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# Activation-tagged tobacco mutants that are tolerant to antimicrotubular herbicides are cross-resistant to chilling stress

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**Abstract**

T-DNA activation tagging was used to generate tobacco mutants with increased tolerance to antimicrotubular herbicides and chilling stress. After transformation, protoplast-derived calli were screened for tolerance to treatments that affect microtubule assembly. In one screen mutants with tolerance to aryl carbamates (a blocker of microtubule assembly) were selected, the second screen was targeted to chilling-tolerant mutants that could survive for several months at 3°C, a third screen combined both factors. The resistance of these mutants to aryl carbamates or chilling was accompanied by resistance of microtubules to these factors. The carbamate tolerant mutants were cross-resistant to chilling stress. This was mirrored by an adaptive reorganization of microtubules and a reduction of microtubule dynamics in response to chilling. The analysis of these mutants suggests (1) that microtubule dynamics limit the tolerance to chilling and EPC, and (2) that the cold sensitivity of microtubules limits chilling tolerance in tobacco.

*Abbreviations:* EPC – Ethyl-*N*-phenylcarbamate.

## Introduction

Low temperatures limit crop yield in temperate climates. The reason is that the cold sensitivity of growth is more pronounced than that of photosynthesis. As a consequence, the retarded unfolding of leaves confines productivity during the spring season (Watson, 1952). However, outside the temperate regions sensitivity to low temperature is an important issue as well. Most crop species of tropical or subtropical origin such as cucumbers, melons, cotton, rice, tobacco and many tropical fruits suffer severely, when they are exposed to cool temperatures that are still far above the freezing point. Chilling sensitivity can vary between different species and even between different cultivars of the same crop. An extreme example is the fertility of rice that drops dramatically, when temperatures during flower development fall below 18°C. This so-called chilling damage has to be distinguished from the generally known freezing damage, since it is likely to involve completely different mechanisms. These mechanisms are still far from being understood.

Microtubules disassemble in response to low temperature and are therefore primary targets for chilling stress (for review see Nick, 2000). In fact, in various crops the cold stability of microtubules has been found to correlate with chilling sensitivity, and treatment with blockers of microtubule assembly was observed to increase chilling sensitivity (Rikin et al., 1980). On the other hand, a mounting body of evidence indicates that microtubules are more than simple targets of chilling stress, but might play a role as modulators of cold sensing: When microtubule disassembly in response to low temperature was suppressed by taxol, the long-term adaptation to low temperature (so-called cold hardening) is impaired (Bartolo & Carter, 1991) indicating that microtubule disassembly is necessary to induce efficient acclimation to a cold shock. The primary trigger of cold signaling is generally believed to be a transient rise of intracellular calcium concentration (Knight et al., 1991). This could be

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elegantly demonstrated by transgenic plants expressing aequorin, where the bioluminescence in response to a cold shock could be monitored. When this calcium peak is suppressed pharmacologically, cold hardening is suppressed (Monroy et al., 1993). Disassembly of microtubules was found to stimulate the activity of voltage-dependent calcium channels (Ding & Pickard, 1993; Thion et al., 1996) and specifically, of cold-induced calcium fluxes (Mazars et al., 1997).

These data led to a model (Nick, 2000), where chilling-induced microtubule disassembly capacitates calcium influx through cold-sensitive ion channels which triggers cold hardening and (among other targets) the formation of a cold-resistant microtubular cytoskeleton. According to this model, activation of genes that control assembly or disassembly of microtubules is predicted to modulate chilling resistance in a chilling sensitive species. To test this prediction on the functional level, one would need to overexpress candidate genes and test for altered chilling tolerance. However, these genes are not known.

The generation of dominant (gain of function) mutants by *T-DNA-activation tagging* allows to overcome this drawback and, in addition, to isolate such genes. To isolate unknown genes based on their function, mutant approaches are widely used, often in combination with a gene-tag (in plants often a T-DNA sequence) that permits isolation of the mutated gene. The insertion of a T-DNA sequence into a transcriptional unit routinely results in a recessive mutation by virtue of gene disruption or inactivation. In diploid plants, such mutations become manifest only after repeated selfing of the mutagenized population, which is especially cumbersome for species with higher ploidy (e.g., many Solanaceae). However, the frequency of dominant mutations with a particular phenotype is usually extremely low, and it would require a screen saturating for the respective genome, which is hard to achieve even for a small genome such as that of *Arabidopsis*. Dominance of a trait is conventionally interpreted as a gain-of-function phenotype and can either be caused by mutations in the coding region that lead to constitutive activation of the resulting protein or by mutations that stimulate the expression of the respective gene (Chang et al., 1993).

This consideration stimulated the development of an approach based on activation tagging (Hayashi et al., 1992; Koncz et al., 1994). This technique is based on the application of multiple (four) transcriptional enhancer elements originating from the CaMV-35S promotor fused to the complete CaMV-35S promotor linked to the left border of the transformation plasmid. The right border of the insert harbors a hygromycin resistance gene as a marker to select transformed plant cells. In addition, an ampicillin resistance gene along with an *E. coli* origin of replication are located between the hygromycin resistance gene and the multiple enhancers allow selection in bacteria during plasmid rescue into *E. coli* (Feldman, 1991). Upon integration into the plant genome, this insert is expected to cause dominant *cis*-activation of genes located in the vicinity of the T-DNA integration site. Thus, the insertion of the tag should activate flanking genes as a result of the influence of the multiple enhancers. The overexpression of the flanking gene should produce dominant traits such that selection would be possible directly in the primary transformants. Activation tagging has been successfully employed to identify a histidin-kinase from *Arabidopsis* that can bypass the requirement for cytokinin during shoot regeneration (Kakimoto, 1996) or to isolate mutants with altered morphogenesis or constitutive pathogen resistance (Weigel et al., 2000).

Antimicrotubular compounds are often designated as 'microtubule-disrupting drugs'. This implies that these agents destroy assembled microtubules. This impression is wrong, however, as all microtubule blockers known so far, bind to tubulin heterodimers and prevent their incorporation into the growing end of the microtubule (Mizuno & Suzaki, 1991, for a recent review see Vaughn, 2000). Resistance to assembly blockers might either be caused by reduced affinity of tubulin for the inhibitor or simply by reduced dynamics of assembly and disassembly (Nick et al., 1994). A microtubule with high turnover will be more sensitive to reduced assembly, because disassembly will continue at a high rate. Conversely, a microtubule with low turnover will tolerate a reduced rate of assembly and will persist in the presence of the inhibitor. For instance, the sensitivity of growth to ethyl-*N*-phenylcarbamate (phenyl urethane, EPC) has been shown to be elevated after addition of auxin, although the affinity of α-tubulin for this antimicrotubular herbicide is reduced by auxin (Wiesler et al., 2002). This indicates that, under these conditions, not the affinity of the inhibitor, but the turnover of microtubules limits the tolerance of microtubules against EPC.

The stability of microtubules is generally believed to depend on the activity of structural microtubule-associated proteins (MAPs) that decrease the frequency of microtubule destruction (Bin-Bing & Kirschner, 1999; Caudron et al., 2000). Again, so far

no real homologues of well-known structural MAPs such as Tau have been found in plants, indicating that assembly and disassembly are either controlled by completely novel proteins or by proteins that carry different functions in animal cells. To find genes carrying the MAP-function in plants, a screen for mutants that tolerate antimicrotubular herbicides could be used. However, this screen would yield also mutants that are altered in the tubulin molecule itself (Nick et al., 1994).

These considerations led us to design a screen, where activation-tagging is combined with a selection with a microtubule-assembly blocker. In contrast to mutagenesis techniques that produce point mutations, the insertion of a large tag into the host DNA is very unlikely to reduce the affinity of a target tubulin by changing the binding site of the inhibitor. Activation tagging, followed by a screen for herbicide tolerance should be specific for mutants with reduced turnover of microtubules. This might arise by elevated expression of structural MAPs due to activation of their promoters or due to activation of factors that regulate the expression or activity of these MAPs. In the present screen we used the assembly blocker ethyl-*N*-phenylcarbamate (phenyl urethane, EPC). It binds to the carboxyterminus of α-tubulin with the terminal tyrosine that is crucial for the affinity (Wiesler et al., 2002). In parallel, we screened for mutants with increased chilling tolerance or accelerated cold acclimation. This allowed us to ask, whether cold tolerance can be achieved through a manipulation of microtubule turnover. In the current study we describe the generation, screening and phenotype of these mutants and show that mutants that have been generated by T-DNA activation and are tolerant to EPC, are mostly cross-tolerant to chilling. This suggests that the dynamics of microtubule assembly and disassembly is responsible for chilling sensitivity in tobacco.

## Materials and methods

### *Plant material and protoplasts*

The streptomycin-resistant, diploid tobacco line SR1 (*Nicotiana tabacum* cv. Petit Havana, Maliga et al., 1973) was used for T-DNA activation tagging. Seeds were sterilized with 70% ethanol for 2 min, followed by an incubation for 5 min in sodium hypocloride containing 0.1% Triton X-100. They were then washed five times with sterile water and dried under sterile conditions. Sterilized seeds were germinated on solid MS medium (Murashige & Skoog, 1962) with 2% sucrose. Germinated seedlings were transferred to plastic containers (PS-68.0/110 mm, Greiner Labortechnik, Germany) on MS medium and cultured at 24°C in a 16:8 h light-dark cycle in a phytotrone (Certomat®, BS-1, Germany). Shoot tips were excised and subcultured every 4–5 weeks. For protoplast isolation only well-rooted plants were used that had undergone at least three subcultures *in vitro* and then grown for 5–6 weeks. Mesophyll protoplasts were isolated according to Potrykus and Shilito (1986) with minor modifications: fully expanded leaves were placed upside down in a sterile petridish on 6 ml of CNT-medium [KAO medium (Kao & Michayluk, 1975) with 1% cellulase (Onozuka-R, Yakult, Japan), 1% macerozyme (Serva, Heidelberg, Germany) and 0.4 M sucrose], the midrib was removed and the leaf blade cut into pieces of 0.5–1 $cm^2$ area in the liquid medium. Then additional 6 ml of CNT-medium were added, and the leaves were incubated overnight at 26°C in the dark. Protoplasts were released from the digested tissues by careful passage through a 10 ml pipette with a large opening. Subsequently this mixture was passed through a sterile stainless steel mesh sieve (mesh size 100 μm, Saulas Francois, Montreuil, France). This filtered protoplast suspension was very carefully overlaid with 1 ml PNT medium (KAO medium including 0.4 M glucose) on top of the suspension and then centrifuged for 5 min at low speed (Eppendorf centrifuge No. 5403, 1000 rpm, 25°C). Intact protoplasts were collected from the interphase and transferred into a new tube using a 1 ml sterile micropipette with a cut tip. Ten microliters of fresh PNT medium were added and mixed gently, followed by a second centrifugation under the same conditions. This washing step was repeated and a small aliquot of the washed protoplasts was used for the estimation of cell density in a hematocytometer (Blau Brand, Germany). The supernatant was carefully removed and the isolated protoplasts resuspended in PNT medium to a density of $2 \times 10^5$ protoplasts $ml^{-1}$. This protoplast suspension was then transferred into a sterile, 10 cm petri-dish, sealed with parafilm and incubated at 26°C in the dark for 7–8 days, when most of the protoplasts passed through the second division.

### *Transformation*

For transformation, the *Agrobacterium tumefaciens* strain GV 3101 containing the helper plasmid

pMP90RK, harboring the activation vector pPCV Tac7 was used (Koncz et al., 1989, 1990). This vector contains a full-length CaMV-35S promoter complemented with four enhancer sequences from the 35S RNA promoter linked to the left border (LB) of the T-DNA. The right border (RB) was linked to a hygromycin-resistance gene as selectable marker and to a *nos* terminator sequence. The T-DNA also contains an *E. coli* origin of replication and an ampicillin resistance gene that allows selection of *Agrobacterium* on carbenicillin. For routine maintenance, the bacteria were subcultured every month on fresh YEB medium (Walden et al., 1995) complemented with 100 mg $l^{-1}$ carbenecillin. The mesophyll protoplasts were cultivated with *Agrobacterium* 7–8 days after protoplast isolation when most of the protoplasts passed through the second division. The protoplast culture was incubated with freshly cultivated *Agrobacterium* in YEB medium with 100 mg $l^{-1}$ carbenicillin at a density of 100 bacteria per individual protoplast, corresponding to $100/(1.8 \times OD_{600})$ µl of bacterial suspension per $10^6$ protoplasts. After cocultivation in the dark at 26°C for 48 h, the protoplasts were washed three times with W5 medium (Walden et al., 1995) at low speed (1000 rpm, 3 min, 25°C) and resuspended in the same volume of PNT medium. These cells were then directly subjected to the different screening protocols (see below). Aliquots were used to determine plating and transformation efficiency. After 2 days in liquid medium, the dividing cells were resuspended in half the volume of double-concentrated PNT medium. This was mixed very gently with hand-warm 1.6% (w/v) Sea Plaque Agarose (Duchefa, The Netherlands) in double-concentrated PNT medium. The mixture was solidified in small petri-dishes (3 cm diameter) for 30 min, the solidified agarose containing the dividing protoplasts cut into halves and transferred to large petri dishes (10 cm diameter) containing 20 ml of AA1 medium (Caboche, 1980) and further cultivated in the phytotron as described above. All media contained 150 mg $l^{-1}$ of cefotaxim and 75 mg $l^{-1}$ ticarcillin (both Duchefa, The Netherlands) to suppress bacterial growth. For the estimation of plating efficiency no other antibiotic was added; for the screens and for the estimation of transformation efficiency 15 mg $l^{-1}$ of hygromycin were used throughout. Unless stated otherwise, all cells were incubated at 24°C in a phytotron under dim white light with a 16:8 h light-dark cycle.

*Screening for tolerance to aryl carbamates and chilling*

The following screening strategies were employed:

1. Reduced dynamics of microtubule assembly and disassembly: this was selected by tolerance to the aryl carbamate ethyl-*N*-phenyl carbamate (phenylurethane, EPC) that was added at 300 µM. After 1 month, the concentration of EPC was raised to 500 µM. From this screen, seven lines could be recovered that were designated as *Activation-Tagged EPC-Resistant* (ATER) lines.
2. Chilling tolerance: this was selected by incubating the cells at 3°C over several months. Three mutant lines could be recovered from this screen that were designated as *Inuit* lines.
3. Chilling tolerance that is based on reduced microtubule dynamics: this was selected by combining the two selective pressures, that is, the cells were incubated at 3°C on 300 µM of EPC. Only one line could be recovered from this screen that was designated as *EPC-Inuit 1*.

*Regeneration of transformants and propagation of the lines*

After selection of small, putatively transformed calli were transferred to solid MS-morpho medium (Caboche, 1980) for the initiation of shoots. For the ATER lines this was possible after 6–8 weeks and for the *Inuit* or *EPC-Inuit* lines after 8 months. For the ATER and the *EPC-Inuit* lines, the selective pressure by EPC was maintained by adding 300 µM of EPC to the regeneration medium. Regenerating shoots were excised under sterile conditions and transferred to solid, hormone-free MS medium for rooting. Well-rooted plants were later transplanted into soil and raised in the greenhouse till seed set.

*Leaf disc assay for EPC and chilling tolerance*

To assay tolerance to EPC or chilling, sterile leaf discs from plants that had been grown *in vitro* for 3 weeks were plated in petri dishes on MS-10 medium (MS-medium containing 0.03 ppm NAA and 1 ppm kinetin) solidified with 0.9% agar. In the assay for EPC-tolerance, various concentrations of EPC (0–500 µM) were added into the agar and the plates incubated at 25°C for 2 months. In the assay for chilling tolerance, no EPC was added, but the plates

were incubated at 3°C for 5 months. Images were recorded at the onset of the experiment and at various time intervals during incubation using a digital camera (Coolpix 990, Nikon). Discs from the SR1-wild type plants were included as internal standard into each plate. The increment in leaf area was quantified using a software for quantitative image analysis (Image J, National Institute for Health, downloadable under http://list.nih.gov/archives/imagej.html. The data shown in Figure 2 represent mean values from two to four independent series including four to six individual leaves.

*Visualization of microtubules*

Small aliquots of mutant callus were either challenged by various concentrations of EPC or by chilling stress (by incubation on ice water) and then collected on microcontainers equipped with a nylon mesh (Nick et al., 2000). The cells were fixed in two steps in 3.7% (w/v) of fresh paraformaldehyde in microtubule-stabilizing buffer (MSB: 50 mM PIPES, 2 mM EGTA, 2 mM $MgS0_4 \cdot 7H_20$, pH 6.9) for 45 min at room temperature with gently shaking. This was followed by a second fixation for 20 min under the same conditions, in the presence of 1% Triton X-100. In case of the chilled callus, the fixation was performed on ice. After fixation, the calli were washed under gentle shaking with MSB three times for 5 min. The cell wall was digested for 30 min at 25°C with 1% (w/v) Macerozyme and 0.2% (w/v) Pectolyase in MSB and the cells washed again three times for 5 min with MSB. Then, the cells were transferred to coverslips coated with poly-L-Lysine (Sigma, Deisenhofen, Germany), and allowed to settle for 20 min in MSB with 1% Triton X-100. In the next step, the cells were incubated for 5 min with PBS (PBS: 8 g/l NaCl, 0.2 g/l KCl, 0.158 g/l $KH_2PO_4$ and 2.31 g/l $Na_2HPO_4$) and then blocked for 30 min with PBS containing 0.5% (w/v) bovine serum albumin. The cells were then incubated for 1 h at 37°C with a monoclonal mouse antibody directed against a conserved epitope of α-tubulins (DM1A, Sigma, Deisenhofen, Germany) diluted 1:100 in PBS and then washed three times 10 min with PBS. The secondary antibody (anti-mouse IgG-fluorescein-isothiocyanate, Sigma, Deisenhofen, Germany) was administered at a dilution of 1:50 in PBS for 1 h at 37°C in the dark. After incubation, the cells were washed twice 10 min, sealed with nailpolish and directly analyzed under an epifluorescence microscope (Axioplan, Zeiss, Oberkochen, Germany) using a filter set with excitation at 470 nm, a beam-splitter at 493 and emission between 505 and 530 (filterset 13, Zeiss, Oberkochen, Germany). Pictures were acquired using a CCD-Color-Camera (Axiocam, Zeiss, Oberkochen, Germany), connected to a PC equipped with the corresponding imaging software (Axiovision, Zeiss, Oberkochen, Germany).

*Western analysis*

Total protein extracts were obtained and analyzed by SDS-PAGE and western blotting as described in detail in Nick et al. (2000). Total α-tubulin was probed with the monoclonal DM1A antibody directed to a conservative epitope (Sigma, Deisenhofen, Neu-Germany), whereas tyrosinated tubulin was probed with the ATT antibody that specifically recognizes a carboxyterminal epitope comprising the terminal tyrosine (Kreis, 1987). The DM1A antibody was used in a dilution of 1:150, the ATT antibody in a dilution of 1:300. Polyclonal antisera against mouse IgG conjugated to horseradish peroxidase were used as secondary antibodies at a dilution of 1:2000 (Sigma, Deisenhofen, Germany). The signal was visualized by bioluminescence as described in Nick et al. (2000). Figure 3(K) shows representative samples from a set of four to seven independent time courses.

## Results

*Selection of activation-tagged cells yields few, but clearly distinct mutants*

Although a large number of protoplast was used for each experiment, only a small number of mutants could be recovered from each screen (Table 1). To test, whether this was caused by a high number of false positive cells that survived the cocultivation with *Agrobacterium* without harboring the plasmid, equal aliquots of the cocultivated protoplasts were either plated on medium without hygromycin (−hygro) or with hygromycin (+hygro) and the number of surviving cells was quantified after growth for a further 3 weeks. This number was identical in both cases (Table 1), indicating that all cells that had survived the cocultivation carried the hygromycin resistance marker and therefore also the activation-tagging vector. The low number of putative mutants therefore

*Table 1.* Quantitative data on transformation efficiency and screening stringency[a]

| Input | Transformation control | | Selection | | Recovered colonies | Putative mutants |
|---|---|---|---|---|---|---|
| | −hygro | +hygro | Cells | Screen | | |
| $42.5 \times 10^6$ | $4.4 \times 10^6$ | $4.4 \times 10^6$ | $33.7 \times 10^6$ | EPC-tolerance | 61312 | 7 |
| $7 \times 10^6$ | $5 \times 10^5$ | $5 \times 10^5$ | $6 \times 10^6$ | Chilling tolerance | 6574 | 3 |
| $6 \times 10^6$ | $5 \times 10^5$ | $5 \times 10^5$ | $5 \times 10^6$ | EPC- and chilling tolerance | 8036 | 1 |

[a] The initial number of protoplasts used for each experiment is indicated under 'input'. After cocultivation, aliquots were grown with or without hygromycin to estimate the number of transformed cells. From these numbers, the transformation efficiency can be estimated to be somewhat lower than 10%. The number of colonies recovered immediately after the screen is indicated as well as the number of putative mutant plants that could be obtained from those colonies under continuous selective pressure.

indicated that the screening protocol employed was very stringent, such that tolerance must be considered as a highly significant trait. This was supported by the observation that not a single wild-type protoplast survived and formed calli under selective pressure that was milder compared to that employed for the screen of the mutants. The number of putative mutants was even smaller when the two selective pressures (chilling and EPC tolerance) were combined, emphasizing the specificity of the tolerance trait.

### *Microtubules and growth are tolerant to EPC in the ATER lines*

The ATER lines had been selected for tolerance to the aryl carbamate EPC. The regenerating calli were subdivided into equal aliquots and then cultured on various concentrations of EPC (Table 2, Figure 1(A)). Whereas callus growth was significantly reduced at 50 μM of EPC in the wild type, all seven ATER lines were able to grow vigorously on concentrations up to 300 μM. In some of the lines growth seemed even to be stimulated by EPC as compared to controls grown without this microtubule inhibitor. Some of the ATER lines were even able to cope with 500 μM of EPC. Cross-resistance studies showed that the ATER lines were cross-resistant to other aryl carbamates (such as isopropyl-*N*-phenylcarbamate, pronamide) and to drugs with different binding sites on the tubulin molecule, such as oryzalin (data not shown).

*Table 2.* Growth of primary calli from the ATER lines in the presence of the aryl carbamate EPC[a]

| Mutants | EPC concentration (μM) in the medium | | | | |
|---|---|---|---|---|---|
| | 0 | 50 | 100 | 300 | 500 |
| SR1 | +++ | ++ | + | − | − |
| ATER 1 | +++ | +++ | +++ | +++ | ++ |
| ATER 2 | ++ | +++ | +++ | +++ | ++ |
| ATER 3 | ++ | +++ | +++ | +++ | + |
| ATER 4 | +++ | +++ | +++ | +++ | n.d. |
| ATER 5 | ++ | +++ | +++ | +++ | n.d. |
| ATER 6 | +++ | +++ | +++ | +++ | n.d. |
| ATER 7 | +++ | +++ | +++ | +++ | n.d. |

[a] −: no growth, +: very slow growth, ++: moderate growth, +++: vigorous growth, n.d.: not determined.

To investigate, whether tolerance manifested as growth was accompanied by a corresponding tolerance of microtubules, calli from different ATER lines were challenged with increasing concentrations of EPC and then analyzed by immunofluorescence to determine the response of microtubules (Figure 1(B)). In unchallenged cells (wild type) cortical arrays of parallel microtubule bundles prevailed. Already for 100 μM of EPC, these microtubules vanished completely within 2 h and were replaced by a diffuse fluorescence distributed all over the cytoplasm. At higher concentrations, this fluorescence gradually accumulated on the surface of nuclei. As shown for ATER 6 and ATER 2 (Figure 1(B)), unchallenged cells of the ATER mutants typically exhibited scarce, fine strands of cortical microtubules that contrasted with the well-visible bundles observed in unchallenged wild type. In response to 50–100 μM of EPC, numerous vesicular structures appeared that were strongly fluorescent and seemed to be focal points, from where numerous short, but delicate microtubule strands emanated. At a concentration of 300 μM, these vesicles were present as well, but they were smaller and interconnected by long, more distinct bundles of microtubules. The resulting array was clearer and better developed than the arrays found in unchallenged mutant cells. In some of the lines, such as ATER 2, well-ordered and completely intact microtubular arrays could be observed

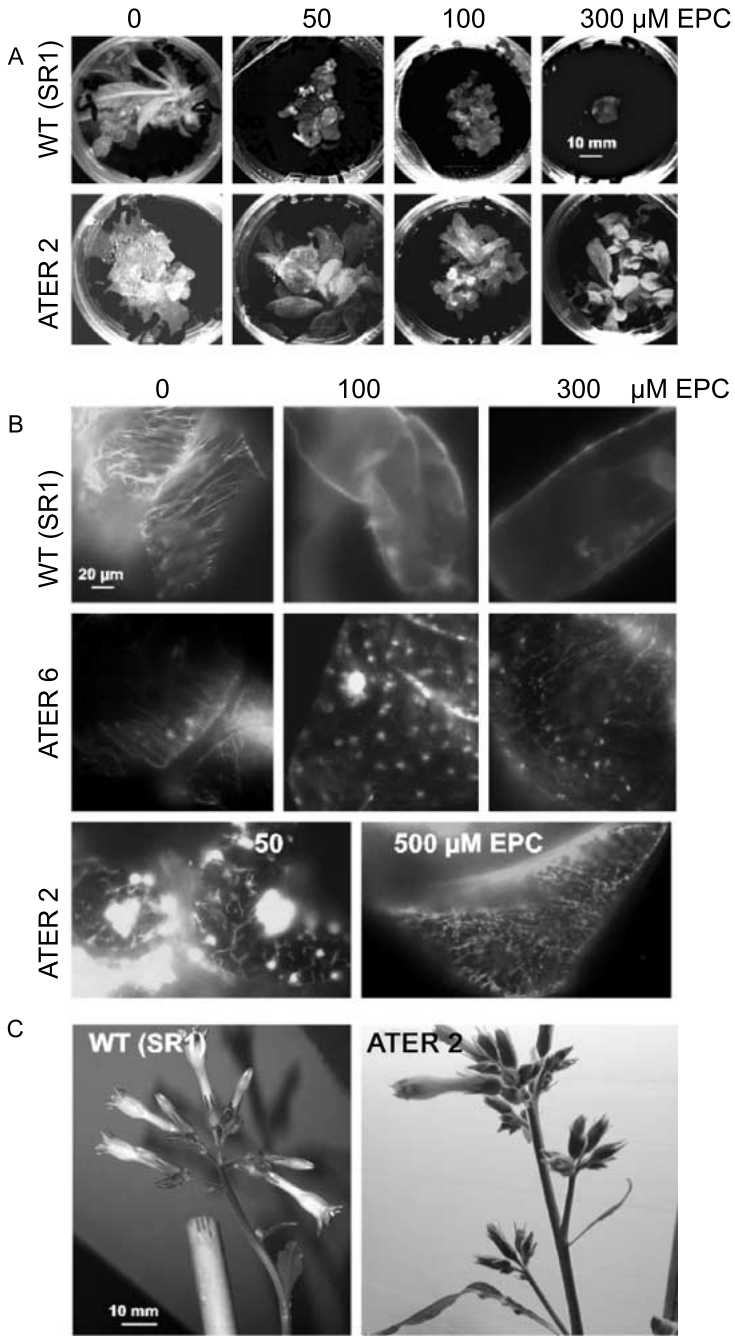


*Figure 1.* Phenotype of the ATER mutants. (A) Callus growth of wild type SR1 and the ATER 2 mutant on different concentrations of the microtubule-assembly blocker EPC. (B) Response of microtubules in wild type SR1 and the mutant lines ATER 6 and ATER 2 to incubation with different concentrations of EPC. (C) Bushy appearance of the inflorescence in the ATER 2 mutant caused by the formation of supernumerary axillary flowers.

in the presence of 500 µM of EPC (Figure 1(B)). This pattern of a rudimentary cortical array in unchallenged cells, that is, replaced by a more distinct array at higher concentrations of EPC accompanied by vesicular nucleation foci for intermediate concentrations was observed in all of the ATER lines with some variation in the amplitude of this unique response.

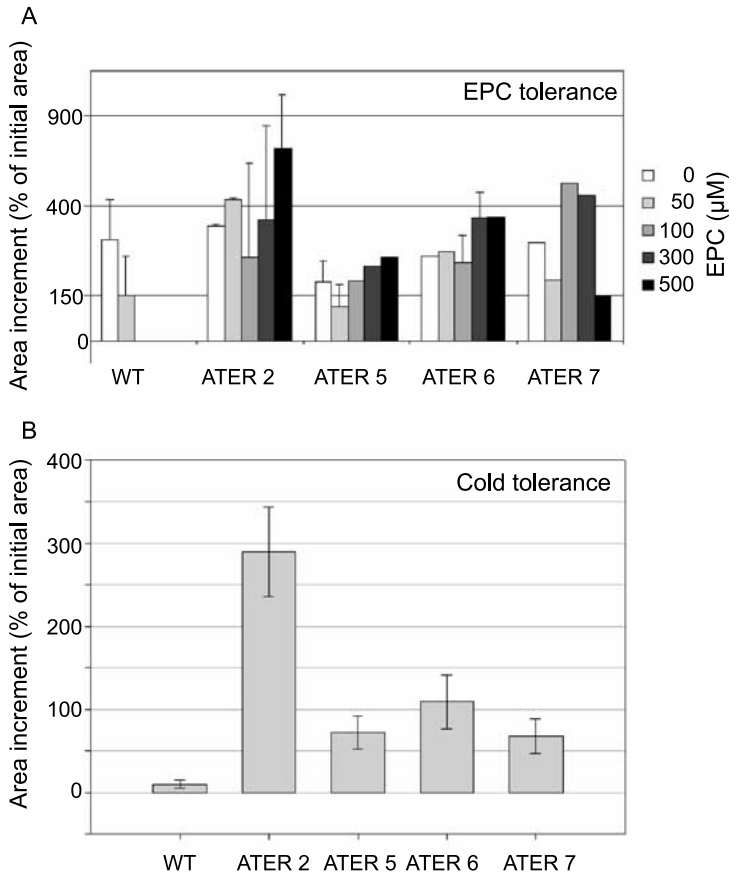


*Figure 2.* Cross-resistance of growth in the ATER mutants to factors that affect microtubules. (A) Areal growth in leaf discs exposed to EPC in wild type SR1 and different ATER mutants. (B) Areal growth in leaf discs under continuous chilling (3°C) in wild type SR1 and different ATER mutants.

The primary transformants were grown to maturity and seeds were germinated in the presence of hygromycin (to select for transformants harboring the T-DNA tag) and EPC (to select for the ATER trait). The two traits were found to be linked through three generations thus far. Two lines that were presumably homozygous for the ATER trait and contained the T-DNA tag were obtained and were shown to be otherwise completely normal in terms of growth, leaf formation and fertility. Preliminary segregation and Southern analyses (data not shown) indicate that the T-DNA tag had been inserted once. However, the number of axillary flowers was increased as compared to the wild type, whereas only one axillary flower was observed (Figure 1(C)). This trait was observed in all of ATER lines.

To assess, whether tolerance to carbamates was genetically stable, a leaf-disc assay was developed. The increment in surface area of leaf discs on solid medium complemented with increasing concentrations of EPC was followed over time. In the absence of EPC (Figure 2(A), white bars), the increase areal was comparable between the wild type and the ATER-mutants with a tendency of the mutants to grow slower. However, even at 50 µM of EPC, growth was reduced to about 50% in the wild type and was completely arrested at higher concentrations. In contrast, growth in the ATER mutants either persisted (ATER 5) or was even stimulated (especially pronounced in ATER 2) when the concentration of EPC was increased. The concentration of EPC had to be raised up to 500 µM in order to inhibit growth in line ATER 7, whereas in the other ATER lines the stimulation just reached its maximum. This demonstrates that the tolerance of the ATER mutants to EPC is increased at least by one order of magnitude compared to the wild type.

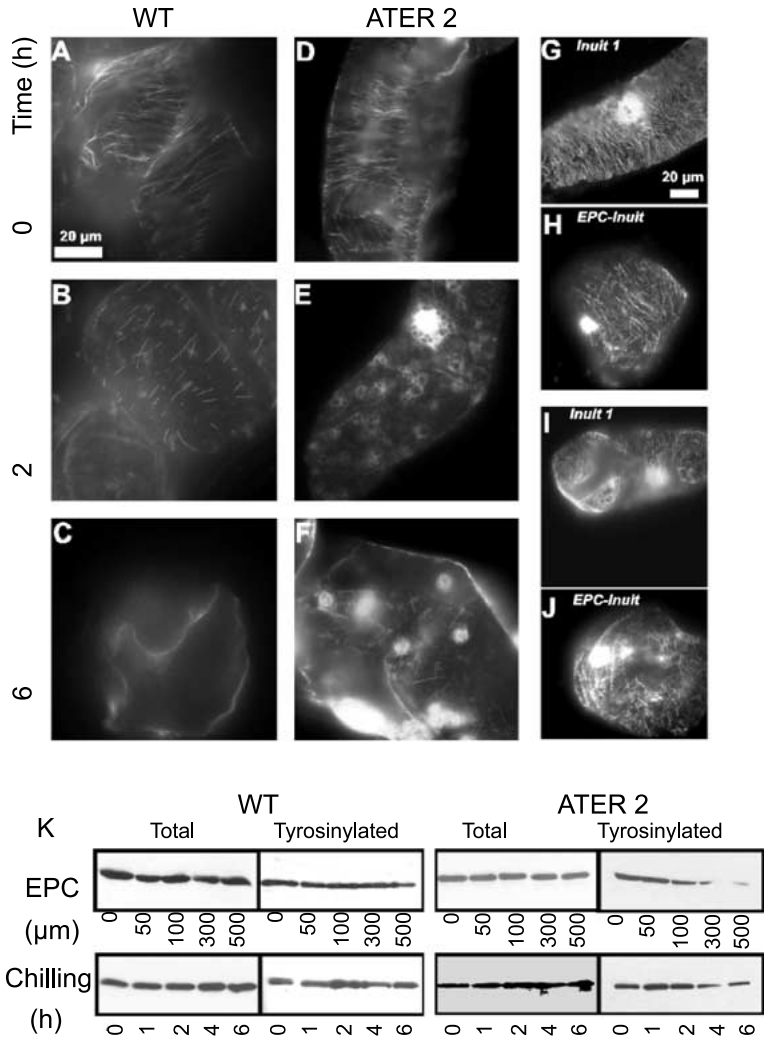


*Figure 3.* Microtubular response to chilling. Cortical microtubules in wild type SR1 ((A)–(C)), ATER 2 mutant ((D)–(F)), the *Inuit-1* mutant ((G), (I)), and the *EPC-Inuit* mutant ((H), (J)) are shown at different stages during chilling (0°C). The relative abundance of tyrosinylated tubulin decreases in the ATER 2 mutant in response to chilling for the indicated time intervals or incubation with different concentrations of EPC for 3 h (K).

*The ATER lines are cross-tolerant to chilling*

Does the tolerance of the ATER lines to aryl carbamates, blockers of microtubule assembly, result in a tolerance to other agents that cause an elimination of microtubules? To answer this question, the ATER lines were analyzed for chilling tolerance using the leaf disc assay described earlier. For this assay, the leaf discs were cultured in the absence of EPC, but under continuous chilling. Only traces of residual growth could be observed in the wild type under these conditions (Figure 2(B)), whereas growth persisted in the ATER lines, which was especially pronounced in lines ATER 2 and ATER 6 (Figure 2(A)). Comparison with the patterns obtained for EPC-tolerance indicated that the ATER lines with pronounced EPC-tolerance also expressed a pronounced chilling tolerance (for example ATER 2 and ATER 6).

To understand whether the chilling tolerance in terms of growth was related to chilling tolerance of microtubules, mesophyll cells of wild type and ATER mutants were stained for microtubules by indirect immunofluorescence (Figure 3). In the wild type, the cortical microtubules became fragmented within the first two hours (Figure 3(A) and (B)) after the onset of chilling and were completely eliminated from 4 to 6 h after chilling (Figure 3(C)). The chilling response differed dramatically in the mutants as shown

for example in ATER 2 (Figure 3(D)–(F)). Here, the parallel bundles of cortical microtubules present prior to chilling, where replaced by vesicular structures in the cell cortex. These vesicles were most abundant between 1 and 3 h after the onset of chilling. Upon prolonged chilling radial arrays of fine microtubules emanated. This vesicle formation differed in the exact timing and amplitude between lines but was present in all lines (data not shown). Thus, whereas microtubules in the wild type are fragmented and eliminated completely, in the ATER mutants transient vesicular structures were formed that seemed to be organized in a new microtubular network.

This chilling response of microtubules seems to be specific for the ATER mutants, since in mutants that had been selected directly for increased chilling tolerance (such as *Inuit 1*) or combined tolerance to EPC and chilling (*EPC-Inuit*), microtubules persist, when they are challenged by low temperature and do not show significant changes even after 6 h of chilling (Figure 3(G)–(J)), when the microtubular cytoskeleton is already completely eliminated in the wild type (Figure 3(C)).

In the ATER mutants, factors that impair microtubule assembly (EPC, chilling) produced a transient state, where vesicular structures seemed to organize new microtubule arrays that emanate from these vesicles (Figures 1(B) and 3(E)). The resulting microtubular cytoskeleton appears to be very stable against inhibition of assembly. This might be caused by a generally reduced dynamics of microtubules, such that the lifetime of individual microtubules is increased. To test this hypothesis the degree of α-tubulin tyrosination was followed as marker for microtubule dynamics (Wiesler et al., 2002). Leaf discs from ATER 2 plants, where the formation of tubulin vesicles was especially pronounced were analyzed for the response to increasing concentrations of EPC or chilling (Figure 3(K)). In the wild type, the amount of total α-tubulin as well as the abundance of tyrosinylated α-tubulin was found to be constant after treatment with EPC or in response to chilling. In the ATER 2 mutant, the amount of total α-tubulin was found to be fairly constant as well. However, the abundance of tyrosinylated α-tubulin decreased dramatically, when the concentration of EPC exceeded 100 μM or when the chilling lasted longer than 2 h. This suggests that the dynamics of the microtubular cytoskeleton exhibit downregulation in the mutant in response to factors that impair microtubule assembly.

### *Chilling tolerance in the* Inuit *mutants is based on different mechanisms*

The phenotype of those mutant lines that had been screened directly for either increased chilling tolerance, or for a combination of EPC and chilling tolerance, differs dramatically. In those mutants, microtubules did not show the transient formation of vesicles, nor the gradual organization of a new microtubular cytoskeleton. In contrast, in *Inuit 1* or *EPC/Inuit*, microtubules simply persist when the cells are subjected to chilling stress. Even after 6 h of chilling microtubules displayed only small indications of partial disassembly (compare Figure 3(I) and (J) with (G) and (H)) and persisted up to 8–12 h (data not shown). Preliminary immunoblot experiments indicate that there are no changes in the relative abundance of tyrosinylated α-tubulin in those mutants (data not shown). The phenotype of the *Inuit* and *EPC-Inuit* lines is characterized by extremely slow development accompanied by semi-dwarfism (Figure 4(A)) due to reduced internode length, and by male sterility (Figure 4(B)). Therefore, these mutant lines have to be maintained as heterozygotes and have still to be propagated in order to get enough material for more detailed studies.

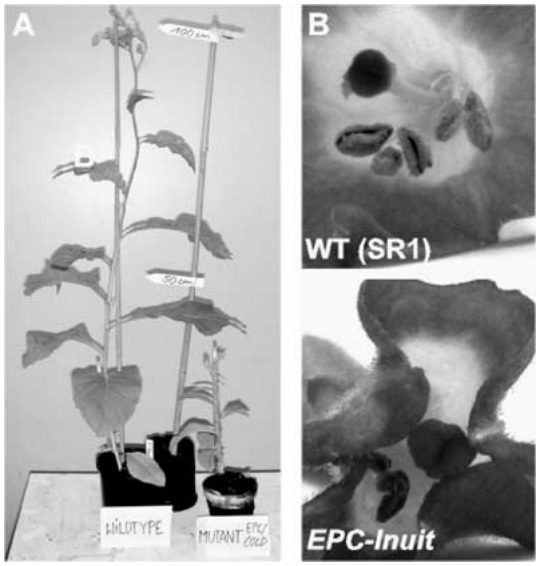


*Figure 4.* Phenotype of the *EPC-Inuit* mutant. (A) Semidwarfism of an adult *EPC-Inuit* plant in comparison to an adult wild type at the time of flowering. (B) Flower morphology in *EPC-Inuit* in comparison to the wild type. Note the closed anthers and the lack of pollen grains in the mutant.

## Discussion

### *Can activation tagging yield mutants of cytoskeletal dynamics?*

So far, screens for tolerance to antimicrotubular compounds have recovered only few cytoskeletal mutants in plants (for a recent review see Baird et al., 2000) that were usually affected in the affinity of tubulin for these compounds leading to increased tolerance. Since all antimicrotubular compounds investigated so far act as blockers of microtubule assembly (Mizuno & Suzaki, 1991), tolerance or sensitivity of a given microtubule will depend on its turnover. Thus, reduced microtubule dynamics should result in increased tolerance to antimicrotubular compounds, whereas induction of microtubule dynamics is expected to render microtubules more sensitive. In fact, the sensitivity of microtubules to assembly blockers can be modulated through altering their dynamics through auxin (Wiesler et al., 2002). A screen for mutants with increased tolerance to blockers of microtubule assembly should therefore recover, in addition to mutants with reduced affinity of tubulin for the inhibitor, mutants with reduced turnover of microtubules. However, classical mutagenesis has not recovered such mutants so far. This may be for two reasons:

1. Selection of mutants that have been generated by classical mutagenesis leading to point mutations will produce a high number of mutants, where missense or nonsense mutations in the coding sequence will alter the binding of the assembly blocker to tubulin.
2. Mutants affected in factors changing the dynamics of microtubules will be overlooked in the first generation, because the intact version of this factor will maintain microtubule dynamics to a degree that no selectable change of microtubule dynamics can be observed.

A screen for mutants, where the dynamics of microtubules is reduced or where the signaling towards this dynamics is affected, must therefore meet two conditions:

1. The mechanism of generating mutants should avoid point mutations.
2. The phenotype should be screenable in the first generation, that is, the phenotype should be dominant.

Both conditions are met by T-DNA activation tagging. Insertion of the tag into a promotor region is expected to activate a neighboring gene. The elevated expression of this gene would therefore result in a dominant phenotype that can be screened in the first generation. Since the mutagenesis occurs by insertion, point mutations of the coding sequence leading to affinity mutants of tubulin are avoided. Insertion of the tag into the coding sequence of a gene should result in a knockout phenotype that in most cases would not manifest in the first generation and thus will not be recovered in this screen. The combination of T-DNA activation tagging with selection for resistance to blockers of microtubule assembly should therefore lead to the isolation of mutants with reduced dynamics of microtubules, either because associated proteins that confer microtubule stability are induced or because signaling components that activate such proteins are upregulated. The screen for chilling tolerance (*Inuit*) is expected to yield mutants where the chilling response of genes involved in the adaptation to low-temperature stress is activated. These genes may or may not be related to cytoskeletal signaling. To isolate those genes that are part of the chilling-induced signaling towards the microtubules, the screens for chilling tolerance or accelerated cold acclimation were combined with selection for increased tolerance to the microtubule assembly blocker EPC (*EPC-Inuit* line).

The number of mutants recovered from these screens was very small indicating that the selective pressure was very stringent. In fact, the dose–response of leaf-disc growth (Figure 2(A)) shows that the ATER mutants can tolerate EPC-concentrations that are about one order of magnitude higher than the dose that is lethal for the wild type. Conversely, the *Inuit* and *EPC/Inuit* mutants, survived under continuous chilling lasting for several months. In one experiment (data not shown), callus from the *Inuit* line was maintained in the refrigerator for 8 months and upon rewarming regenerated extremely vigorously a high number of plantlets. This extent of perseverance is especially extraordinary in the background of an otherwise highly chilling-sensitive species as tobacco. Although only few mutants were collected from the different screens, the phenotype of these mutants is highly significant.

To achieve a high transformation efficiency, the protoplasts were co-cultivated with *Agrobacterium* when most cells passed through second division. This period of optimal transformation competence was reached about 6–8 days after the isolation of protoplasts. Using a different tagging vector, other groups

reported maximal transformation efficiency for co-cultivation 4–5 days after protoplast isolation, when the cells entered the first cell division (Walden et al., 1995; Zubko et al., 2002). The transformation efficiency in our system as estimated from plating aliquots of the co-cultivated population on hygromycin, was in the range 5–10%, which seems to be near the maximum that can be achieved for activation tagging. Earlier reports that had claimed 15–20% of transformation efficiency (Walden et al., 1995) were later retracted (Schell et al., 1999).

Summarizing, the combination of activation tagging with selection protocols that aimed on alteration of microtubule responses to depolymerizing factors such as EPC or chilling, permitted isolation of a few, but highly tolerant mutants. This shows that activation tagging is a feasible approach to generate mutations that affect either microtubular dynamics or the signal control of such dynamics.

### *In ATER 2, increases of the dimer pool suppress microtubule dynamics*

Tolerance to EPC that otherwise would cause a breakdown of the microtubular cytoskeleton could be achieved by a constitutively elevated synthesis of tubulin that would compensate for the loss of dimers bound by EPC. However, in ATER 2, where the EPC tolerance is particularly pronounced (Figure 2(A)), the abundance of α-tubulin is not significantly different from the wild type (Figure 3(K)), discounting a tolerance mechanism based on tubulin overexpression. This is consistent with published reports that tubulin constitutes a highly buffered system that will compensate any imbalance between α- or β-tubulin or any change in the general abundance of tubulins (Anthony & Hussey, 1998; Anthony et al., 1999). Moreover, tubulin overexpression would be a way to escape the action of drugs that remove tubulin dimers from the assembly equilibrium, but not a mechanism to overcome the effect of chilling. The ATER 2 mutant is highly cross-tolerant to chilling, however (Figure 2(B)).

This points to an alternative scenario, where the factors that ensure the dynamic equilibrium of the microtubular system display elevated sensitivity to changes in the pool of unassembled tubulin. Increases in the abundance of tubulin heterodimers are very efficiently sensed in the ATER 2 mutant and trigger a response culminating in reduced microtubule dynamics. When the abundance of tyrosinylated α-tubulin is followed in response to either EPC or chilling (Figure 3(K)), it is found to decrease in ATER 2. Since tyrosinylated α-tubulin is a marker for dynamic microtubules (Wiesler et al., 2002), this finding suggests that microtubules in this mutant compensate for the blocked assembly by increasing the lifetime of individual microtubules. It will be interesting to investigate, whether this is caused by a hypersensitive sensing of tubulin disassembly, a mechanism that is well known in animal cells (for review see Cleveland, 1988) or whether a step within the effector chain triggered by this sensory mechanism is activated.

Preliminary findings with the *Inuit 1* and the *EPC/Inuit* mutant do not give indications for altered abundance of tyrosinylated α-tubulin, suggesting that in these mutants chilling tolerance is based on a different mechanism.

Summarizing, our data suggest that in ATER 2 (and possibly in the other ATER mutants as well), the strong cross-tolerance of growth to EPC and chilling (Figure 2) is based on a strong inducible response of microtubules that will downregulate microtubule dynamics in response to increases in the pool of unassembled tubulin heterodimers.

### *Microtubule dynamics define chilling tolerance in tobacco*

As other crops originating from subtropical or tropical regions, tobacco is a typical example of a chilling sensitive plant. This is mirrored by a rapid elimination of microtubules (Figure 3(A)–(C)) accompanied by a drastic inhibition of growth (Figure 2(B)), when the cells are challenged by chilling. The chilling sensitivity of a crop has been reported to correlate with the cold tolerance of microtubules (Jian et al., 1989) suggesting that it is the tolerance of microtubules that will define the degree of chilling tolerance of the whole plant. Consistent with this conclusion, elimination of microtubules by colchicine increased low-temperature damage in cotton, a classical chilling-sensitive crop (Rikin et al., 1980).

The exact mechanism, by which the microtubular network breaks down in response to chilling stress is far from understood. Theoretically, chilling could reduce the assembly of microtubules – chilling tolerance would then be achieved by maintaining a high rate of assembly under low temperature. Alternatively, chilling could stimulate the disassembly of microtubules – tolerance would then be caused by suppression of this stimulation. The exact mechanism is difficult to assess because assembly and disassembly are usually

balanced resulting in a more or less dynamic equilibrium. Through a highly complex interaction between expression of tubulin genes and the assembly state of microtubules (for a recent review see Breviario & Nick, 2000), any change in the assembly rate is expected to be balanced by an antagonistic response of disassembly (and *vice versa*), as long as the buffering capacity of the regulatory system is not exceeded. Irrespective of this complexity, the cross-tolerance of the ATER mutants to chilling stress (Figures 2 and 3) allows to draw four conclusions:

1. Reduced dynamics of microtubules causes resistance of microtubules to chilling-induced elimination.
2. Resistance of microtubules to chilling-induced elimination causes chilling tolerance of growth.
3. Microtubule dynamics defines chilling tolerance in tobacco, a chilling-sensitive crop.
4. By manipulation of microtubular dynamics it should be possible to engineer general chilling tolerance in such crops.

*Outlook: cold-induced signaling to the microtubular cytoskeleton*

The signaling cascade triggering plant adaptation to cold stress is still far from being understood, but 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 adaptive proteins. This cascade somewhere merges or cross-talks with abscisic-acid induced signaling, but this might well be relatively downstream of this cascade, for instance at the target promotors of CBF.

It is even less clear, where signaling to the microtubules branches from this general cascade. However, several findings indicate that calcium is a central player in the cold-induced signaling towards microtubules. For example, the cold-induced elimination of microtubules was reported to be blocked by lithium, suggesting that the phosphoinositide pathway might be involved (Bartolo & Carter, 1992). Through interaction with potential MT-associated proteins such as the elongation factor EF-1α (Durso & Cyr, 1994) or by direct binding to tubulin (Kumagai & Nishida, 1979), calcium/calmodulin could modulate the microtubules (Fisher et al., 1996). Alternatively, calcium could act on microtubule dynamics through the actin cytoskeleton, since some of the calcium-dependent protein kinases have been shown to interact with actin (Putnam-Evans et al., 1989). As expected from the emerging model of cold signaling (Thomashow, 2001), not only calcium/calmodulin, but also kinase signaling seems to be involved, because kinase inhibitors such as 6-dimethylaminopurine or staurosporin can render microtubules chilling tolerant in cultured tobacco cells (Mizuno, 1992). This would favor a model, where the bifurcation between general cold signaling and signaling to the microtubular cytoskeleton would occur downstream the kinase cascade that culminates in the inhibition of HOS1.

What are the microtubular responses to this signaling chain that will shift the assembly/disassembly equilibrium towards the soluble heterodimers? The phenotype of the ATER mutants and especially the progressive decrease of tyrosinylated α-tubulin suggests a rapid downregulation of tubulin dynamics. This might be accompanied and stabilized by changes in the composition of tubulin isotypes as repeatedly described for cold acclimation (rye roots, Kerr & Carter, 1990; *Arabidopsis*, Chu et al., 1993). It is worth investigating whether the chilling-tolerance of the *Inuit* and *EPC/Inuit* mutants is caused by an *a priori* altered pattern of isotypes.

To isolate molecular components of this signaling chain, the tagged genes will be isolated and cloned from the ATER mutants through plasmid rescue. The criterion for a true element of the signaling chain would be a phenocopy of the mutant phenotype by overexpression of the rescued gene in the wild type background. This would demonstrate that the phenotype is caused by the activation of the tagged gene (and not by long-term activation of a neighboring non-tagged gene). A first genomic fragment isolated from the ATER 6 mutant contains a so-called SH3-domain that is characteristic for a group of actin-binding proteins (Kioka et al., 1999) suggesting that a functional approach based on activation tagging will yield genes that are novel and/or not expected from *a priori* considerations.

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