is sufficient to trigger cold acclimation*

The correlation between partial and transient microtubule disassembly during the early phase of cold acclimation and the efficiency of acclimation indicates a causal relationship but does not prove it. We therefore asked what happens when this disassembly is artificially introduced into the otherwise freezing-sensitive cultivar Bezostaya 1, using pronamide (Fig. 4) at the onset of acclimation. We observed that the pronamide treatment can mimic all tested aspects accompanying the efficient acclimation in the freezing-tolerant cultivars. This includes resistance of root growth to a challenging freeze shock and the recovery of root growth (Fig. 4C), as well as the formation of freezing-resistant microtubules (Fig. 4D, E), and the down-regulation of tubulins of the TUBA2 group (Fig. 5C). In other words, the pronamide treatment can replace cold acclimation.

The pronamide experiments show that the initial, partial microtubule disassembly is sufficient for an efficient induction of cold hardiness.

*Acquired cold stability of microtubules is related to changes in isotype composition*

Using antisera that discriminate between the three isotypes of α-tubulin in rice, changes in the expression of different epitopes could be observed during acclimation. Due to the high similarity between the more numerous, different isotypes present in wheat, it is not possible to discriminate between the isotypes individually without cross-reaction. Isotypes of the TUBA2 group are down-regulated during cold acclimation in Albidum 114 and Mironovskaya 808, whereas they remain more or less constant in Bezostaya 1. In contrast, isotypes of the TUBA3 group that either remain constant or decrease in the freezing tolerant-cultivars, are conspicuously up-regulated in Bezostaya 1. In Albidum 114 and Mironovskaya 808, these changes (Fig. 5B) occur at the time when freezing resistance of growth (Fig. 1A, B) and microtubules (Fig. 2C, D) becomes manifest. This suggests that the cold stable microtubules, characteristic of fully acclimated roots, are depleted of isotypes of the TUBA2 group. In Bezostaya 1, a strong increase in isotypes of the TUBA3 group is observed in response to chilling. The temporal pattern of these changes in isotype composition places them downstream of acclimation. These changes are clearly the effect of cold acclimation, not the cause. They

might, however, be markers for altered binding of microtubule-associated proteins (MAPs) that act as stabilizers. The increase of fluorescence intensity in the microtubules of the freezing-tolerant cultivars (Fig. 2G, H) might be caused by increased microtubule bundling. We plan to test this idea by a combination of electron microscopy and immunolocalisation with heterologous antibodies against plant MAPs.

*Microtubule dynamics increase during the early phase of cold acclimation*

In contrast to the changes in isotype composition that occur relatively late, the relative abundance of the epitope recognized by the ATT antibody increased already during the first day of acclimation (Fig. 5D). The ATT signal probably represents the tyrosinylated portion of α-tubulins, although it cannot be excluded that the reactivity of this antibody with different isotypes differs. To check this possibility, we tested whether the rapid increase visualized by the ATT antibody is also observed with the YL1/2 antibody (Kilmartin et al. 1982), whose specificity predominantly depends on the terminal tyrosine and an acidic residue (glutamate or aspartate) at position 450. Since a rapid increase of the signal is observed for YL1/2 as well (Fig. 5D), we do not think that it is not due to changes in the isotype pattern (moreover, we cannot observe such changes with the TUBA1-3 antibodies during this early phase) but by changes in the proportion of tyrosinylated α-tubulin. This response is observed in all three cultivars. However, it is interesting that the abundance of tyrosinated α-tubulin is a priori highest in Albidum 114, somewhat lower in Mironovskaya and much lower in Bezostaya 1 (Fig. 5E). The tubulinyl carboxypeptidase that is responsible for the detyrosination of tubulin, preferentially acts on tubulin that is assembled into microtubules. Although originally thought to be the cause of microtubule stability, detyrosination was later found to be its consequence (Skoufias and Wilson 1998). The shorter the life time of a given microtubule, the higher its content in tyrosinylated α-tubulin that escaped cleavage by the tubulinyl carboxypeptidase. Thus, the relative abundance of tyrosinylated α-tubulin can be used as a marker for differences in microtubule dynamics even within a given cell (Wiesler et al. 2002). Thus, the increase in tyrosinylated α-tubulin represents the manifestation of increased microtubule dynamics as an early event in cold acclimation. The high efficiency of cold acclimation in Albidum 114 and Mironovskaya 808 correlates with the high dynamics of microtubules already prior to the onset of acclimation. This is supported by the finding that microtubules in Albidum 114 and Mironovskaya 808 are more susceptible to oryzalin than in Bezostaya 1 (Olinevich et al. 2002). Since oryzalin blocks the addition of tubulin heterodimers to a growing microtubule, differences in microtubule turnover will result in differential susceptibility to this drug (Wiesler et al. 2002). The increased microtubule dynamics might be a precondition for the partial disassembly observed in the tolerant cultivars. However, it is apparently not sufficient, since it is also observed in Bezostaya 1, where it is not followed by partial disassembly. This indicates that chilling must induce a second, unidentified factor that, in combination with the increased turnover of microtubules, produces partial disassembly. This factor would then be absent or inactive in Bezostaya 1.

*Microtubules as "thermometers"?*

We have shown in this study that (1) freezing tolerance is correlated with the efficiency of cold acclimation; (2) efficient cold acclimation is accompanied by an initial, partial disassembly of microtubules; (3) this disassembly is sufficient to trigger efficient cold acclimation; (4) efficient cold acclimation is correlated to high a-priori dynamics of microtubules; (5) microtubules with reduced content of isotypes belonging to the TUBA2 group are characteristic of fully acclimated cells.

These findings suggest that microtubules, in addition to their role as effectors of the cold response, must have a function related to the efficient sensing of low temperature, culminating in the induction of the acclimation machinery. The activity or opening of cold-activated calcium channels, that are generally believed to be the triggers for cold adaptation (for review, see Thomashow 2001), is amplified by disassembly of microtubules. In higher plants, the activity of voltage-dependent calcium channels in general (Ding and Pickard 1993, Thion et al. 1996) and, in particular, chilling-induced calcium channels (Mazars et al. 1997) has been shown to be enhanced by microtubule disassembly. In this conceptual framework, the dynamic microtubules in the cultivars Albidum 114 and Mironovskaya 808, in combination with a second factor that triggers disassembly, would ensure an efficient function of cold-activated calcium channels leading to efficient triggering of the signal pathway, culminating in acclimation. The a priori less dynamic microtubules in Bezostaya 1 and the lack of the disassembly factor would be responsible for a sluggish cold response of calcium influx, such that acclimation is triggered at lower efficiency. The changes in the isotype composition observed during advanced stages of acclimation are part of the effector system that stabilizes microtubules against freezing – they cannot be part of the sensing mechanism. The sensing is related to the transient, partial disassembly observed in the freezing-tolerant cultivars. This, in turn, seems to depend on a high innate dynamics of microtubules on the one hand, the activity of a disassembly factor on the other. Both preconditions point to differences in the activity of microtubule-associated proteins. For instance, the chilling-induced calcium influx could trigger a kinase-cascade that would culminate in the activation of microtubule-severing MAPs, such as katanin, or in the inhibition of microtubule-stabilizers, such as MOR1 (for review, see Wasteneys 2002). Alternatively, unidentified factors that participate in the nucleation or in the disassembly of microtubules might be modulated.

In other words, microtubules would act as modulators of cold-activated calcium channels, i.e. they would function as "thermometers". The efficiency of this "thermometer" func-

tion would depend on the degree of microtubule dynamics and the activity of MAPs.

In the future, we want to demonstrate this "thermometer" function directly by monitoring cold-induced calcium fluxes in vivo utilizing wheat lines that express aequorin.

## Materials and Methods

*Plant material*

Caryopses of winter wheat (*Triticum aestivum* L. cvs. Albidum 114, Mironovskaya 808 and Bezostaya 1) were soaked in water for 2 h at room temperature, and then sown equidistantly, embryo up, on moistened double-layered filter paper in Plexiglas boxes (95 mm × 95 mm × 60 mm, 20 seeds in each box). In order to obtain well-grown roots, cultivation took place in complete darkness: The Plexiglas boxes were placed in light-tight black boxes covered with black cloth and kept in a dark room at 25°C for variable time intervals until the seedlings were used for the experiments. Each data point represents the mean from 20–50 individual roots measured in two to four independent time-course experiments.

*Cold treatments*

All treatments took place in the dark. For cold acclimation, the seedlings were cultivated in complete darkness at 4°C (±1°C) from day 2 after sowing through 7 consecutive days. As a challenging cold shock, treatment for 2 h at –7°C using a temperature-controlled incubation chamber (Fryka Kältetechnik, Esslingen, Germany) was utilized. In unacclimated roots, this treatment caused an irreversible inhibition of growth (data not shown).

*Visualization of cortical microtubules by immunofluorescence*

The analysis focussed on segments, 8 mm long (taken from 2 to 10 mm from the root tip), comprising the proximal elongation and a part of the distal differentiation zone (for details, see Wang and Nick 2001). Frequency distributions over microtubule orientation showed that microtubules were mostly transverse or slightly oblique in this zone. Although several tissue layers were examined, the study focussed on the subepidermal cells of the outer cortex layer, where the microtubule response to signals that control root growth is most sensitive (see for instance, Blancaflor and Hasenstein (1993) for gravity, Baluška et al. (1996) for auxin) and where in our hands the response to cold was observed to be most sensitive.

The segments were fixed by 3.7% w/v paraformaldehyde in microtubule stabilizing buffer (MSB: 50 mM PIPES, 5 mM EGTA, 1 mM $MgSO_4$, 1% v/v glycerol, 0.25% v/v Triton-X100, pH 6.9) for 1 h at the respective temperature depending on the treatment prior to fixation.

After fixation, tangential sections were obtained using a vibratome as described in Waller and Nick (1997). The sections were collected and placed on a glass slide in a drop of 1.2% w/v agar dissolved in MSB and then incubated with 5% v/v normal goat serum (Sigma, Neu-Ulm, Germany) in Tris-buffered saline (TBS: 150 mM NaCl, 20 mM Tris-HCl, 0.25% v/v Triton-X100, pH 7.4) for 20 min to block nonspecific interactions. After incubation for 1 h at 37°C or overnight at 4°C with mouse monoclonal antibodies directed against α-tubulin (DM1A, Sigma, Neu-Ulm, Germany; dilution 1 : 100 in TBS), the sections were washed 3× for 5 min with TBS, and reincubated for 1 h at 37°C or overnight at 4°C with a fluorescein-isothiocyanate-conjugated secondary antibody (anti-mouse immunglobulin G from goat; Sigma, Deisenhofen, Germany) diluted 1 : 20 in TBS. Sections were thoroughly washed 5× for 5 min in TBS and then mounted in anti-fade medium (Moviol containing 0.1% *p*-phenylenediamine). Slides were analyzed by confocal microscopy.

*Confocal microscopy and quantification of microtubular responses*

Microtubule images were collected by confocal laser scanning microscopy (DM RBE; Leica, Bensheim, Germany) using the 488-nm line of an argon-krypton laser for excitation, a beam splitter at 510 nm and a bandpass filter at 515 nm, and a line algorithm averaging eight individual scans. Each cell layer was optically sectioned into eight slices and these individual sections were then projected into one stack. The principle of the method is shown in Fig. 3D. To quantify microtubule frequency, images were processed using the Scion Image software (Scion Corporation, Frederick, MD, U.S.A.), using the complete population of cells that were not obviously damaged during the processing or where microtubules were out of focus: After transformation into binary images to eliminate differences in overall intensity, the images were filtered using the Find Edge algorithm. The result was an image where a profile across each microtubule yielded the same integrated density, irrespective of the thickness of the microtubule or its original fluorescence intensity. Under these conditions, it was possible to obtain a linear function between integrated density along a line intersecting the microtubule array, perpendicular to the orientation of individual microtubules and the number of microtubules intersected by this line. This function was used to calibrate the sample data. To obtain the sample data, a lattice of five equally spaced parallel lines, 8 pixels thick, was laid over each individual cell, so that the lines were oriented perpendicular to the microtubule array and did not touch the cell wall. The integrated density along each line was then determined with the Analyze algorithm, averaged for each cell and corrected for background measurements obtained from the same image. Microtubule frequency (defined as the number of microtubules that are intersected by a line 100 μm long) was calculated from these measured values for integrated density, by means of the calibration function. Each value for microtubule frequency represents the average of 200 individual cells from 20–30 individual roots).

*Protein analysis*

Subapical root segments (2–10 mm from the tip) were excised under green safelight (550 nm; Heim and Schäfer 1982) and harvested directly into liquid nitrogen. Total protein extracts were obtained and analysed by SDS-PAGE, Western blotting and visualization by peroxidase-dependent bioluminescence as described in detail by Nick et al. (2000). Equal loading of lanes was verified by staining equally loaded parallels with Coomassie Brilliant Blue-250 (Sigma, Neu-Ulm, Germany).

*Antibodies*

The mouse monoclonal antibody DM1A (Sigma) detecting the epitope AALEKDYEEVG (amino acid residues 426–436; Breitling and Little 1986) present in all known α-tubulins was used to detect total α-tubulin in a dilution of 1 : 300 for Western blotting, in a dilution of 1 : 100 for immunofluorescence. The mouse monoclonal antibody ATT (Kreis 1987, purchased from Sigma) and the rat monoclonal antibody YL1/2 (Kilmartin et al. 1982; purchased from Biozol, Eching, Germany) were used to detect tyrosinylated α-tubulin in a dilution of 1 : 500 for Western blotting. Peroxidase-conjugated antibodies against mouse and rat IgG were used at a dilution of 1 : 2,500 to visualize tubulin in Western blots, fluorescein-isothiocyanate-conjugated anti-mouse IgG antibodies (Sigma) were used in a dilution of 1 : 25 for immunofluorescence. C-terminal peptides for the three α-tubulin isotypes of rice (Qin et al. 1997) were synthetized and conjugated to keyhole-limpet hemocyanin (Pepscan Systems, Lelystad, The Netherlands) and used for the production of polyclonal mouse antisera (Bessler Laboratory, Freiburg, Germany) that specifically rec-

ognized the three isotypes of rice α-tubulin (P. Nick et al. in preparation). These antibodies were used at a dilution of 1 : 300 for Western blotting.

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## References

Baluška, F., Barlow, P.W. and Volkmann, D. (1996) Complete disintegration of the microtubular cytoskeleton precedes its auxin-mediated reconstruction in postmitotic maize root cells. *Plant Cell Physiol.* 37: 1013–1021.

Bartolo, M.E. and Carter, J.V. (1991) Effect of microtubule stabilization on the freezing tolerance of mesophyll cells of spinach. *Plant Physiol.* 97: 182–187.

Blancaflor, E.B. and Hasenstein, K.H. (1993) Organization of cortical microtubules in gravi-responding maize roots. *Planta* 191: 231–237.

Breitling, F. and Little, M. (1986) Carboxy-terminal regions on the surface of tubulin and microtubules. Epitope locations of YOL1/34, DM1A and DM1B. *J. Mol. Biol.* 189: 367–370.

Burke, M.J., Gusta, L.V., Quamme, H.A., Weiser, C.J. and Li, P.H. (1976) Freezing and injury in plants. *Annu. Rev. Plant Physiol.* 27: 507–528.

Chen, H.H., Li, P.H. and Brenner, M.L. (1983) Involvement of abscisic acid in potato cold acclimation. *Plant Physiol.* 71: 362–365.

Ding, J.P. and Pickard, B.G. (1993) Mechanosensory calcium-selective cation channels in epidermal cells. *Plant J.* 3: 83–110.

Guy, C. (1999) Molecular responses of plants to cold shock and cold acclimation. *J. Mol. Microbiol. Biotechnol.* 1: 231–241.

Heim, B. and Schäfer, E. (1982) Light-controlled inhibition of hypocotyl growth in *Sinapis alba* seedlings: fluence rate dependence of light pulses and continuous irradiation. *Planta* 154: 150–155.

Jian, L.C., Sun, L.H. and Liu, Z.P. (1989) Studies on microtubule cold stability in relation to plant cold hardiness. *Acta Bot. Sinica* 31: 737–741.

Kerr, G.P. and Carter, J.V. (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.

Khokhlova, L., Olinevich, O., Pahlich, E., Tarakanova, N., Asafova, E. Volovnic, I. (1997) Effect of tubulin protein modifiers on the water exchange of non-hardened and cold hardened plants of winter wheat. *Acta Agro Hungarica* 45: 377–389.

Kreis, T.E. (1987) Microtubules containing detyrosinylated tubulin are less dynamic. *EMBO J.* 6: 2597–2606.

Kilmartin, J.V., Wright, B. and Milstein, C. (1982) Rat monoclonal antitubulin antibodies derived by using a new non-secreting rat cell line. *J. Cell Biol.* 93: 576–582.

Lyons, J.M. (1973) Chilling injury in plants. *Annu. Rev. Plant Physiol.* 24: 445–466.

Mäntylä, E., Lång, V. and Palva, E.T. (1995) Role of abscisic acid in drought-induced freezing tolerance, cold acclimation, and accumulation of LTI78 and RAB18 proteins in *Arabidopsis thaliana*. *Plant Physiol.* 107: 141–148.

Mazars, C., Thion, L., Thuleau, P., Graziana, A., Knight, M.R., Moreau, M. and Ranjeva, R. (1997) Organization of cytoskeleton controls the changes in cytosolic calcium of cold-shocked *Nicotiana plumbaginifolia* protoplasts. *Cell Calcium* 22: 413–420.

Mazur, P. (1963) Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing. *J. Gen. Physiol.* 47: 347–369.

Medina, J., Bargues, M., Terol, J., Pérez-Alonso, M. and Salinas, J. (1999) The Arabidopsis *CBF* gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temperature but not by abscisic acid or dehydration. *Plant Physiol.* 119: 463–469.

Mohapatra, S.S., Poole, R.J. and Dhindsa, R.S. (1988) Abscisic acid-regulated gene expression in relation to freezing tolerance in alfalfa. *Plant Physiol.* 87: 468–473.

Molisch, H. (1897) *Untersuchungen über das Erfrieren der Pflanzen*. Gustav Fischer Verlag, Jena, p. 73.

Monroy, A.F., Sarhan, F. and Dhindsa, R.S. (1993) Cold-induced changes in freezing tolerance, protein phosphorylation, and gene expression. *Plant Physiol.* 102: 1227–1235.

Nick, P., Heuing, A. and Ehmann, B. (2000) Plant chaperonins: a role in microtubule-dependent wall formation? *Protoplasma* 211: 234–244.

Nick, P., Schäfer, E., Hertel, R. and Furuya, M. (1991) On the putative role of microtubules in gravitropism of maize coleoptiles. *Plant Cell Physiol.* 32: 873–880.

Olinevich, O.V., Khokhlova, L.P. and Raudaskoski, M. (2002) The microtubule stability increases in abscisic acid-treated and cold-acclimated differentiating vascular root tissues of wheat. *J. Plant Physiol.* 159: 465–472.

Pihakaski-Maunsbach, K. and Puhakainen, T. (1995) Effect of cold exposure on cortical microtubules of rye (*Secale cereale*) as observed by immunocytochemistry. *Physiol. Plant.* 93: 563–571.

Qin, X., Gianí, S. and Breviario, D. (1997) Molecular cloning of three rice alpha-tubulin isotypes: differential expression in tissues and during flower development. *Biochim. Biophys Acta* 1354: 19–23.

Rikin, A., Atsmon, D. and Gitler, C. (1980) Chilling injury in cotton (*Gossypium hirsutum* L.): effect of antimicrotubular drugs. *Plant Cell Physiol.* 21: 829–837.

Rikin, A., Atsmon, D. and Gitler, C. (1983) Quantitation of chill-induced release of a tubulin-like factor and its prevention by abscisic acid in *Gossypium hirsutum* L. *Plant Physiol.* 71: 747–748.

Sachs, J. (1865) *Handbuch der Experimental-Physiologie der Pflanzen*. Engelmann Verlag, Leipzig, p. 514.

Skoufias, D.A. and Wilson, L. (1998) Assembly and colchicine binding characteristics of tubulin with maximally tyrosinylated and detyrosinylated alpha-tubulins. *Arch. Biochem. Biophys.* 351: 115–122.

Steponkus, P.L., Uemura, M., Joseph, R.A. and Gilmour, S.J. (1998) Mode of action of the *COR15a* gene on the freezing tolerance of *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA* 95: 14570–14575.

Thion, L., Mazars, C., Thuleau, P., Graziana, A., Rossignol, M., Moreau, M., Ranjeva, R. (1996) Activation of plasma membrane voltage-dependent calcium-permeable channels by disruption of microtubules in carrot cells. *FEBS Lett.* 393: 13–18.

Thomashow, M.F. (2001) So what's new in the field of plant cold acclimation? Lots! *Plant Physiol.* 125: 89–93.

Tucker, E.B. and Allen, N.S. (1986) Intracellular particle motion (cytoplasmic streaming) in staminal hairs of Setcreasea purpurea: effect of azide and low temperature. *Cell Motil. Cytoskeleton* 6: 305–313.

Waller, F. and Nick, P. (1997) Response of actin microfilaments during phytochrome-controlled growth of maize seedlings. *Protoplasma* 200: 154–162.

Wang, Q.Y. and Nick, P. (2001) Cold acclimation can induce microtubular cold stability in a manner distinct from abscisic acid. *Plant Cell Physiol.* 42: 999–1005.

Wasteneys, G.O. (2002) Microtubule organization in the green kingdom: chaos or self-order? *J. Cell Sci.* 115: 1345–1354.

Watson, D.J. (1952) the physiological basis of variation yield. *Adv. Agron.* 4: 101–145.

Wiesler, B., Wang, Q.Y. and Nick, P (2002) The stability of cortical microtubules depends on their orientation. *Plant J.* 32: 1023–1032.

Woods, C.M., Reids, M.S. and Patterson, B.D. (1984) Response to chilling stress in plant cells. I. Changes in cyclosis and cytoplasmic structure. *Protoplasma* 121: 8–16.

Yu, X.M. and Griffith, M. (2001) Winter rye antifreeze activity increases in response to cold and drought, but not abscisic acid. *Physiol. Plant.* 112: 78–86.

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