Signaling to the microtubular cytoskeleton
during abiotic stress in plants

PhD thesis

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Preface:

The following text consists of a general introduction and four published articles, which I submit together as a PhD thesis in partial fulfillment of the requirements for the degree of PhD. The aim of this general introduction is to put my research on the role of plant microtubules during abiotic stress into a broader context of the role of plant microtubules in signal transduction. Therefore, the following text has a form of a review, summarizing our present knowledge about the role of plant microtubules in the receiving and transducing of signals from the extracellular space. I try to summarize several hypotheses, according to which microtubules are an integral component of signal transduction pathways in plants.
1 Introduction:

The dynamic cytoskeleton, consisting of microtubules and actin filaments, represents an important structure that fulfills many roles within all eukaryotic cells. Among other roles, the cytoskeleton is known to mediate the transduction of various external or internal signals into the cell that are needed for cell morphogenesis or response to the environmental conditions.

In plant cells, actin filaments are known to participate in signal transduction. Plant actin filaments interact with evolutionarily conserved actin regulating factors involved in signaling pathways as ROP GTPases (Ying et al. 2004), Arp2/3 complex (Mathur 2005) or formins (Cvrčková et al. 2004). At least ROP GTPases are known to participate in the transduction of environmental stimuli such as the plant defense response into the cell (Agrawal et al. 2003). Actin filaments are probably a component of signal transduction machinery or a target of signalization from the environment during the self-incompatibility response in pollen tubes, during the infection of roots with symbiotic bacteria *Rhizobium*, during the response to pathogens as fungal infections, during light-induced chloroplast movement or in the regulation of stomata opening. However, no full cascade of events or intermediates from signal to cytoskeletal response has been elucidated for any system (reviewed in Staiger 2000). Even much less is known about the role of plant microtubules during the signal transduction of environmental stimuli. According to new findings, however, also plant microtubules can act as elements of signal transduction pathways. Therefore, this work focuses on this newly emerged role of plant microtubules in mediating signals. Several hypothetical models of cascade of events that include the microtubular cytoskeleton will be discussed.

Microtubular network is actually extremely well designed for transmission of signals within a cell as well as for signal transduction from the cellular surroundings into the cytoplasm. Its linear elements span the cytoplasm, creating the three-dimensional network that connects distant regions of the cell. Microtubules provide the cell with substantial surface for protein-protein interaction and directional flow of information. In a model animal cell, proliferating interphase fibroblast, there is about 1000 μm² of protein surface on microtubules. This is about the same protein surface area as the plasma membrane (Gundersen and Cook 1999). Such characteristics are very probably common in both animal and plant cells. Microtubular scaffold of animal and plant cells shows characteristic dynamics that ensures its sustained ability to change immediately in a response to any signal and cellular requirements. In case of plant microtubules, the position of plant interphase cortical microtubules near the
plasma membrane (Sonesson and Widell 1998; Collings et al. 1998), is ideal for receiving or transducing of information.

In animal cells, microtubules are known to cooperate in transduction of signals mediated by the Rho family of monomorphic GTPases (Hollenbeck 2001), the hedgehog signaling pathway, mitogen-activated protein kinase (MAPK) pathway, Wnt signaling pathway, and tubulin has been shown to act as a substrate for G-protein coupled receptor kinases (GRKs) (reviewed in Gundersen and Cook 1999). Unlike animal cells, only limited information on interaction of signaling molecules with microtubules either directly or indirectly is available for plant cells. However, Chaong et al. (2004) used tubulin affinity chromatography and proteomic characterization approach for a large-scale identification of tubulin binding proteins in Arabidopsis. This study provided a new insight into the microtubular functioning in plant cell, identifying a number of proteins involved in the signaling that were able to bind to tubulin.

One of the first direct evidences on interaction of plant microtubules with signaling components came from Gardiner et al. (2001), who showed that phospholipase D (PLD), a membrane associated protein, binds cortical microtubules. PLDs are enzymes that hydrolyze phospholipids to produce phosphatidic acid (PA) and free choline. PA acts primarily as a second messenger in signaling cascades. It has been shown that upon the inhibition of PLD-dependent formation of PA by n-butanol, microtubules were released from the plasma membrane and partially depolymerized in BY-2 cells expressing GFP-MAP4 (Dhonukshe et al. 2003). In Arabidopsis, treatment with n-butanol resulted in root growth inhibition and cell swelling in the elongation zone (Gardiner et al. 2003). Thus, PLD represents both physical and functional interconnection between microtubules and the plasma membrane in plants, and a component of a signaling pathway interacting directly with microtubules as the microtubule-associated protein at the same time.

The calcium ion (Ca$^{2+}$) represents an universal intracellular second messenger in plants. An increase of cytosolic free Ca$^{2+}$ concentration couples a diverse array of signals and responses. Evidence exists that the activity of depolarization-activated plasma membrane Ca$^{2+}$ channels is affected by the microtubular cytoskeletons (Thion et al. 1996; Thion et al. 1998). Thus, microtubules are probably directly involved in the generation of Ca$^{2+}$ signals in plant cells.

The aim of this work is to address the roles of microtubules in transducing signals triggered by abiotic stress, namely low temperatures and toxic levels of aluminum, and to summarize the present knowledge about underlying mechanisms. Low temperatures and influence of toxic levels of aluminum represent only two examples of environmental stimuli, where evidence exists that microtubules play a role in their signal transduction. Our results on the changes of the structure and potential role of plant microtubules in response to toxic influence of Al$^{3+}$ ions and low temperature are discussed.
2 Aluminum toxicity

Aluminum (Al) is the most abundant metal in the earth's crust. Naturally it occurs in the form of insoluble compounds. However, upon solubilization under low pH conditions, the toxic form of Al is released to the soil solution, thus limiting growth of plants in these soils. Therefore, Al toxicity represents an important limiting factor for crop productivity on acid soils (pH<5.5) that comprise about 40% of the arable land in the world, being abundant in tropical and subtropical regions in particular (De la Fuente-Martinez and Herrera-Estrella 1999). Despite its special importance for the ecology and agriculture, the molecular base of Al toxicity in plants remains elusive so far. However, a progress has been made in recent years that helped us understand better the mechanism of Al tolerance and specify possible candidates for prime targets of Al toxicity.

Al has been shown to interact with many intracellular processes and components. In this chapter, the attention will be paid only to those effects that could be connected with the growth cessation induced by Al. Since the inhibition of root growth occurs within minutes of Al treatment, it is obvious that only very early effects of Al toxicity could be taken into consideration as possible candidates for the prime target of Al. In this respect, the most important Al-induced effects are: plasma membrane damage including interaction with phosphoinositide signal transduction pathways, xeroxia of membranes and production of reactive oxygen species (ROS), disturbance of calcium homeostasis, and disruption of the cytoskeleton. Two present hypotheses will be discussed, where the plasma membrane, calcium homeostasis and microtubules are considered to act in one signal transduction pathway during Al toxicity, leading possibly to the cessation of root growth.

2.1 Al speciation

Before any discussion about their toxic effects, it is necessary to mention specific features of Al ions. Al undergoes a complex speciation, it is, the state of Al ion in which it occurs in a solution depends on many factors, namely pH and the activity of other ions. In solutions more acidic than pH=5, Al ions exist as the octahedral hexahydrate Al(H2O)6^{3+}, often called Al^{3+} or "free" Al ion. In solutions that are less acidic, Al(H2O)6^{3+} undergoes deprotonations and forms Al(OH)2^{2+} and Al(OH)3^{2+}. In neutral solutions, Al exists as Al(OH)3 and in solutions of pH=7.4, all Al ions occur as Al(OH)3 (Mcdonald and Martin 1988). To predict Al speciation can be difficult even in simple solutions. This was the reason why the identification of the rhizotoxic form of Al had not been obvious for a long time. In the work of Kinraide (1991), Al^{3+} and Al_{13}, a polynuclear species formed under specific conditions, were considered as the most toxic forms of Al. Al(OH)_{2}^{2+} and Al(OH)_{3}^{2+} forms appeared to be toxic for dicotyledonous plants as well, although it is possible that their toxicity was attributable rather to formation of Al^{3+} or Al_{13}.

2.2 What is the primary effect?

The primary physiological consequence of Al toxicity is the cessation of root growth, a very rapid phenomenon that occurs within minutes of treatment (Sasaki et al. 1997b; Blancaflor et al. 1998). The root tip with actively dividing and elongating cells seems to be most sensitive to Al, with both cell division and cell elongation being affected (Clarkson 1965). More recently, Sivaguru and Horst (1998) found out that in maize, the distal part of the transition zone of the root apex, where cells undergo a preparatory phase for the elongation, is the most Al-sensitive part of the root tip zone.

To answer the question what cellular structure or process is altered or disrupted upon interaction with Al, causing almost immediate root growth inhibition (thus, the primary effect of Al toxicity), it is highly important to know if Al enters apoplastic and symplast and how is it transported through the plasma membrane. Studies carried out to elucidate the target of Al action in plant tissues and cells demonstrated that Al enters and binds to the apoplastic (Horst 1995) and affect the plasma membrane (Wagatsuna et al. 1995). Most of the apoplastic Al is bound to negatively charged pectin components of the cell wall (Chang et al. 1999). Al probably enters the symplast of root cells, although our knowledge about Al accumulation in the cytosol is still not satisfying due to technical barriers. Characteristically, Al accumulates in the epidermis and outer cortex of the root, but experimental results depend on the different materials and techniques used by investigators (Matsumoto 2000). Accumulation of Al in the plant cytosol has been reported by several authors. For example, Vitorello and Haug (1996) reported that Al uptake of suspension tobacco cells was dependent on the cell growth. In contrast to the negligible uptake of stationary cells, growing cells accumulated Al in detectable amounts within 5 minutes of treatment. Greatest portion of cytosolic Al is expected to be inactivated by the nearly neutral pH of the cytosol and by binding to cytosolic compounds. Nevertheless, the residual amount of free Al in the cytosol could be sufficient to affect intracellular components (Macdonald and Martin 1988). It is not clear how Al is transported through the plasma membrane. It is generally accepted that the transport of highly charged molecules as Al^{3+} is very slow. Several hypotheses for Al transport have been proposed. They include endocytosis and transporter-dependent uptake. An Al pump, a
specific Al carrier protein or a channel protein are supposed in the latter case. However, direct evidence for each mechanism is missing (Matsumoto 2000).

2.2.1 Effects on plasma membrane

Plasma membrane depolarization

Depolarization of the plasma membrane was reported to occur in maize (Zeae mayes L.) in response to Al treatment (Sivaguru et al. 1999a). Ahn et al. (2001) reported a zone-specific depolarization of the plasma membrane surface potential coupled with inhibition of H+-ATPase activity in squash (Cucurbita pepo L. cv Tetsukabuto) roots during first hours of exposure to Al. Masumoto et al. (1996) showed that the electrical imbalance on the plasma membrane occurred during short-term effect (24 hours) of Al in pea roots under low pH conditions. The level of abscisic acid (ABA) increased and ATP and PPI-dependent H+ transport on tonoplast was activated. Since the effect occurred in the time course of 24 hours, the authors suggest that these mechanisms could participate in the mechanism of Al tolerance rather than signaling. The evidence on very quick changes on the plasma membrane came from Sivaguru et al. (2003). Within minutes of treatment, authors reported increased calcium influx in Al-treated cells that was followed by disorganization of microtubules and depolarization of the plasma membrane in Arabidopsis roots. The same effect was shown to be induced also by treatment with glutamate. Authors suggested a hypothesis that a glutamate receptor, the ligand-gated cation channel, is a part of the transduction pathway involved in the toxic effect of Al. Glutamate receptors are known to function as ligand-gated ion channels that control signaling across neural synapses in invertebrates and vertebrates. In Arabidopsis, a family of putative glutamate receptor genes was newly identified (Laconbe et al. 2001). During Al stress signaling in plants, Al could open the channel through which glutamate effluxes. Subsequently, glutamate could bind its receptor, a ligand-gated calcium channel at the plasma membrane, and to trigger calcium influx. This would result in the membrane depolarization, the microtubule depolymerisation and the root growth cessation. This hypothesis is discussed in the chapter 2.2.5 in detail.

Disruption of ion fluxes

Al clearly affects ion fluxes on the plasma membrane (Matsumoto 2000). Potassium in particular. Al treatment led to the increase of K+ influx after 24 hours of treatment with increasing concentration of Al in wheat (Triticum aestivum L.) (Zsoldos et al. 1999). On the contrary, Al inhibited plant inward K+ channels by blocking from the cytoplasmic side of the plasma membrane within few minutes of treatment (Liu and Luan 2001). Treatment with Al almost instantaneously inhibited K+ outward and inward currents and activated inward Cl- channel in maize (Zeae mayes) hybrid South American 3 (Pineros and Kochian 2001). Etherton et al. (2004) reported that inward K+ currents were activated at lower concentrations of Al (10μM), but inhibited at higher concentrations (80μM).

Callase formation

During plant growth and development, callose (1,3-β-D-glucan) can be normally found in various plant cells and tissues. For example, it is a part of the newly formed cell plate during cell division or is found as a structural component of plasmodesmata. Callose deposition between the plasma membrane and the cell wall also occurs after the exposure of plants to a range of abiotic and biotic stresses, including wounding, desiccation, metal toxicity, and microbial attack. Al-induced increased synthesis and deposition of callose is one of the best characterized effects of Al toxicity in the apoplast and has been used as a marker of Al toxicity in plants (Wissemeyer et al. 1992). Callose is synthesized by a membrane-bound, wound activated 1,3-β-D-glucan synthase that uses UDP-glucose as a substrate. 1,3-β-D-glucan synthase represents a stress-activated enzyme and Ca2+ at micromolar concentration is required for its activation (Bhuya et al. 2004). It has been suggested that Al-induced elevations of Ca2+ concentration in the cytosol ([Ca2+]cyt), reported by several authors (see thereafter), could be responsible for callose production. However, the case is not that simple. First of all, Al-induced decrease of [Ca2+]cyt has been also reported, which suggests that Al-induced disturbance of calcium homeostasis needs further research. More importantly, [Ca2+]cyt increase is not the only factor modulating synthesis and deposition of callose (Wissemeyer et al. 1992; Bhuya et al. 2004). In the latter study, authors suggest several hypotheses explaining Al-induced formation of callose. It is possible that callose is synthesized by the enzyme cellulose synthase, whose function is altered by the interaction with Al; second possibility is that Al induces metabolic changes that lead to increased cytosolic concentration of precursors utilized by 1,3-β-D-glucan synthase, thus increasing formation of callose in the apoplast.

Since 1,3-β-D-glucan synthase as well as cellulose synthase are membrane-bound enzymes, it is possible that the increase of formation of callose is the result of Al-induced changes of the plasma membrane properties that could influence membrane-bound enzymes activities. Indeed, plasma membrane, metabolism of lipids and activity of some enzymes
associated with the plasma membrane have been shown to be altered upon treatment with Al: *in vitro*, Al increased membrane surface potential, promoted the loss of membrane fluidity and changed lipid lateral arrangement in liposomes (Verstraeten and Oteiza 2002).

### 2.2.2 Interaction with components of phosphoinositide signal transduction

Components of signal transduction pathways were shown to be affected by Al treatment in several studies, which seems to be well consistent with the fact that the response to Al is very quick. For example, phospholipase D (PLD) is recognized as an important signal-transducing enzyme (Li and Fleming 1999). The major substrate for PLD is phosphatidyl cholín (PC). Al inhibited different forms of PLD in rat submandibular extracts, acting via a membrane-associated target (Li and Fleming 1999). This finding is very interesting, considering the important role of PLDs in plant cells. Further, Jones and Koehan (1997) reported strong interaction between Al and lipid components of the plasma membrane; the strongest interaction occurred with phosphatidylinositol 3,5-bisphosphate (PtdInsP_2). In the same study, enzymatic activities of several membrane proteins were tested and only phospholipase A_2 showed interaction with Al. Phospholipase C could be also considered as a target of Al; Jones and Koehan (1995) showed that PLC was inhibited by Al in wheat roots. Verstraeten et al. (2003) reported that Al induced rigidification of membranes, but on the contrary to Jones and Koehan (1995), Al inhibited the activity of prokaryotic phospholipase C-phosphatidyl inositol (PI-PLC) after 20 min of treatment *in vitro*, but not prokaryotic PLC.

Al induced changes in the metabolism of lipids involved in signal-transduction mechanism in Coffea arabica cells (Martinez-Estvez et al. 2003). During first minute of treatment with Al, the activity of PLC and IP_3 formation increased two fold. Prolonged cultivation, however, resulted in the inhibition of the activity of PLC. Authors also reported that lipid kinase activities increased during treatment with Al. These results suggest that PLC may act as a signaling molecule during early phases of Al toxicity.

Therefore, the phosphoinositide pathway could be influenced by Al at several points, because more than one component of this pathway was shown to interact with Al. Some reports, however, seem to be rather contradictory and it is necessary to consider differences in experimental materials (plant, animal or prokaryotic), experimental conditions (*in vitro* or *in vivo*) and time intervals (early phases or late phases of Al toxicity) used in studies mentioned above. At present, available data do not allow us to build a hypothetical cascade of events that could be triggered by Al-interaction with a component of the phosphoinositide pathway. Although the main role of the phosphoinositide signaling pathway is to transduce signals, the evidence that this pathway is involved in transduction of signals during toxic influence of Al ions in plants is missing.

### 2.2.3 Effect on calcium homeostasis

The cytosolic concentration of Ca^{2+} ions ([Ca^{2+}]_{cyt}) is maintained at a very low level in plant cells, which ranges between 100-200nM. By contrast, concentration of Ca^{2+} ions is high in the cell wall and several organelles. The main role of cytosolic calcium ions is to act as the second messenger in transduction of various signals. Therefore, upon activation of Ca^{2+}-permeable channels in the plasma membrane and endomembranes, Ca^{2+} ions move down the steep electrochemical gradient into the cytosol, resulting in transient and very rapid increase of [Ca^{2+}]_{cyt}. Disruption of any process participating in maintaining of low levels of [Ca^{2+}]_{cyt} or in generating of transient peaks of [Ca^{2+}]_{cyt} during signaling results in serious cellular disorders.

It is generally accepted that early phases of Al toxicity are accompanied by a quick increase of [Ca^{2+}]_{cyt} (for review see Rengel and Zhang 2003) and much attention was paid to this due to the importance of Ca^{2+} homeostasis in plant cells. For example, Zhang and Rengel (1999) showed that Al induced an increase in the cytoplasmic Ca^{2+} concentration in intact wheat roots. Further, Ma et al. (2002) reported that [Ca^{2+}]_{cyt} increased by 46% in 10 minutes of treatment with 100μM Al in rye roots. Among several studies reporting increased [Ca^{2+}]_{cyt} after exposure to Al, only Jones et al. (1998) reported that Al exposure resulted in a prolonged reduction in [Ca^{2+}]_{cyt} in tobacco BY-2 cells. However, it is important to note that plant systems tested responded quickly to Al stress by altering the cytosolic Ca^{2+} homeostasis.

Debates continue about the role of a concrete Ca^{2+} permeable channel. In their review, Rengel and Zhang (2003) summarized that in several studies, hyperpolarization-activated Ca^{2+} channels were shown to be blocked by Al, but depolarization-activated Ca^{2+} channels were shown to be inhibited only partly by Al. Thus, depolarization of the plasma membrane occurring in response to Al could activate depolarization-activated Ca^{2+} channels, inducing influx of Ca^{2+}. Further, a role of glutamate-gated Ca^{2+} channel was considered. Finally, a role of inhibition of Ca^{2+} efflux pump was discussed as an alternative explanation of increase of [Ca^{2+}]_{cyt} during Al stress. Hypotheses considering the activity of depolarization-activated Ca^{2+} channel and glutamate-gated channel include also a role of microtubules and are therefore discussed in details in the chapter 2.2.5.

Recently, Kawano et al. (2004) showed that at lower concentration of Al, induction of reactive oxygen species (ROS) occurs that activates distinct Ca^{2+} channels. Higher
concentrations of Al induce the inhibition of the same Ca^{2+} channels. Authors identified these ROS-activated Ca^{2+} channels as channels belonging to the TPC1 channel (two-pore channel 1) family. Since TPC1 channels are responsive to reactive oxygen species and also to cryptogein (a fungal elicitor protein), they are possible to play a role in the development of plant defense mechanisms. Al then represents a specific inhibitor of TPC1 (fig. 1). It is interesting to note that Al inhibited also cold-induced Ca^{2+} influx (for cold-induced influx of Ca^{2+} see the chapter 3.4.2), suggesting a role of Al-sensitive Ca^{2+}-channel in cold sensing (Lin et al. 2005). However, this possibility needs further investigation.

Although the dynamics of Ca^{2+} fluxes in plant cells under Al stress needs further studies, it is clear that due to its rapid response to Al, Ca^{2+} homeostasis represents one of the most interesting candidates for the prime target of Al. It becomes evident that calcium acts as a key signaling molecule during normal plant growth and development as well as in transduction of abiotic signals (for review see Sanders et al. 1999; Reddy 2001). Therefore, some frequently observed Al-induced effects as callose formation (as discussed previously), oxidative stress or changes in the cytoskeleton (as will be discussed later) are well consistent with the theory that disturbances in calcium homeostasis could lie in the beginning of the Al-stress signal transduction.

2.2.4 Oxidative stress

Al toxicity is clearly related to oxidative stress. What is oxidative stress, how it arises and what can oxidative stress cause within a cell? Major organelles producing ROS (as superoxide anion O_{2}^{-}, or hydrogen peroxide H_{2}O_{2}) under normal conditions are mitochondria. The leakage of electrons from the mitochondrial electron transport chain generates O_{2}^{-} radicals, which are converted to H_{2}O_{2} non-enzymatically and/or by manganese superoxide dismutase (MnSOD) localized in mitochondria. H_{2}O_{2} diffuses across membranes and is converted to H_{2}O by various types of peroxidases and catalases. If not converted, O_{2}^{-} and H_{2}O_{2} react to form hydroxyl radicals OH, highly reactive oxygen radical in the presence of transition metal ions as Fe and Cu. Hydroxyl radicals cause dangerous injuries in cells as lipid peroxidation or protein oxidation. Besides antioxidant enzymes, cells possess molecules such as glutathione (GSH), ascorbate (ASA) and α-tocopherol, which are used as a hydrogen donor to convert H_{2}O₂ to H_{2}O enzymatically or as non-enzymatical scavengers of ROS (Devì et al. 2003). Under normal circumstances, this defense system efficiently reduces ROS. Conditions of increased ROS production represent the oxidative stress. Oxidative stress leads to the oxidation of biomolecules and destruction of cellular structures.

Lipid peroxidation, ROS production, and expression of genes whose products act in cellular defense systems against oxidation occurred simultaneously in the course of Al treatment in several plants. In barley, treatment of roots with Al for 3-12 hours led to the formation of ROS and caused the death of root cells after 8 hours (Pan et al. 2001). In pea roots, Yamamoto et al. (2001) reported Al-induced lipid peroxidation. Nevertheless, despite the fact that lipid peroxidation was a relatively early event, authors reported that it was not the prime cause of root growth inhibition. Similarly, Boscolo et al. (2003) showed that Al-induced oxidative stress accompanied by formation of ROS, activation of superoxide dismutase (SOD) and peroxidases (PX) activities and protein oxidation in maize Al-sensitive line was not the primal cause of the root growth inhibition, since the inhibitory effect of Al on the root growth took place earlier. The mitochondrion is possibly the target of Al toxicity, although it is not known whether the earlier or one of the later one, and its damage could lead to oxidative stress. Yamamoto et al. (2002) reported that Al induced quick repression of processes that take place in mitochondria in cultured tobacco cells. Later, after 12 hours of treatment, ROS production, respiration inhibition, ATP depletion, and the loss of growth capability was triggered almost simultaneously. The authors suggested that Al accumulation on the outer plasma membrane affects mitochondrial functions by an unknown signal transduction pathway. Alternatively, Al could interfere with mitochondria directly in the cytoplasm.

The capability of plant cells to prevent effectively the oxidative stress confers, however, the ability to tolerate Al to them. In the study of Devì et al. (2003), Al tolerant (ALT301) and sensitive (SL) tobacco cell lines were compared. Lipid peroxidation was lower in Al-tolerant cell line, which authors explain by higher amounts of ASA and GSH in these cells. Thus, higher antioxidant status in ALT301 cell line contributed to Al tolerance of this cell line. Typically, the overexpression of genes whose products act in cellular defense against oxidative stress has been shown to confer Al resistance (Ezaki et al. 2000, Ezaki et al. 2001).

Therefore, the role of oxidative stress in toxicity of Al ions requires further research. As was discussed in the chapter 2.2.3, a ROS-activated Ca^{2+} channel TPC1 is specifically
blocked by Al. The oxidative and Al stress thus probably interact with each other on the level of Ca\textsuperscript{2+} homeostasis, but the role of this interaction in signaling triggered by Al as well as in the disruption of Ca\textsuperscript{2+} homeostasis is not known. At present, data suggest that the formation of ROS is not directly involved in sensing of Al stress, but rather represents a consequence of toxic influence of Al ions.

2.2.5 Effect on cytoskeleton

As mentioned above, plant microtubules in the cortical cytoplasm of a cell play important roles in the cell growth and the polarity maintenance. The hypothesis that Al-induced disruption of cortical microtubules could result in the cessation of root growth and the cell swelling belongs to earliest hypotheses concerning the primary site of Al-action. For microtubules speaks also the fact that drugs influencing microtubular dynamics (oryzalin and taxol) induce very similar changes (root growth inhibition and cell swelling) when applied to plant roots (Fig. 2; Baskin et al. 1994). Almost 20 years ago, Macdonald et al. (1987) showed in their pilot study that in vitro, the association constant for Al ion-GTP-tubulin ternary complex was 10\textsuperscript{7} times higher than that for Mg\textsuperscript{2+} ions, physiological mediators of microtubule assembly. Further, Al ions competed effectively with Mg\textsuperscript{2+} ions in the process of tubulin polymerization. And finally, although Al-induced microtubules were structurally indistinguishable from those produced by Mg\textsuperscript{2+} ions, Al-microtubules showed lower susceptibility to Ca\textsuperscript{2+}, a physiological regulator of microtubular depolymerization. Since the dynamics represents one of the most important features for proper functioning of microtubules in all eukaryotic cells, even small changes of the sensitivity to physiological modulators of their dynamics as Ca\textsuperscript{2+} ions could lead to serious injuries in these cells.

One of the first studies showing the disruption of plant microtubules in root cells in response to Al was conducted on wheat (Sasaki et al. 1997a; Sasaki et al. 1997b). Cortical microtubules in the root elongation zone were shown to be disrupted after prolonged exposure to Al (3 hours), but treatment for 1-2 hours did not result in any change of structure of cortical microtubules. One year later, maize roots were subjected to a detailed study by Blancaflor et al. (1998) and microtubules of the elongation zone were recognized as the target of Al. Both microtubular and actin cytoskeletons were visualised during Al treatments (0-24 hours) and their reorganization and stability studied. The authors showed that microtubules and actin reoriented after 3 and 5 hours of treatment, respectively, and that the response was tissue-specific. Whereas microtubules in cells of the inner cortex and stele changed their orientation after 3 hours of treatment, microtubules in the outer cortex did not reorient. Actin cytoskeleton in cells of the inner cortex and stele reoriented after 5 hours of treatment. Actin in cells of the outer stele did not reorient. Microtubules and actin in cells of the outer stele, however, were found to be stabilized against auxin- and cold-induced reorientation and depolymerization. More importantly, this increased stabilization was detectable during less than 1 hour of treatment.

As mentioned in the chapter 2.2, the distal part of the transition zone (DTZ) of maize roots, where cells switch from the division to the elongation and undergo a preparatory phase for rapid elongation, was shown to be the most Al-sensitive site (Sivaguru and Horst 1998). High Al-sensitivity, as compared to other root zones, was attributed to DTZ on the basis of its highest ability to induce growth inhibition when exposed to Al, to form callose and accumulate Al. Therefore, Sivaguru et al. (1999a) studied early effects of Al on the cytoskeleton in DTZ in maize using immunofluorescent techniques. They found out that microtubules in DTZ were depleted after 1 hour of treatment with Al, whereas no change in their structure was detected in the elongation zone (the latter feature is in agreement with findings of Blancaflor et al. 1998). Both effects were accompanied by the plasma membrane
depolarization and callose formation. Actin cytoskeleton was found to be altered as well, as judged from decreased amounts of F-actin in DTZ. Authors therefore concluded that the cytoskeleton in DTZ cells is especially sensitive to Al and can be considered as the primary target of Al toxicity leading to root growth inhibition.

Cytoskeletal changes caused by Al ions have been observed in the rapidly dividing and elongating cells of the root tip rather than in the differentiated cells of mature tissues. This might either reflect a decrease of Al-sensitivity during the cell differentiation or, alternatively, an influence of positional information within the tissue. To discriminate between these two possibilities, it was necessary to employ an alternative experimental system. Plant cell cultures provide more or less physiologically homogenous population of cells, where the position in the cell cycle can be controlled experimentally and where the position effects are almost eliminated. Suspension-cultured tobacco cells were used to investigate the effect of Al on microtubules by Sivaguru et al. (1999b). Authors showed that the response of microtubules was dependent on the position of cells within the cell cycle: depolymerization of microtubules after 6 hours of treatment with Al was observed in exponentially growing cells, whereas microtubules in cells of stationary phase were stabilized upon Al treatment. These results were in good agreement with previous reports on the effect of Al on microtubule-behavior in various root zones and their cell types. However, the time interval of 6 hours was too long considering almost direct Al-induced root growth inhibition and thus only later effects of Al treatment were observed in this study. We have reported a more rapid effect of Al on microtubules in tobacco plant cells (Schwarzerová et al. 2002) and showed that 1-hour Al treatment induced formation of additional microtubular bundles in the cortical region of exponentially growing cells and that these microtubular bundles were thinner compared to the control. An alternative explanation is that observed changes were attributable to partial microtubule depolymerization and release of single microtubules from cortical microtubular bundles. These rapid changes were accompanied by a transient increase of tubulin content. Later, after 6 hours, Al induced disorganization and depolymerization of cortical microtubules. Whereas later changes as disorganization and depolymerization were in agreement with observations of Sivaguru et al. (1999b), rapid changes observed during 1 hour of treatment in our study suggested that microtubules could represent one of the earliest targets of Al toxicity. Rapid, Al-induced changes in the organization of cortical microtubules, which are considered as an important factor in the control of the cell shape, could lie in the beginning of the root growth inhibition. The transient increase of tubulin content observed during first hour of Al-treatment suggests that not only organization of microtubules, but also de-novo synthesis of tubulin was a part of adaptive processes in tobacco cells. It is possible that de-novo synthesis of tubulin occurred in the response to the decreased microtubule dynamics that was accompanied by formation of additional microtubule bundles or reduced microtubule depolymerization during early phases of toxic influence. The prolonged exposure to Al (6 hours) resulted in the reduction of such adaptive processes and the depletion of microtubules occurred.

Our results on the rapid changes in the organization of cortical microtubules in response to Al are well consistent with results of Sivaguru et al. (2003). Authors provided a new insight into responses of plants to Al, using GFP-MBD (microtubule-binding domain) expressing Arabidopsis plants, suggesting a possible molecular mechanism, by which Al could induce rapid root growth inhibition. The most important contribution of this study was that microtubular behavior was observed in vivo, and that the very early response of microtubules to Al was studied (in a range of minutes). Surprisingly, authors described rapid depolymerisation of microtubules in the lateral root cap cells during first 30 minutes of the treatment with Al that was accompanied by the plasma membrane depolarization. Authors also referred that microtubules repolymerized after several hours, and after 12-24 hours, microtubules were found to be disorganized and disrupted. The latter is in accordance with previous studies describing disruption of microtubules during prolonged exposure to Al. Thus, this study allowed the authors to discriminate between two phases of the microtubular response during Al toxicity: the earliest one (within 30 minutes) that probably includes also signal transduction at the beginning of response to Al, and later phase (hours) that reflects downstream effects. If partial and transient depolymerization of microtubules is really a part of signal transduction triggered by Al (which seems to be supported by the rapidity of microtubular response), a question arises how actually microtubules act in the signal transduction pathway and what kind of signal pathway is it. Sivaguru et al. (2003) showed that glutamate treatment mimicked Al treatment, inducing microtubules to depolymerize and plasma membrane to depolarize. Ion channels gated by glutamate are called glutamate receptors and they belong to the group of animal non-selective cation channels, functioning as ligand-gated ion channels that control signaling across neural synapses in invertebrates and vertebrates. In the Arabidopsis genome, 20 genes encoding plant glutamate-like receptors were found (Lacomb et al. 2001). A specific antagonist of neuronal glutamate receptor, 2-amino-5-phosphonopentanoate (AP-5) prevented glutamate- and Al-induced depolymerization of microtubules in the work of Sivaguru et al. (2003), which suggested that a glutamate receptor might be included in Al-triggered signal transduction. Further, trivalent
cations that are known to block calcium channels prevented glutamate- and AI-induced depolymerization of microtubules in MBD-GFP expressing Arabidopsis plants, suggesting the role of calcium influx as well.

Authors presented a hypothesis that during AI treatment, AI opens a channel through which glutamate effluxes. Glutamate binds to its receptor, a ligand-gated calcium channel, at the plasma membrane, and triggers calcium influx. This initiates subsequent downstream signal events including partial and transient depolymerization of microtubules and plasma membrane depolarization. The hypothesis integrates best-known effects of AI toxicity into one signal transduction pathway. However, several questions remain to be answered. For example, a discrepancy exists concerning the differential sensitivity of root zones to AI. If the distal part of the elongation zone (DTZ) was shown to be the most sensitive part of the root in maize, what is then its role in roots during AI stress and do microtubules in cells of DTZ response in the same way to AI and glutamate as lateral root-cap cells? Alternatively, is it possible that the sensing of AI occurs in other root zones than the AI-induced growth inhibition? Further, the identity of the channel gated by AI, through which glutamate effluxes, remains unknown. And finally, the exact mechanism by which microtubules depolymerize in response to Ca²⁺ (direct effect of Ca²⁺ on microtubules or its action mediated by Ca²⁺-regulated microtubule associated protein) is not known at present.

Alternatively, another AI signaling pathway including microtubules, plasma membrane depolarization and Ca²⁺ influx could be raised. Plasma membrane depolarization-activated Ca²⁺ channels (DACCs) have been described in a number of cell types (for review see Hetherington and Brownlee 2004). The activity of these channels is regulated by the microtubules cytoskeleton; the disruption of microtubules resulted in the activation of DACCs in carrot (Daucus carota L.) protoplasts (Thion et al. 1996) and in Arabidopsis thaliana protoplasts (Thion et al. 1994). Therefore, at the beginning of the signal transduction, AI-induced disruption of microtubules under the plasma membrane might activate DACCs in the plasma membrane, resulting in the elevation of Ca²⁺ concentration in the cytosol. Since AI was shown to induce depolarization of plasma membrane alone (Matsumoto et al. 1996; Sivaguru et al. 1999a; Ahn et al. 2001; Sivaguru et al. 2003), the increased activity of DACCs could be further enhanced also directly by AI (Rengel and Zhang 2003).

2.3 Signaling during AI stress - conclusion

The most important AI-induced effects that possibly lead to quick root growth inhibition were discussed. The role of AI interaction with phosphoinositide signaling pathway components as well as production of ROS and lipid peroxidation during signaling triggered by AI remains unknown due to the lack of information. However, two hypotheses integrating effects of AI on the plasma membrane potential, calcium fluxes and cortical microtubules into one signal transduction pathway through which plants may sense AI toxicity, resulting in root growth inhibition, could be raised (see Fig. 4). The glutamate hypothesis is based on results of Sivaguru et al. (2003), who showed that a glutamate receptor is activated by AI. This induces Ca²⁺ influx and transient depolymerization of microtubules, leading to downstream effects such as root growth inhibition. The DACCs hypothesis is based on the existence of plasma membrane depolarization-activated Ca²⁺ channels, which are modulated by microtubules (Thion et al. 1996; Thion et al. 1998). As was suggested by Rengel and Zhang (2003), AI-induced microtubule depolymerization could lead to the activation of DACCs and increased Ca²⁺ influx, resulting in plasma membrane depolarization and downstream effects. The activity of DACCs could be enhanced further by AI-induced plasma membrane depolarization. Although both theories must be further confirmed, it is clear that they represent the first model of cascade of events that could lead to AI-induced growth inhibition, and where microtubules act as a component of this signal transduction pathway.

![Fig. 4: A schematic depiction of sequence of events during sensing of AI toxicity in the hypothesis considering the role of a glutamate receptor (glutamate hypothesis; Sivaguru et al. 2003) or depolarization-activated Ca²⁺ channels (DACC hypothesis; Rengel and Zhang 2003). Dashed line indicates possible direct effect of Al ions on plasma membrane depolarization, enhancing the activity of Ca²⁺ channels.](image-url)
3 Low temperature

Microtubules are known to be cold-sensitive - that is, they depolymerize at low temperatures. However, the effect of low temperatures is associated with many other changes within cells and whole plants. In some plants, the influence of low, non-freezing temperatures is even required for the successful trigger of cold acclimation, a process during which plants become tolerant to freezing temperatures. Although the nature of the plant “thermometer” (or the cold sensor) remains elusive so far, several results indicate that changes in membrane fluidity stand at the very beginning of the cold sensing. In higher plants, cooperation of membrane rigidification with Ca²⁺ fluxes and cytoskeleton remodeling are supposed to play a role in cold sensing. In this chapter, recent theories about the role of microtubules in cold signaling in plants will be discussed.

3.1 Differential sensitivity of microtubules to cold

The threshold temperature, at which microtubules depolymerize, differs among organisms (Wallin and Stromberg 1995). For example, poikilotherm animals such as Antarctic fishes are adapted to very low temperatures; their microtubules must be able to polymerize at temperatures lower than 4°C, whereas mammalian microtubules disassemble at temperatures lower than 20°C. Plant microtubules seem to be generally more cold-stable when compared to animal microtubules, possibly reflecting higher developmental plasticity of plants (Nick 2000). In plants, cold sensitivity of microtubules could be regulated by plant hormones such as abscisic acid (Wang and Nick 2001) or by some physiological processes as cold acclimation (Abdakhamanova et al. 2003). Cold sensitivity can also change in respect to the age of cells and position within the cell cycle (Mizuno 1992) so that microtubules of actively dividing cells are more sensitive to cold when compared to non-dividing cells. Moreover, the subpopulations of microtubules within one cell could differ in cold-sensitivity; a subpopulation of cold-resistant microtubules that did not disassemble even after several hours of treatment with 0°C was found in tobacco cells (Hasecawza et al. 1997). In our study, changes in the structure of the microtubular cytoskeleton in tobacco cells at 0°C were studied in detail (Pokornà et al. 2004). We have shown that after 12 hours at 0°C, a small set of polymerized microtubules remained in the cortical region, suggesting the presence of highly cold-resistant microtubules in tobacco cells. On the contrary, microtubules forming division spindles and phragmoplasts were extremely cold labile, depolymerizing after minutes of treatment with low temperature. Interestingly, microtubules repolymerized extremely quickly (within 1 minute) after removal of the cells from 0°C. Microtubules reconstituted after cold stress were nucleated at the cortical region and from the nuclear envelope. In conclusion, above-mentioned facts indicate that there exist regulatory factors that modulate sensitivity of microtubules to low temperatures.

3.2 Factors modifying cold sensitivity of microtubules

Tubulin isoforms

The first on the list of factors modulating sensitivity of microtubules to low temperature is undoubtedly the structure of tubulin molecule itself. A gene family encodes tubulin in both animals and plants. Therefore, different isoforms of tubulin exist whose expression can be modulated by a cell under various conditions. As already mentioned, it has been shown that tubulins of Antarctic fishes are able to assemble at temperatures as low as -2 – +4°C. Both α- and β-tubulin of these fishes were shown to contain small sets of unique or rare amino acid residue substitutions that could contribute to high cold-resistance of assembled microtubules (Pucciarelli et al. 1997; Detrich et al. 2000). The pattern of tubulin transcript changes also in plant organisms that are exposed to low temperature (Chu et al. 1993a); transcripts of preferentially expressed tubulins in Arabidopsis plants were more stable at 4°C.

Post-translational modifications

The fact that plant tubulin is extensively post-translationally modified was showed in tobacco cells by Smertenko et al. (1997), suggesting that such modifications participate in generation of tubulins with various functions. Thus, post-translational modifications of tubulins represent the second factor that could correlate with the cold stability of microtubules. Phosphorylation is a rare post-translational modification of tubulins, but is found extensively in β-tubulins of cold-adapted organisms (Pucciarelli et al. 1997). In plants, Mizuno (1992) showed that treatment with protein kinase inhibitors resulted in increased cold stability of microtubules in tobacco cells. The author suggested that phosphorylation and dephosphorylation of proteins, possibly also tubulin itself, could play a role in induction of cold stability of microtubules in tobacco. Acetylation of α-tubulin rendered microtubules of maize protoplasts more stable when exposed to low temperature (Huang and Lloyd 1999). Nevertheless, acetylation, however extensively was found in Atlantic fishes living at low temperatures, did not correlate with cold stability of microtubules in these organisms.
according to studies of Modig et al. (1994) and Rutberg et al. (1995). This indicates that the role of acetylation in cold stability of microtubules has to be further studied.

**Microtubule-associated proteins**

Microtubule-associated proteins represent the third factor that could modulate the sensitivity of microtubules to cold. Among number of plant proteins associated with microtubules that are identified to date, several has been shown to contribute to cold stability of microtubules. Two carrot kinesins, DoKRP120-1 and DoKRP120-2 co-sedimented with cold-stable fraction of microtubules, indicating that the physical interaction between microtubules and associated proteins could play a role in microtubule cold-stabilization (Barroso et al. 2000). Further, tobacco microtubule-associated protein MAP65-1 belongs to a family of plant microtubule-associated proteins that induce microtubule bundling (Smertenko et al. 2000). MAP65-1b protein has been shown to bind to microtubules, to bundle them and to stabilize them against cold-induced depolymerization (Wicker-Planquart et al. 2004).

**Cooperation with other cellular structures**

Finally, cooperation of the microtubular cytoskeleton with other cellular structures may play a role in their cold-sensitivity. We have shown that the endomembrane system may act as a supporting structure for the repolymerization of the cytoskeleton and provide it with the spatial information, important for formation of three-dimensional network (Schwarzerová et al. 2003). Moreover, mutual cooperation between microtubular and actin cytoskeleton plays a role as well. Stabilization of cortical microtubules resulted in the formation of thicker bundles of transversal cortical actin filaments (Blancaflor 2000). Similar evidence on actin and microtubular interaction came from experiments with isolated plasma membrane that retained proteins associated with it, so called membrane ghosts. Membrane ghosts isolated from cells treated with taxol, a microtubule-stabilizing drug, retained more actin filaments that were more aligned with microtubules than in untreated cells (Collinge et al. 1998). Chu et al. (1993b) reported that the treatment of rye roots with taxol (a microtubule-stabilizing drug) resulted in increased freezing-stability of actin cytoskeleton. Therefore, a mutual stabilization or destabilization of both cytoskeletons may be important for their sensitivity to cold-induced depolymerization.

### 3.3 Cold acclimation – a unique feature of plants

Some plants of temperate climate zone are capable to go through a complex process called cold acclimation during the onset of the winter. As the result, these plants become tolerant to freezing temperatures of the oncoming winter. Cold acclimation is defined as a prolonged exposure to chilling, but non-freezing temperatures and is associated with novel gene expression, de-novo protein synthesis and many other physiological changes. Among other changes associated with cold acclimation, microtubules could become more resistant to low temperatures (Wang and Nick 2001; Abdakhmanova et al. 2003). The molecular mechanism or mechanisms leading to increased cold-resistance of acclimated plant microtubules remains unknown, but it is highly probable that some of mechanisms mentioned above could be involved. Indeed, Abdakhmanova et al. (2003) showed that cold acclimation leading to the stabilization of microtubules against cold is related to changes of tubulin isotype composition and the increase of tubulin tyrosination in freezing-tolerant cultivars of wheat.

It seems that for efficient cold acclimation, transient depolymerization or destabilization of microtubules is required, because treatment with taxol decreased the tolerance of plants to low temperature (Kerr and Carter 1990; Bartolo and Carter 1991). Initial microtubule disassembly was even considered as a marker for efficient cold acclimation in wheat (Abdakhmanova et al. 2003). There are two possible explanations of this feature. The first is that transient disassembly reflects higher dynamics within the microtubular net that is needed for the adjustment of the composition of tubulins towards the decrease of the cold-sensitivity. The second explanation involves the role of microtubules in sensing of low temperature. The possible role of microtubules in cold sensing is discussed in the next chapter. The theory supposes that transient disassembly of microtubules could trigger downstream effects and the transduction of low temperature signal leading to efficient cold acclimation.

### 3.4 Cold sensing and signalization in plants

How do plants sense low temperature and how they transduce these signals to activate complex changes needed for cold acclimation? As already mentioned, cold acclimation represents a process during which almost every cellular process is altered. Many genes were shown to be up-regulated during cold acclimation (for review see Thomashow 2001; Browse and Xia 2001) and some of these genes were useful in investigating the signaling pathways associated with cold acclimation. It becomes obvious that cold acclimation includes multiple
signaling pathways, whose complexity is far from being understood (Browse and Xin 2001). However, question is what is the nature of the plant "thermometer". According to some published results, changes in membrane fluidity could act as the primary signal of low temperature.

**Cold sensing in cyanobacteria**

A sensor of low temperature is pretty well described in the cyanobacterium *Synechocystis*. Here, the cold sensor histidine kinase 33 (Hik33) was originally identified as a regulator of the cold-inducible expression of the desB gene, which encodes the enzyme desaturase (Los and Murata 2004). Fatty acid desaturases are encoded by 4 genes in *Synechocystis* (called desA - desD) and they synthesize fatty acids with four double bonds. Unsaturated fatty acids increase membrane fluidity that is necessary for survival at low temperature, because low temperature induces dangerous membrane rigidification (Fig. 5).

Hik33 contains two transmembrane domains in the N-terminal region. It has been suggested that Hik33 perceives changes in membrane fluidity as the primary signal of cold stress. Membrane rigidification leads to dimerization and auto-phosphorylation of Hik33 (Los and Murata 2004) that triggers downstream effects, such as induction of expression of other cold-inducible genes. Nevertheless, Hik33 does not represent the only temperature sensor - at least one additional temperature sensor must exist in *Synechocystis*, because mutations in Hik33 greatly reduced induction of desB and desD genes, but had no effect on desA gene (Browse and Xin 2001).

**Cold sensing in higher plants**

Hik33, however, does not have homologues in higher plants, although it is interesting for future studies to note that Arabidopsis genome encodes 12 potential histidine kinases of two-component system (Browse and Xin 2001). In plants, various stresses including cold stress are known to activate mitogen-activated protein kinase (MAPK) cascades. Different MAPK pathways respond to different stimuli and cold and salt stress was recently shown to activate specifically the MKK2 pathway in Arabidopsis (Teige et al. 2004). Cold-activated MKK2 pathway consists of the stress-induced MAPK kinase kinase MEKK1, which activates MAPK kinase MKK2, which subsequently activates MAP kinases MPK4 and MPK6. Question is what is the cold sensor that activates MEKK1. The study of Sangwan et al. (2002) proved that the cold-activated MAPK called SAMK is activated by changes in membrane structure and that these changes can be mimicked at 25°C by cytoskeleton destabilizers or inhibited by blocking of Ca²⁺ influx.

Thus, similarly to cyanobacteria, temperature perception is associated with changes in membrane fluidity also in plants (Murata and Los 1997), indicating that membrane rigidification could act as the "thermometer". Indeed, it has been shown that membrane fluidization prevents calcium influx and freezing tolerance induction in alfalfa (Medicago sativa) (Orvar et al. 2000). Another feature associated with cold stress in higher plant cells is the rapid influx of Ca²⁺ ions (Woods et al. 1984; Knight et al. 1996; for recent review see Thomashow 2001). It is obvious that Ca²⁺ represents an important second messenger during cold stress signaling, which controls various Ca-regulated effector proteins such as calmodulin, calcium-dependent protein kinases and calcium-regulated phosphatases (for review see Knight and Knight 2001). Further, microtubules have been of special interest in low temperature research due to their high sensitivity to cold. Since they respond to low temperature immediately, they are also supposed to play a key role in cold sensing and their role as the plant "thermometer" has been suggested by Abdakhmanova et al. (2003). In the study of Mazars et al. (1997), disruption of microtubules in tobacco (Nicotiana plamaginifolia) protoplasts led to the additional increase of Ca²⁺ influx during cold shock, suggesting that microtubules modulate the intensity of calcium signals.

It seems that membrane rigidification, Ca²⁺ influx and microtubules together are probable to play a low temperature sensing. In the study of Sangwan et al. (2001), the gene expression under low temperature was studied. As a marker of low-temperature induced gene expression was used gene BN115, whose expression is known to be induced by cold and that represents an orthologue of the Arabidopsis cor15 gene. Expression of gene BN115 was strongly inhibited at low temperature, when the membrane fluidizer benzyl alcohol was applied at the same time. At 25°C, the expression was induced by membrane rigidification using dimethylsulfoxide. The cold induction of BN115 was strongly inhibited also by microtubule-stabilizer taxol, and mimicked at 25°C by microtubule-depolymerization drugs oryzalin and colchicine. Cold activation of BN115 further required Ca²⁺ influx, because the activation of BN115 was inhibited when Gd³⁺, a specific blocker of mechanosensitive Ca²⁺

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channels, was applied. Authors concluded that for activation of BNII5 expression, rigidification of membranes, followed by depolymerization of the cytoskeleton was needed, that induced activation of Ca^{2+} channels. Similarly, cold activation of stress-activated MAP kinase (SAMK) required membrane rigidification in alfalfa, and could be mimicked at 25°C by microtubule-destabilization or blocked by inhibiting influx of Ca^{2+} (Sangwan et al. 2002). It is important to mention that in both studies, a role of actin was noted as well.

The hypothesis considers influx of Ca^{2+} ions into the cytoplasm as a result of cytoskeleton remodeling. Question remains what kind of Ca^{2+} channel could be involved in this response. Mechano-sensitive Ca^{2+} channels are activated by low temperatures as was demonstrated by Ding and Pickard (1993). Since the cytoskeleton is known to be attached to the plasma membrane (Sonesson and Widell 1998; Collings et al. 1998), changes in the membrane fluidity could be transmitted into the cell via altered interaction of plasma membrane with the microtubular or actin cytoskeleton attached to Ca^{2+} channels. The existence of such mechanosensitive Ca^{2+} channels that are modulated by the cytoskeleton remains to be elucidated. However, the evidence that depolarization-activated plasma membrane Ca^{2+} channels are controlled by the microtubular cytoskeleton exists (Thion et al. 1996, Thion et al. 1998, see the chapter 2.2.5), suggesting that microtubules or actin could be directly involved in the generation of Ca^{2+} signals in plants.

3.5 Cold-induced nuclear tubulin - a new aspect of the role of tubulin during the cell cycle

Our studies on changes of the plant cytoskeleton in response to low temperature led to a detailed description of microtubule depolymerization and repolymerization processes (see the chapter 3.1). Apart from them, a completely new phenomenon was observed tobacco cells. During low temperature treatment, increasing number of cells with tubulin accumulated in interphase nuclei was observed. When these cells were removed from 0°C, tubulin was quickly excluded from nuclei and repolymerized into microtubules. Therefore, we have suggested a new role of plant tubulin besides its more or less "classical" roles as will be discussed in this chapter.

In contrast to animal and plant cells, some protists, fungi and algae perform so-called closed mitosis, where nuclear division takes place within an intact nuclear envelope (for review see Heath 1980). In cells with closed mitosis, the entry of tubulin and microtubule-nucleating components into the nucleus at the onset of mitosis, as well as their exclusion at the end, must be under strict cell cycle control. For example, in Saccharomyces cerevisiae, microtubules of the mitotic spindle are organized inside the nucleus by a specialized structure, the spindle pole body (SPB) (Pereira et al. 1998). It has been shown that the SPB assembles in the cytoplasm and is transported in a cell cycle-dependent manner into the nucleus via the nuclear localization sequence (NLS) of Spc98p protein (Pereira et al. 1998). Interestingly, α- and β-tubulin, the basic components of the mitotic spindle, are not found in the nucleus of cells with closed mitosis during interphase. Therefore, in organisms with closed mitosis, tubulin has to be transported into and out of the nucleus. Indeed, it has been shown in Aspergillus that tubulin enters the nucleus immediately before the onset of closed mitosis and is quickly excluded at the end (Ovechkina et al. 2003). Although the molecular mechanisms of nuclear tubulin transport remain to be elucidated, these results suggest that the movement of tubulin through the nuclear envelope during the cell cycle is an active and well regulated process.

The mitotic spindle microtubules of animals, higher plants (Embryobionta) and charophycean green algae (Charophyceae) interact with chromosomes after the nuclear envelope has disintegrated at the onset of mitosis (Heath 1980). Consequently, directed movement of tubulin through the nuclear envelope is not required for formations of the mitotic spindle. After nuclear envelope disintegration, the microtubular spindle can exploit the pool of cytoplasmic tubulin that is no longer withdrawn by compartmentalization. The spindle microtubules are nucleated from centrosomes in animal and algal cells (Wiese and Zheng 1999, but from rather diffuse microtubule-organizing centers (MTOCs) in the acentriolar cells of higher plants (Baskin and Cande 1990; Shimamura et al. 2004). In addition, the kinetochores of both animal and plant cells are endowed with a microtubule nucleating activity (Cande 1990). γ-tubulin, a minus-end nucleator of microtubule assembly, is an indispensable component of both centrosomes and plant MTOCs (Pereira and Schiebel 1997; Stoppin-Mellet et al. 2000).

In plant cells, the association of γ-tubulin with the prospective kinetochore sites occurs already during the G2 phase and thus precedes the disintegration of the nuclear envelope (Binarová et al. 2000). This indicates that, like the SPB of Saccharomyces cerevisiae, the microtubule-nucleating component γ-tubulin must be actively transported through the intact nuclear envelope before mitosis. Again, neither the molecular mechanism of γ-tubulin transport into the nucleus is known nor whether other components of MTOCs persist in the nucleus during the whole cell cycle or have to be imported as well.

We have shown for the first time and by means of several methods that cold-induced disintegration of microtubules is accompanied by the entry of tubulin into interphase nuclei.
and its gradual accumulation there. Upon re-warming, tubulin was quickly excluded from nuclei, and immediately polymerized into cytoplasmic microtubules such that free tubulin became undetectable in most nuclei within a few minutes of incubation at 25°C. This was supported by the identification of five putative signatures for nuclear export in sequences of plant α- and β-tubulin (Schwarzerová et al. 2005). The incidence of tubulin in interphase nuclei is explained by a hypothesis that the integrity of the nuclear membrane during interphase, when nuclear pores control the transport of molecules, is the main mechanism that ensures the sequestration of tubulin to the cytoplasm during interphase, because its presence in the interphase nucleus could induce serious disorders in the cell-cycle regulation. On the other hand, the interaction of tubulin with chromatin is crucial during mitosis. For closed mitosis, tubulin is imported into the nucleus by an unknown mechanism, whereas for open mitosis, no tubulin transport through the nuclear membrane is required since the nuclear envelope breaks down at the onset of mitosis. However, when daughter nuclei are formed at the end of mitosis, new nuclear envelopes have to be reestablished and the pool of tubulin molecules from the disintegrated spindle has to be removed from the karyoplasm. At this stage, tubulin is excluded from nuclei, possibly utilizing the nuclear export signals conserved in all tubulins. The integrity of the nuclear envelope and the active transport through the nuclear pores are impaired during cold treatment, resulting in intranuclear accumulation of tubulin.

3.6 Signaling during low temperature - conclusion

We could assume that for low temperature sensing in higher plant cells, membrane rigidification, cytoskeleton remodeling and induction of Ca²⁺ release are necessary. According to the hypothesis, the cascade of events starts with membrane rigidification, leading to cytoskeleton remodeling that triggers activation of Ca²⁺ channels (Sangwan et al. 2001; Sangwan et al. 2002; summarized in Fig. 6). The identity of Ca²⁺ channel involved in low temperature remains unknown, but several facts indicate that this channel is mechanosensitive in its nature.

We have reported about the incidence of cold-induced plant tubulin in the interphase nucleus. The discovery of cold-induced nuclear tubulin enabled us to build a hypothesis that the transport of tubulin through the nuclear envelope occurs during the cell cycle that might be functionally relevant for the dynamic changes in the nuclear envelope at the beginning and the end of mitosis (Schwarzerová et al. 2005). The dynamics of plant tubulin during the cell cycle progression, the role of potential NESes and the role of nuclear tubulin in signalization will be further studied, with special emphasis on in vivo visualization techniques.
Conclusion

The role of plant microtubules in the sensing of ambient events has been documented on two examples of abiotic stressors, toxic concentrations of Al³⁺ ions and low temperature. It is possible to find several important characteristics common for signalization during both stresses. First of all, Ca²⁺ ions seem to act as the second messenger in the transduction of signal triggered by Al³⁺ ions as well as by low temperature. The Ca²⁺ channel involved in signaling during Al stress is either an ion channel gated by glutamate (glutamate receptor) or possibly depolarization-activated Ca²⁺-channel (DACC). The Ca²⁺ channel involved in low temperature signaling remains unknown. The opening of Ca²⁺ channels is considered to be mediated by glutamate binding or by depolymerization of microtubules in case of Al stress, and by depolymerization of microtubules in case of low temperature. Thus, for sensing of both abiotic stressors, interphase cortical microtubules as modulators of Ca²⁺ channels or regulators of downstream effects are necessary. It is obvious that close interaction of cortical microtubules with the plasma membrane is of great importance for the efficient trigger of the signal.

Many aspects remain to be elucidated and all hypotheses raised in this work need further confirmation. However, it is possible that in the future, in respect to the nature of microtubules and their interaction with other cellular structures and processes, the role of microtubules also in other signal transduction pathways will be defined.

A new aspect of the role of plant tubulin emerged from studies on cold-induced reorganization of microtubules in BY-2 cells. Here, a transient incidence of tubulin in interphase nuclei was described for the first time. Further studies on the potential role of plant nuclear tubulin in the cell cycle regulation or signalization as well as its relevance in animal cells will possibly reveal its new, so far unknown role.

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List of original publications, on which this PhD thesis is based on:


List of other original publications:


Aluminum-Induced Rapid Changes in the Microtubular Cytoskeleton of Tobacco Cell Lines

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Aluminum (Al) is a major factor that limits plant growth in acid soils. It causes a cessation of root growth and changes in root morphology suggesting a role of the root cytoskeleton as a target of Al-toxicity. Here we report a rapid effect of Al on the microtubular cytoskeleton of the suspension tobacco cell lines BY-2 and VLB-0. viability studies showed that the cells were more sensitive to Al during exponential phase as compared to stationary cells. During the first hours of exposure, Al induced the formation of additional bundles of cortical microtubules (cMTs), whereas the thickness of the individual bundles decreased. Prolonged exposure resulted in disorientation of cMTs. These changes of cMTs preceded the decrease of cell viability by several hours and were accompanied by an increase in the levels of α-tubulin (in its tyrosinated form) and elements of the tubulin-folding chaperone CCT. These findings suggest that the microtubular cytoskeleton is one of the early targets of Al toxicity.

Key words: Aluminum — BY-2 — CCT — Microtubules — Nicotiana tabacum — VLB-0.

Abbreviations: cMTs, cortical microtubules; DISC, differential interference contrast; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; MBB, microtubule-stabilizing buffer; MTS, microtubules; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PFES, piperoxane-1,4,6,8-tetraoxononic acid.

Introduction

Aluminum (Al) ions released from soils under low pH conditions have been recognized as a major factor limiting plant growth in acid soils. The molecular basis of Al toxicity is still far from being understood, but the most important physiological consequence of Al toxicity is a cessation of root growth, a very rapid phenomenon that occurs within less than 1 h of exposure (Sasai et al. 1997b, Blancaflor et al. 1999).

The root tip with actively dividing and elongating cells seems to be the most sensitive to Al treatment with both cell division and cell elongation being affected.

Studies carried out to elucidate the target of Al action in plant tissues and cells demonstrated that Al3+ ions enter and bind to the apoplastic (Horef 1995), and change the properties of the plasma membrane (Wagatiwana et al. 1995). Most of the apoplastic Al is bound to the pectic components of the cell wall (Chang et al. 1999). Since there exists a continuum between cell wall, plasma membrane and cytoskeleton (Wyatt and Carpita 1993), it was suggested that Al could affect intracellular events without even permeating through the plasma membrane (Horef et al. 1999). Moreover, the greatest proportion of cytotoxic Al should be inactivated by the nearly neutral pH of the cytosol and by binding to cytosolic compounds. Nevertheless, the residual amount of free Al in cytosol could be sufficient to affect intracellular processes (Macdonald and Maris 1988).

Al-induced effects on root growth have been attributed to putative interactions between Al ions and the cytoskeleton, since Al3+ ions were shown to affect microtubular polymerization and dynamics in vitro (Macdonald et al. 1987). More recently, microtubules (MTs) in the elongation zone of wheat roots have been observed to be disrupted after 3 h of exposure to Al (Sasai et al. 1997b). Similarly, the cytoskeleton of maize roots was reorganized and stabilized in a cell-specific manner in response to Al exposure (Blancaflor et al. 1998). This process was rapid (after 3 h of treatment) and coincided with the time course of growth inhibition. In the root apex of the same species, MTs were found to be disrupted or stabilized depending on cell position and the time of exposure to Al (Sivaguru et al. 1999a).

Generally, these cytoskeletal changes caused by Al3+ ions have been observed in the rapidly dividing and elongating cells of the root tip rather than in the differentiated cells of the mature root tissues. This might either reflect a decrease of Al sensitivity during cell differentiation or, alternatively, an influence of positional information within the tissue. To discriminate between these two possibilities it is necessary to change the experimental system.

Plant cell cultures provide a more or less physiologically homogeneous population of cells, where the position in the cell cycle and the differentiation of cells can be controlled experimentally and where position effects are almost eliminated. Suspension cultures have therefore been used for the investigation of Al-toxicity on plant cells (Chang et al. 1999, Kergaovs et al.).

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![Graph showing viability and phenotype](image)

Fig. 1. Viability and phenotype of tobacco cell line during the exposure to Al. (A) Viability of exponential and stationary cells during 24 h of exposure to 0.1 mM AlCl₃, pH 4.5 in 3% sucrose. (B) Phenotype of exponential cells after 1 h of exposure to full (MS) medium (pH = 5.8), 3% sucrose (pH = 4.5) and 0.1 mM AlCl₃, in 3% sucrose (pH = 4.5), respectively. (C, D) Phenotype of stationary cells after 11 h of exposure to the full (MS) medium (pH = 5.8), 3% sucrose (pH = 4.5) and 0.1 mM AlCl₃, in 3% sucrose (pH = 4.5), respectively. Nomarski DIC. Bar, 50 μm.

1998, Jones et al. 1998, Kataoka et al. 1997, McDonald-Stephens and Taylor 1995, Vitorcellio and Hug 1999). MTs were reported to be depleted in suspension-cultured tobacco cells after 6 h of Al-treatment, when the culture was rapidly dividing. In contrast, during stationary phase, Al induced a stabilization of MTs (Srivaguru et al. 1999b). The physiological background of these observations remains enigmatic, however.

Cell lines that are highly homogenous and tightly controlled in terms of physiology are a precondition to understand the cytoskeletal effects of Al. Therefore, for the present study, the tobacco lines BY-2 (Nagata et al. 1992) and VBI-0 (Oppermann and Oppermann 1995) were chosen, where distinct phases of cell division, cell elongation phase, and differentiation can be followed during the subculture interval. Both cell lines have been widely used in numerous physiological studies and are well characterised with respect to the cytoskeleton (reviewed for BY-2 in Nagata et al. 1992). For VBI-0, the effect of heat stress on the microtubular organisation (Smertenko et al. 1997b), the distribution of post-translationally modified tubulins (Smertenko et al. 1997a), and the co-localisation of MTs with HSP90 (Petříček et al. 1998) or elements of the tubulinfolding chaperone CDT (Nick et al. 2000) have been characterized in detail.

Here we report for tobacco cell cultures two rapid effects of Al exposure: (1) Al induces additional bundles of cMTs; (2) Al causes these bundles to become thinner, presumably reflecting a reduced number of individual MTs per bundle. These effects precede any effect on cell viability and are accompanied by an increase in the levels of α-tubulin, tyrosinated α-tubulin, and CDT (a subunit of the tubulin-folding chaperonin complex CDT). In contrast, prolonged time of exposure to Al ions resulted in extensive disorganisation of cMTs and decreased levels of α-tubulin, tyrosinated α-tubulin, and CDT.

Results

Al sensitivity is more pronounced during exponential phase

The effect of Al on actively dividing cells was compared to that on cells in the stationary phase of the culture. Actively dividing cells that formed multi-cellular fibre of daughter cells were used as indication for the exponential phase (Fig. 1B), whereas the stationary phase of the culture was defined by a disintegration of cell fibres into two-cellular fragments (Fig. 1E).

Since Al is complexed in the complete media used for the culture, the cells had to be transferred to a minimal medium (3% sucrose, pH 4.5) for the Al-treatment. This transfer per se did not affect the viability of the cells. Even after 24 h in a minimal medium without Al, the viabiliy was still 100% in the stationary cells (Fig. 1A), and in the exponential cells it had decreased only slightly to about 80%. Granular structures in exponential cells were observed after prolonged time of exposure to the minimal medium (Fig. 1C, D and F, G).

In contrast, the viability decreased considerably, when 100 μM of AlCl₃, were added to the minimal medium (Fig. 1A). This decrease initiated earlier (from 2 h after the addition of Al) and was more pronounced in exponential cells as compared to stationary cells. But 10 h after the addition of Al, the cells were observed to remain perfectly viable maintaining morphology and intracellular structure (Fig. 1D, F).

Al affects microtubular organisation and abundance in a two-phase pattern

During exponential phase, characteristic transverse cortical MTs prevailed in both cell lines (Fig. 2A, 3A). Even prolonged cultivation on the minimal medium did not affect the microtubular pattern (compare Fig. 3C for 1 h, Fig. 3F for 10 h on minimal medium to the control shown in Fig. 3A). This supports the data on cell viability and morphology (Fig. 1) that within a time window of 10 h the transfer to minimal medium per se did not cause any changes of intracellular structure at all.

Treatment with Al was observed to cause two major effects in the structure of cortical MTs. The number of cMT bundles increased within the first hours of exposure to Al. This effect was observed in both cell lines (VBI-0: Fig. 2B, BY-2: Fig. 3B) and could be quantified (Fig. 4A) to about 20–25% additional MT bundles as compared to the control. In parallel, MTs appeared to become thinner in response to Al (BY-2: Fig. 3B, D, E). This effect was especially pronounced in BY-2, where the estimated number of individual MTs dropped from around 12 to less than 8 (Fig. 4B), whereas in the somewhat larger VBI-0 cells the MT bundles are already thinner in the controls (about 10 individual MTs per bundle) and decreased to around 8 after Al treatment (Fig. 4B).

Later, from about the sixth h of exposure, cMTs became
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Fig. 3 MTs in the exponential BY-2 cells after the exposure to AI. (A) Cells grown in the full medium, (B, D and E) Cells grown in 0.1 mM AICl, for 1, 4 and 10 h, respectively. (C and F) Cells grown in 3% sucrose for 1 and 10 h, respectively. Cells were stained with anti-α-tubulin antibody and FITC-conjugated secondary antibody. Bar, 50 μm.

progressively disoriented in AI-treated cells, and were found to be very thin (Fig. 3D, E). Additionally, a perinuclear diffuse tubulin signal, which was hard to be detected in controls grown on minimal medium, became prominent with prolonged exposure to AI (data not shown).

In contrast to the cMTs, no evident changes of mitotic microtubular structures such as division spindles were observed during the first 10 h of exposure (data not shown).

Microtubules remain longer after oryzalin-treatment

To test the possibility that the formation of more fine and abundant cMTs results from altered microtubular stability, we studied the effect of 1.5 μM oryzalin, an inhibitor of MT assembly, on cells that had been treated for 1 h with AI. Within 15 min of treatment, microtubular bundles were found to be affected in both control and AI-treated cells (Fig. 5). However, in the AI-treated cells (Fig. 5B), the MTs appeared to be less bundled, longer and more abundant as compared to the control (Fig. 5A). Nevertheless, they remain sensitive to oryzalin to a certain degree.

The level of α-tubulin transiently increases in response to AI

To understand the AI-induced increase in MT number (Fig. 2A, B), the level of α-tubulin and the tyrosinated form of α-tubulin were assayed by immunoblotting loading equal amounts of total protein extracts (Fig. 6D) from AI-treated cells versus cells that had been cultivated for the same time intervals on the minimal medium (Fig. 6A, B). During cultivation on the minimal medium, the amount of α-tubulin decreased with time, whereas the amount of tyrosinated α-tubulin remained constant. Within 1 h of exposure to AI both the amount of α-tubulin and the amount of tyrosinated α-tubulin increased in comparison with the control (Fig. 6A, B). This rapid increase was transient with time the level of both α-tubulin and tyrosinated α-tubulin dropped back to that observed in the controls cultivated without AI.

Does AI-treatment increase the number of MT-nucleating sites?

The increase in the number of MT bundles in response to AI could be accompanied by an increase of MT-nucleation in the cortical cytoplasm. To test this possibility, we used a subunit of the tubulin-folding CCT complex as marker for sites of MT nucleation (Nick et al. 2000). The level of this subunit decreased with time of cultivation on the minimal medium (Fig. 6C). Interestingly, it increased transiently within the first h of exposure to AI and gradually returned to the levels observed in the controls cultivated on minimal medium without AI.

Discussion

The inhibition of root growth by AI ions seems to be based mainly upon the toxicity of AI to cell division and elongation in the root tip (reviewed in Dolnikov and Ryan 1994 and Kochian 1995). The cytoskeleton has been shown to be affected in the roots treated with AI (Grabski and Schindler 1995, Sasaki et al. 1997a), and this effect occurs even after short exposure to AI (Blucotte et al. 1998). However, the effect depends on the position of the target cell within the tissue, which makes it difficult to approach the cellular base of AI toxicity. The influence of adjacent cells or cellular complexes as well as remote correlative regulation could impact the response of a single cell to an examined factor. Therefore, the cytosmotically well-defined plant cell culture BY-2 and VBI-6 were used as an experimental material for evaluation of role of cytoskeleton in AI toxicity.

Because the complex growth media make it difficult to predict what AI-species occur in what effective concentration, we used a minimal medium at low pH such that Al³⁺ ions were the prevalent species. Consistent with previous results for VBI-
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Fig. 5 MTs in exponential BY-2 cells treated with 1.5 μM oryzalin after 1 h of exposure to the Al-elicited medium. (A) Control cells grown in full medium treated with 1.5 μM oryzalin for 15 min. (B) Cells treated with 0.1 mM AlCl3 for 1 h and subsequently with 1.5 μM oryzalin for 15 min. Cells were stained with anti-α-tubulin antibody and FITC-conjugated secondary antibody. Bar, 20 μm.

0 (Zazimalová et al., 1995), we observed that in BY-2 neither viability and intracellular structure (Fig. 1) nor the microtubular cytoskeleton (Fig. 3) were affected during the time period relevant for the present study, i.e., the first 10 h after exposure to Al (Fig. 1).

The time course of Al-dependent cell death (Fig. 1) was dependent on the age of the cell population. Rapidly cycling cells were more sensitive than stationary cells which is consistent with observations published previously (Kataoka et al., 1997). The lethal effect of AI loss seems to be related to the metabolic activity during the exponential phase supporting previous findings in tobacco suspension cultures (Yamamoto et al., 1994). The sensitivity might reside in the secretory apparatus, since inhibition of Golgi transport by brefeldin A caused a reduction of Al uptake (Vitale and Haag 1999). The reduced sensitivity of stationary cells might be related to their reduced secretory and metabolic activity.

The microtubular response was rapid (Fig. 2, 3) and preceded the decrease of AI on viability by several hours (Fig. 1). Studies on Al uptake in cell culture (Kataoka et al., 1997) demonstrated that cell death occurred several hours after Al has entered the cell and is thus a rather late effect of Al toxicity. It is highly probable that cMTs are one of the first not the only cellular structures influenced by AI. The actual cause for cell death remains obscure so far.

The microtubular response to AI during the first hours of exposure (Fig. 2, 3) involved an increase in the number of M1 bundles (Fig. 4A) and a reduction of bundle thickness (Fig. 4B). Although estimations of MT thickness based on fluorescence images are expected to be affected by fluorescence dispersion, this seems to be not a fundamental problem. The estimated number of individual MTs per bundle observed for the control cells in BY-2 (Fig. 4A) match very well the data obtained by electron microscopy published for bundles of cMT isolated from tobacco cell line (Gonoba et al., 2001). This agreement justifies the conclusion that AI reduces the number of individual MTs within a bundle, an effect which, again, is observed in both cell lines, however, somewhat more pronounced in BY-2. Nevertheless, these bundles were perfectly ordered and displayed no deformations or lesions.

The formation of additional MT bundles was accompanied by an increased level of α-tubulin and tyrosinated α-tubulin (Fig. 6). Simultaneously, an epitope characteristic for tubulin-chaperone CCT complex increased transiently in expression (Fig. 6). These changes in protein expression indicate that the formation of additional MT bundles observed in response to AI (Fig. 2, 3) involves de novo synthesis and folding. Alternatively, the turnover of tubulin subunits might be reduced leading to an increased steady-state level of α-tubulin. If tubulin turnover was reduced, this should lead to a higher resistance of MTs to oryzalin, a drug that blocks the assembly of tubulin dimers into MTs (Marejohn et al., 1987). However, the AI-induced additional MTs remain sensitive to oryzalin (Fig. 5). A reduced turnover of tubulin subunits is expected to decrease the tyrosinisation of α-tubulin. The enzyme responsible for deetyrosination can bind only to assembled MTs (MacRae 1997). This has the consequence that an increased stability of MTs results in a higher the fraction of tubulin subunits that have undergone deetyrosination. The level of tyrosinisation does not decrease, it increases significantly (Fig. 6). It is therefore more likely that the additional MTs formed in response to AI originate from elevated tubulin synthesis rather than increased stability of MTs.

An additional mechanism might be responsible for the additional MT bundles seen in BY-2 cells. The quantification of MT thickness (Fig. 4B) allowed estimation of the number of individual MTs per bundle. The correspondence to the findings by Sonobe et al. (2001) obtained by electron microscopy of cMT bundles isolated from BY-2 cells validates the quantification algorithm and allows the conclusion that AI reduces the extent of cMT bundling. It is therefore possible that AI blocks the activity of unknown factors that regulate the bundling of individual MTs.

As the stress continued for more than 6 h, the adaptive processes probably failed and excessive disorientation and dissolution of MTs occurred. These changes represented the second phase of the cellular response to AI toxicity preceding cell death. The disorientation and disruption of MTs has been reported also in roots of wheat and maize after 3 h of exposure (Sasakii et al., 1997, Bianca et al. 1998) and maize after 6 h of exposure (Sivaguru et al., 1999a). Since the deposition of newly synthesized cellular microtubules into the cell wall is a microtubule-dependent process (Nick 2000 for review), depletion of the microtubular network might result in reduction of the root growth observed during treatment of roots with AI.

This two-phase pattern of the microtubular response to AI toxicity was also observed on the biochemical level with a progressive decrease in the expression of α-tubulin and the tubulin-folding chaperone CCT.

The molecular basis for the increased number of MTs in response to AI remains to be elucidated. The data presented in this study, however, rules out certain mechanisms. AI was discussed to compete with magnesium ions for the GTP-binding site of tubulin (Mackonald et al., 1987), and this should block GTP-hydrolysis resulting in a prolonged cap of GTP at the plus-end of a growing MT and thus an increased lifetime (Hayley et al., 1994). As discussed above, the impaired but still detectable sensitivity to oryzalin (Fig. 5) and more strongly, the increased level of tubulin tyrosination (Fig. 6) are not consistent with any mechanism that prolongs the lifetime of individual MTs.
The most likely scenario would be the induction of tubulin thinning (as seen in the elevated level of β-tubulin) and a modest increase in β-tubulin (as seen in the elevated level of CTT) by ACXs by triggering signaling. The additional tubulin dimers would then be assembled into new MTs. This either means that AI induces additional sites of nucleation or that MT elongation is not limited by nucleation sites, but by the level of available tubulin dimers. These mechanisms seem to be supported by a reduced rate of MT growth from MTs comprised of one bundle (Fig. 4B).

This effect could arise from an interaction of AI with factors that influence organization of MTs in the cortical region. MT-associated proteins that bundle MTs (Chan et al., 1999; Marc et al., 1996; Durao and Cyr 1994, Vanard et al. 1991) or microtubule-associated proteins that connect MTs with the plasma membrane (Nonobe et al. 2001) might play a role in Al-induced changes.

The resulting additional MTs were still dynamic and might represent one of the earliest known adaptive responses of the cell to AI stress. Upon prolonged exposure to AI, the proper organization of these MTs into transverse arrays was progressively lost leading to disoriented MT arrays and a gradual loss of cell axis. Future work will be directed toward understanding the signals that regulate the synthesis of new tubulin subunits and the role of different tubulin isotypes in response to AI stress.

Materials and Methods

Plant material

The tobacco BY2 (Nicotiana tabacum L. cv. Bright Yellow) cell line (Ngata et al. 1992) was cultured in Murashige-Skoog medium (Murashige and Skoog 1962) supplemented with 1 mM glutathione (2,4-D). Every 7-14 days 1.5 mL of cells was transferred to 30 mL of fresh medium and cultured on a horizontal shaking platform at 25 °C.

The tobacco-βI (Nicotiana tabacum L. cv. Virgina Bright Italia) cell line (Oponty and Oponty 1976) was cultured on 3% agar with Glucose, 1% yeast extract, 0.05% modified Miller's medium (Heller 1953) supplemented with 100 μM of L-glutathione (Glu) and 40 μM of 2,4-D. To obtain a suspension culture, an inoculum was suspended in liquid medium with 5% of Glu and 5% of 2,4-D and cultured on a horizontal shaker at 100 rpm at 25 °C.

Evaluation of number of microtubules in the cortical region

Visualization by immunofluorescence detects and distinguishes bundles of MTs rather than individual MTs. In the absence of response of MTs to AI, we used 10 interphase cells with transverse MT arrays that had been labeled by immunofluorescence in 3% agar with Glu and 5% of 2,4-D and cultured on a horizontal shaker at 100 rpm. The dark between two MTs was 14 μm and the interdiction density was 5×10³ cells/mL. The number of cells is the cortical region.

Treatment of cells with AI

The cells are in exponential (2-day-old) or stationary (5-day-old) phase of culture were washed in a solution of 3% acetone in a Nalgene bottle-top filter for 3 min and re-suspended in 3% sucrose solution. The cells were then dispersed into microplates (250 μl) and cultured for 3 min (1 Hz stock solution). The pH was adjusted immediately before the experiments.

Treatment of cells with arylsulfatase

A suspension culture of N9 (Elume products Co, USA) was added directly to a final concentration of 1 μM of arylsulfatase.

Quantification of cell viability

The viability of the cells was assessed using fluorescein diacetate (FDA). Aliquots of 40 μL of a 0.2% w/v stock solution of FDA in acetone were added to the cell suspension in 3% PRO (in case the cells were not in the logarithmic growth phase) and then re-suspended in the acetone from the interphase to the next interphase, or during the lagging cells among the cells of new tubulin from the interphase to the next interphase or within the interphase. Under conditions of linearly increasing and symmetrically different density values, the distance between the zero values of this graph estimate the average thickness of the MTs in the given cell line. The more cells would then be recalculated into thickness in μm. Electron-microscopic studies of bundles of β-tubulin-containing MTs, that were isolated from BY2 cells (Nonobe et al. 2001) show that the MTs of individual MTs range 25 nm in diameter and that the spacing between two individual MTs amounts roughly to 35 nm. Thus, it is possible to express the meaned thickness of a bundle in terms of estimated number of individual MTs per bundle.
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1. Introduction

The eukaryotic cytoskeleton is a highly dynamic and complex structure supporting many basic metabolic processes. Generally, the organisation of cytoskeleton carries the spatial information important for various cellular compartments and moreover secures the mobility of protein-transporting membrane vesicles. One of the important features of cytoskeleton polymers is their high sensitivity to low (non-freezing) temperatures leading to reversible depolymerisation into subunits. However, cold sensitivity of cytoskeletal polymers differs in various plants and plant tissues depending on many interfering factors (Balažák et al., 1993; Egertsson and Kasprzak, 2001; Kerr and Carter, 1990). In addition to that, spatial interconnection of cytoskeleton with endomembrane system plays an important role (for review see Lischicki and Balukha, 2000). Cold-induced depolymerisation of both microtubules (MTs) and actin filaments (AFs) provided us with a tool to investigate the role of the cytoskeleton in the spatial organisation of the plant cell cytoplasm. The tobacco BY-2 suspension culture (Nagata et al., 1992) was used as a model, physiologically homogeneous plant cell line. We have focused on the cortical cytoplasm of BY-2 tobacco cells where structural changes can be easily observed by conventional light and fluorescence microscopy (reviewed in Kumagai and Haszawa, 2001). Together with this, the organisation of the endoplasmic reticulum (ER) was studied in vivo using BY-2 cells expressing a GFP-fusin protein targeted to the lumen of the ER. Protein analysis of cytoskeleton proteins was carried out to understand some cold-induced cytoskeletal changes on the protein level.

2. Results and discussion

In interphase, transverse parallel arrays of MTs (Fig. 1a) were observed in the cortical layer of cytoplasm and this typical organisation did not significantly change during the life cycle. There were no Mts in the cortical layer of cytoplasm during mitosis and cytokinosis. In contrast to Mts, AFs formed at least two different arrays in the cortical cytoplasm in interphase cells: transverse parallel arrays, and filaments without apparent orientation (Fig. 1b). AFs that formed transverse parallel arrays were thin in exponentially growing cells and became more prominent in cells in the stationary phase of growth. During mitosis and cytokinosis, AFs formed a very dense, non-oriented net in the cortical region. In vivo observations using ER-targeted GFP showed a lace-like network of tubules, lamellar sheets and mobile particles in the cortical cytoplasm of interphase cells (Fig. 1c). The net of ER did not show any noticeable structural changes during life cycle of tobacco cells, although it was denser during the exponential phase of growth. During mitosis, the cortical ER re-formed into a lace-like net in which lamellar sheets were absent.

The first effect of cold in the cortical region observed after 20 min of cultivation at 0 °C was the partial disassembly of both Mts and AFs. Progressive depolymerisation occurred during prolonged cultivation (12 h) at 0 °C. The rate of depolymerisation differed in cells found in different stages of life cycle. For example, exponentially growing and actively dividing cells maintained more AFs in the polymerised state for a longer time than elongating cells. After 12 h of cold treatment,
of actin was assembled. After that, polymerised AFs reached other cortical areas to form a uniform net. Taken together, the data presented here suggest that cytoskeletal subunits released from polymers at 0 °C existed in a form immediately available for recovery of polymers. Further, our data indicate that membranes (particularly ER) might provide cytoskeletal monomers with the information important for their spatial organisation during cold treatment and also during subsequent recovery of AFs and MTs at optimal temperature.

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References


The majority of cells did not contain any polymerised cytoskeleton or kept very short fragments of MTs (Fig. 1d) and AFs (Fig. 1e). In contrast to the cytoskeleton, no pronounced damage of ER structure was detected in the cortical layer of cold-treated cells and only subtle extension of lamellar sheets of ER was observed in cells cultivated for 12 h at 0 °C (Fig. 1f).

Although this ultra-low but non-freezing temperature (0 °C) was found to be sufficient to depolymerise the majority of both AFs and MTs, recovery experiments showed that polymerisation competence of subunits of both cytoskeletal structures was preserved during the cold treatment. Immediately after transfer of cells to optimal temperature conditions (25 °C), recovery of both AFs and MTs in all surviving cells was observed. Recovery of cytoskeletal polymers was preceded by the formation of transient structures.

Biochemical analysis revealed that the levels of actin and tubulin in cytosolic and sedimentable protein fractions did not change during cold treatment. Furthermore, although protein synthesis in vivo was strongly inhibited under cold stress conditions, in vitro translation assay indicated that no degradation of particular mRNAs occurred.

The stability of ER structure in the absence of cytoskeletal structures manifests a certain organisational independence of this membranous organelle. It can also be concluded that depolymerisation of the cytoskeleton is not the primary cause of death of cells cultivated at 0 °C. Furthermore, our data suggest that membranes of ER might provide the cytoskeleton with some kind of structural support. For example, actin in cells cultivated at 0 °C was often detected as diffuse signal in the cortical region whose pattern resembled the structure of the ER. When cells cultivated at 0 °C were transferred to a standard temperature (25 °C), first re-polymerisation of AFs occurred only in those places where the diffuse signal
Sites of actin filament initiation and reorganization in cold-treated tobacco cells

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ABSTRACT
Cytoskeletal proteins assemble into dynamic polymers that play many roles in nuclear and cell division, signal transduction, and determination of cell shape and polarity. The distribution and dynamics of microtubules (MTs) and actin filaments (AFs) are determined, among other factors, by the location of their nucleation sites. Whereas the sites of microtubule nucleation in plants are known to be located under the plasma membrane and on the nuclear envelope during interphase, there is a striking lack of information about nucleation sites of AFs. In the studies reported herein, low temperature (8°C) was used to de-polymerize AFs and MTs in tobacco BY-2 (Nicotiana tabacum L.) cells at interphase. The extent of de-polymerization of cytoskeletal filaments in interphase cells during cold treatment and the subcellular distribution of nucleation sites during subsequent recovery at 25°C were monitored by means of fluorescence microscopy. The results show that AFs re-polymerized rapidly from sites located in the cortical region and on the nuclear envelope, similarly to the initiation sites of MTs. In contrast to MTs, however, complete reconstitution of AFs was preceded by the formation of transient actin structures including actin dots, rods, and filaments with a dotted signal. Immunostaining of soluble and sedimentable protein fractions showed no changes in the relative amounts of free and membrane-bound actin or tubulin.

Key-words: Nicotiana tabacum; actin; actin filaments; cold stress; cytoskeleton; microtubules; tobacco cells BY-2; tubulin.

INTRODUCTION
The cytoskeleton is a highly dynamic structure that plays many roles in nuclear and cell division, signal transduction, determination of cell shape and polarity, and cell motility. Two main components of the cytoskeleton, tubulin polymerizing into microtubules (MTs) and actin polymerizing into actin filaments (AFs), are found in all eukaryotic cells. Protein complexes that assist in the nucleation of polymers appear to be composed of evolutionarily highly conserved proteins. For microtubule nucleating sites, tubulin has been identified as a universal nucleator (Wiese & Zheng 1999). It functions in a protein complex belonging to the Sp105/Spc98p protein family originally identified in fungi (Knopf & Schiebel 1997). Their homologues have been identified in animals (Murphy, Urbani & Staehelin 1998) as well as in plants (Ehrhardt et al. 2002). Despite the conserved nature of cytoskeletal proteins, however, MT arrays differ substantially among various organisms and cell types. Plant cells lack centrosomes, which nucleate interphase MTs and organize mitotic spindles in animal cells. Instead, plant MTs are nucleated in a cell cycle-dependent manner from multiple nucleation sites located on the nuclear surface and in the cortical cytoplasm (Itoh et al. 1997).

In contrast to microtubule initiation, eukaryotic cells possess multiple mechanisms of initiation of actin assembly. These include de novo actin nucleation, uncapping of barbed ends, and severing of existing filaments (Condeelis 2001). In animal, fungal and prokaryotic cells, de novo actin polymerization is driven by the actin-related protein 2/3 (Arp2/3) complex, which is a well-understood mechanism (Machesky & Gould 1999). Much less information is available on actin nucleating sites in plant cells. An Arp2/3 complex consists of seven subunits and conserved DNA coding sequences for all of them are present in the Arabidopsis genome (Vantard & Blanchon 2002). Mutations in Arabidopsis Arp2 and Arp3 genes result in actin rearrangement in some cells, accompanied by malformations of cell shape (Mathur et al. 2003). Thus, it seems that Arp2/3-based actin polymerization represents a conserved mechanism, although detailed information about the molecular composition, subcellular localization, and regulation of the Arp2/3 complex in plants remains elusive.

AFs have been shown to form the backbones of cytoplasmic strands in vacuolated plant cells (Eno et al. S. J. & Emons 2000). They form a dense network that co-aligns with microtubules in the cortical cytoplasm, wind around the nucleus forming a basket, and are localized to the...
MATERIALS AND METHODS
Plant material and chemicals
The tobacco cell line BY-2 (Nicotiana tabacum L. var. Bright Yellow 2; Nagata, Nemoto & Haszeawa 1992) was cultured in liquid medium containing 4.3 g L−1 MS salts (Sigma, St. Louis, MO, USA) 15 mM L−1 thiamine, 200 mg L−1 KH2PO4, 100 mg L−1 inositol, 20 g L−1 sucrose and 0.2 mg L−1 (0.9 µM) 2,4-dichlorophenoxyacetic acid (2,4-D), pH 5.8. Every 4–7 days, 15 mL of cells were transferred to 30% of the same volume and cultured in darkness at 25°C on a horizontal shaker (IKA KS501; IKA LaborTechnik, Staufen, Germany; 120 rpm, orbital diameter 30 mm). All chemicals were obtained from Sigma unless otherwise stated.

Cold treatment and recovery experiments
Tobacco BY-2 cells in the exponential phase of growth (3-day-old) were used for all experiments. A standard cell population in this phase of growth consists of 85–95% cells in interphase and 5–15% cells in mitosis and cytokinesis. In all cytological observations, only interphase cells were analyzed. Cultivation conditions and cold treatments were carefully optimized to exclude any additional stress. Cell suspensions in Erlenmeyer flasks were placed in an ice water bath (0°C) and shaken on a horizontal shaker at 100 rpm. (IKA KS501; IKA LaborTechnik) in darkness. For recovery experiments, flasks were removed from the ice water bath after 12 h of cold treatment and the cells were immediately collected by filtering on a nylon mesh (pore diameter 70 µm). The cells were then resuspended in 15 mL of a medium at the control temperature (25°C) and further cultivated at 25°C. During the periods of cold treatment and subsequent recovery at control temperature, samples of cell culture were collected for cytological observations and protein extraction.

Determination of cell viability
Cell viability was assessed with fluorescein diacetate (FDA) according to the method of Wöhlk (1970). Fifty micromoles of 0.2% (w/v) FDA stock solution in acetone were diluted with 7 mL of culture medium, and an aliquot mixed 1:1 (v/v) with cell suspension on a microscope slide. After a 1 min incubation with FDA, the viability was determined from at least 10 optical fields on each of five separate slides as a percentage of the total number of cells. Here we show that after cold-induced dehydration, FDA re-esterified quickly from nucleic acid sites that were located in the closed nuclear envelope, similar to the initial sites of MTs. In contrast to MTs, however, complete reconstitution of AFD was preceded by the development of their characteristic structures including actin rods, dots, and filaments with a dotted signal, and newly formed filamentous actins were always branched. Immunoblotting of actin and soluble protein fractions revealed no changes in the relative amounts of free and membrane-bound actin or tubulin.

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Quantification of cold-induced changes in the arrangement of actin filaments and microtubules
The percentages of cells with distinct cytokinetic arrangements were determined from at least 10 optical fields on each at least three separate slides (a total of around 300 cells were counted in each field of view). A digital camera was used for the experiment. For quantification of structural changes in cytokinetic orientation during cold treatment, the following categories were defined: (1) cells retaining intact cytokinetic filaments or their fragments; (2) cells with totally de-polymerized cytokinetic; (3) cells with cold-induced re-organization of cytokinetic; and (4) damaged cells. The last category included cells with disrupted cytoplasm, broken cell walls, or cells with plasma membrane detached from the cell wall. In controls, the numbers of such cells were approximately 10% and 15% for MTs and AFDs, respectively. In cell culture maintained at 0°C for 12 h, the number of cells with unsatisfactorily visualized cytoskeletons increased to 40–50%. The phase 16 cells within the cell cycle was determined microscopically after staining the nuclei with Hoechst 33258.

Protein electrophoresis and immunoblotting
Proteins were extracted according to Freudentreich & Nick (1998). The experiments were performed on nylon mesh as above, and 1 g of biomas was homogenized immediately in liquid nitrogen with a pestle and mortar. The freezer powder was mixed 1:1 (w/v) with extraction buffer (50 mM MES (2-morpholinoethanesulfonic acid), 3 mM EGTA, 5 mM MgCl2, 1 mM glycerol, 1 mM sodium-guanidine 5′-triphosphate (GTP), 1 mM dithioriboholic acid, 1 mM phenyl methyl sulphonyl fluoride, 1 µM aprotinin, 1 µM leupeptin, 1 µM pepstatin, pH 6.9). The mixture was allowed to thaw on ice. A 50 µL aliquot of the mixture was heat-inactivated as a total protein fraction. The thawed centrifuge was centrifuged at 3000 g for 15 min at 4°C, and the supernatant centrifuged at 15 000 g for 10 min at 4°C. Supernatant was used as a soluble fraction and the pellet represented the sedimentable fraction. The fractions were mixed with denaturing buffer (50 mM Tris-HCl, pH 6.9, 5% (w/v) sodium dodecyl sulfate (SDS); 35% (w/v) urea, 30% (w/v) glycerol; 5% (w/v) β-mercaptoethanol; 0.5% (w/v) bromophenol blue). Protein concentration was determined after staining with Coomassie Brilliant Blue or transferred onto polyvinylidene difluoride (PVDF) membranes by semidry electrophoresis for probing with antibodies. Mouse monoclonal anti-tubulin N356 (Amer- sham Biosciences) and mouse monoclonal anti-tubulin 61D10, clone C4 (BD Biosciences) antibodies were used at 1:4000 dilution. After incubation with...
Results

Organization of cytoplasm and cell viability during treatment at 0 °C and after recovery at 25 °C

When cultivated at the control temperature of 25 °C, interphase cells formed many radially oriented cytoplasmic strands that connected the cortical and perinuclear regions (Fig. 1a). Cold treatment in ice water (0 °C) led to gradual disruption of the cytoplasmic strands, starting in all cells after only 5 min of cold treatment (data not shown). The cytoplasmic strands then gradually disappeared, the vacuolar complex fused into one big central vacuole, and the nucleus shifted into the layer of cortical cytoplasm. After 12 h at 0 °C, virtually no cytoplasmic strands were observed (Fig. 1b). Restoration of cytoplasmic strands in the surviving cells started within 5 min after transfer to 25 °C (Fig. 1c), and numerous cytoplasmic strands reappeared after 20 min (Fig. 1d).

Viability of cells maintained at 0 °C was assessed periodically (after each additional 2 h of recovery at 25 °C) using FDA staining (Witholde 1972). Figure 1e shows the decline in cell viability during the 12 h at 0 °C in a representative experiment. At the end of the 12 h at 0 °C, the cell viability varied between 30 and 50% in four independent experiments.

De-polymerization of actin filaments and microtubules in cells exposed to 0 °C

In tobacco BY-2 cells maintained at 25 °C, AFs were detectable in three cytoplasmic regions in the cortical layer of cytoplasm (Fig. 2a), in the perinuclear region, and in the transvacuolar cytoplasmic strands (Fig. 2b). AFs in the cortical layer of cytoplasm formed a dense network of thick filaments traversing the cortical cytoplasm in all directions. In addition, fine, parallel filaments oriented transversely to the long cell axes were often detectable (Fig. 2a). AFs in the perinuclear region formed a basket-like structure around the nucleus (Fig. 2b). Cytoplasmic strands connecting the...
The first observable effects of the cold treatment, which became apparent after 5 min at 0°C, were the degradation of the cytoplasmic strands and disappearance of the radial AF array (Fig. 2a). After 20 min, the transversely oriented, fine, parallel AFs in the cortex disappeared and actin reformed into a network of disordered, thick and branched AFs (Fig. 2b). This network gradually thinned out during the first 6 h of cold treatment, leaving only a sparse actin network in the cortical region of successfully stained cells (Figs 2c, 2d). Very rarely (in about 1% of the cells) brightly shining actin rods or dots appeared in the cortex and around the nucleus. Such actin rods or dots were never detected in the controls. After 12 h of cold treatment, only 19% of the cells retained any AFs, and these were few in number, short, and sometimes branched AFs (Fig. 2f). However, the number of cells with actin rods or dots in the cortex and around the nucleus (Fig. 2b) increased to 18%. A total of 24% of the cells did not contain any detectable actin filaments (Fig. 2g), and 39% of the cells were damaged. The relative incidence of individual AF categories in controls and cells exposed to 0°C for 12 h is illustrated in Fig. 4a and c.

As expected, MTs in interphase cells at normal temperature formed parallel cortical arrays, oriented transversely or at an oblique angle to the long axis of the cell (Fig. 3a). In contrast to AFs, no MTs were seen in the cytoplasmic strands or around the nucleus. Disassembly of MTs commenced within 20 min of cold treatment (data not shown). After 2-6 h, cortical MTs gradually de-polymerized (Fig. 3b & c). After 12 h, only 5% of the cells contained short MT fragments in transverse orientation (Fig. 3d, arrows), whereas 42% of the cells did not contain any polymerized tubulin and 50% of the cells were damaged. The relative incidence of individual MT structures in controls and in cells exposed to 0°C for 12 h is illustrated in Fig. 4b and d.

Re-polymerization of AFs and MTs during recovery at control temperature

After only 30 s at 25°C, various forms of polymerized actin were detectable in the cortical cytoplasm as well as around the nucleus in all successfully stained cells (54%). The actin forms were: (a) actin rods (Fig. 5a); (b) actin nodules connected with thin AFs (Fig. 5b); (c) actin rods connected with thick branched AFs resembling "beads-on-string" with brightly staining dots often localized to the junction of V-shaped filaments (Fig. 5c & d); and (d) a dense network of thin filaments (Fig. 5e). These structures appeared only transiently during the early phases of the recovery period. After 15 min at 25°C, around 45% of cells re-formed a sparse network of thicker AFs (Fig. 5f) although the transient actin structures were still detectable in about 10% of the cells. After 30 min at 25°C, all successfully stained cells (approximately 60%) re-formed a sparse network of thick filaments in the cortical region. After 1 h at 25°C, no transient actin structures were detectable and stained cells contained control-like AFs network in the cortical cytoplasm (Fig. 5g). Simultaneously with the re-formation of the cortical actin network, reorganisation of the actin cytoskeleton also occurred around the nucleus during the recovery period. Initially, the diffuse signal from de-polymerized AFs that were detectable during the cold treatment re-formed into filaments sometimes decorated with dots. After 5 min at 25°C, AFs formed a dense basket surrounding the nucleus (Fig. 5h, arrows). At the same time, other AFs emerged from the basket, pointing towards the cortical region (Fig. 5h, arrowheads). After 15 min recovery, AFs also re-formed in the fully developed transversal cytoplasmic strands (Fig. 5i & j).

Newly polymerized rods and randomly oriented MTs were detected in the cortical cytoplasm of interphase cells after 1 min of recovery at 25°C (Fig. 6a). After 5 min, the randomly oriented MTs elongated and formed a dense network (Fig. 6b). Within 1 h, the cortical MTs formed parallel, transverse arrays as in the controls (Fig. 6c). In cells at preprophase and early G1, polymerization of MTs was also seen at the nuclear surface (Fig. 6d & e). Polymerization of MTs around the nucleus was transient, however, occurring only during the first few minutes of recovery, simultaneously with the polymerization of MTs in the cortex. After 1 h of recovery, only control-like MT arrays were detected in the cortical cytoplasm of the cells.

**Western blot analysis of cytoskeletal proteins during cold treatment**

To test for possible changes in association of cytoskeletal proteins with membranes during the cold treatment, the total, soluble and sedimentable protein fractions were analysed by SDS-PAGE and immunoblotting of actin and tubulin. Equal amounts of proteins from all fractions taken at 2-h intervals during the 12 h at 0°C were loaded onto gels for the analysis. The immunoblots show that the relative amounts of actin (Fig. 7a) and α-tubulin (Fig. 7b) in the total, soluble, and sedimentable protein fractions did not change substantially during the whole cold treatment.

**DISCUSSION**

Cold-stressed tobacco BY-2 cells proved to be a highly sensitive experimental model for detailed investigation of the processes of de-polymerization and re-assembly of the actin and microtubular cytoskeleton. Exposing the cells to 0°C for only a few minutes affected both their cytoplasmic architecture and particularly the organization of the cytoskeleton. Although the cold treatment affected the viability of many cells, almost half of the cells were able to recover after transfer to normal temperature, thereby facilitating studies of the sites of AF and MT initiation and polymerization.

The results obtained by the FIA viability tests are based on detection of active processes in living cells, namely, enzymatic activities and membrane integrity (Steward et al. 1999). Since cells just released from 0°C generally have very low enzymatic activities as well as cold-damaged membranes (Kapeczka 1999), an immediate use of the FIA test would be unsuitable. Therefore, the viability of cells released from cold treatment was assessed after an additional 1 h of recovery at 25°C. Using this approach, the cell viability varied between 50 and 30% at the end of 12 h at 0°C in four independent experiments. These data are consistent with the number of cells with...
unsuccessfully stained cytoskeleton. The percentage of such cells was 10-25% in the controls but increased to approximately 40% for AFs and 50% for MTs after 12 h at 0°C, suggesting that most of these cells may have died during the cold treatment.

We have found that AFs in the transvacuolar cytoplasmic strands disappeared within 5 min at 0°C, simultaneously with the disintegration of the cytoplasmic strands as seen with the Nomarski DIC. Woods, Reid & Patterson (1984) have observed similar disintegration of cytoplasmic strands in response to cold in various plant species, and suggested that this was the result of disruption of AFs. Indeed, the scolding role of AFs in the formation of transvacuolar cytoplasmic strands has been demonstrated in experiments using actin de-polymerizing drugs. A breakdown of cytoplasmic strands was observed after treating tobacco BY-2 cells with latrunculin B for 60 min (Van Gestel et al. 2003) or root hairs of Vicia sativa with cytochalasin D for 30 min (Ensling et al. 2000). Therefore the disruption of the transvacuolar cytoplasmic strands observed in our experiments is likely to be the result of cold-induced de-polymerization of the constituent AFs. The transverse AFs in the cell cortex were apparently less sensitive to cold, de-polymerizing within 20 min of the cold treatment, at the same time as the first signs of degradation of the cortical MTs. Possibly the destabilization of one resulted in disorganization of the other, in a manner similar to that shown by Blazcaffer (2000) by selective drug-induced stabilization or destabilization.
ization of the actin and microtubular cytoskeleton, suggesting co-operation of actin and microtubules in the cortical cytoplasm. In our experiments, the most resistant actin structures were the thick actin cables that endured in the cortical cytoplasm even after prolonged cold treatment. This is in agreement with the results of Eigler et al. & Kacperski (2001), who demonstrated that thick actin cables in oilseed rape cell suspension were generally more resistant to freezing temperature than the fine filamentous network. Actin rods, which were observed at the end of the cold period in some cells, may represent a population of highly stable, short actin filaments. However, further investigation will be necessary to elucidate the composition of these actin structures and to determine whether they originate by severing of existing filaments or by de novo polymerization at 0°C.

Our study of AFs included a comparison with MTs. Although data concerning the cold-stability or sensitivity of MTs in tobacco BY-2 cells are available in the literature, they are surprisingly inconsistent. For example, high cold-resistance of tobacco BY-2 MTs has been reported by Hackawitz et al. (1993) who showed that the MTs were not completely destroyed by treatment at 0°C for 3 h. In contrast, Mizuno (1992) showed that all cortical MTs in exponentially growing tobacco BY-2 cells (2-day-old) were highly cold-sensitive; treatment for 30 min at 0°C resulted in their complete disappearance. In our results, approximately 8% of cells still contained a few short fragments of tubulin polymers even at the end of 12 h at 0°C, indicating that they are highly cold stable. We did not observe any newly formed, cold-induced microtubular structures similar to those seen in Chlamydomonas reinhardtii (Mizuno, Kameko & Tsurumi 1993).

The immunoblot analysis did not detect any appreciable changes in the amount of soluble and sedimentable actin or tubulin during the whole cold treatment, indicating that cytoskeletal monomers did not undergo any substantial subcellular redistribution after the disintegration of the polymers. We also have found that translational activity was severely reduced by the cold treatment (unnpublished results). Therefore it seems that the pool of cytoskeletal proteins was stable, did not degrade, and was not augmented by newly synthesized proteins. Incubation of cells at 0°C for 12 h was apparently sufficient to de-polymerize most AFs and MTs. After transfer back to 25°C, a rapid re-polymerization of actins into small dots and rods occurred within 30 s of recovery. As these structures appeared both in the cortical region and around the nucleus, independent nucleation sites are presumably located in these two regions. The formation of the unique transient actin structures during the early stages of actin re-polymerization is of particular interest. To our knowledge,

![Figure 7. Western blot analysis of cytoskeletal proteins in the total, soluble, and sedimentable protein extracts from BY-2 cells incubated 0-12 h at 0°C. Immunoblots were probed with actin (a) and anti-tubulin (b) antibodies.](image-url)

<table>
<thead>
<tr>
<th>Region</th>
<th>25°C</th>
<th>12h at 0°C</th>
<th>12h at 0°C 2hmin at 25°C</th>
<th>12h at 0°C 1h at 25°C</th>
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<tr>
<td>Cortical</td>
<td>Interphase AFs</td>
<td>Partial de-polymerization and formation of actin rods</td>
<td>Complete de-polymerization</td>
<td>Recovery based on existing fragments</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>Interphase MTs</td>
<td>Partial de-polymerization</td>
<td>Complete de-polymerization</td>
<td>Recovery of MTs</td>
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![Figure 8. A model summarizing important stages in the reorganization of cytoskeleton during cold treatment at 0°C and subsequent recovery at 25°C in interphase BY-2 cells. Arrows indicate proposed course of cytoskeletal reorganization. Cold treatment resulted in complete de-polymerization of AFs in the perinuclear region and in the cytoplasmic strands, and complete or partial de-polymerization in the cortical region. Polymerization of actin into dots in the cell cortex and in the perinuclear region during early phases of recovery probably occurred in those cells in which cold-induced de-polymerization was complete. The cells surviving short fragments of actin at the end of the cold treatment were able to polymerize actin into net immediately without formation of actin dots. In the perinuclear region, an actin basket was formed around the nucleus during early phases of recovery. AFs emerged from it pointing towards the cortical region, reforming actin in the cytoplasmic strands. In some cells, transient actin structures were also formed in the perinuclear region during early phases of recovery. As with AFs, cold-induced complete or partial de-polymerization of MTs. During early phases of recovery, the elongating MTs in the cortical region were always disoriented. Their transversal orientation was established later during recovery. Excepting during the G2 phase of the cell cycle, MTs were not detected in the perinuclear region in interphase control cells. However, re-polymerization of microtubules initiated at the nuclear envelope was detected in some cells during early phases of recovery.](image-url)
the incidence of similar transient actin structures in plant cells has not been described. We propose that the formation of actin dots and rods in the cortex and around the nucleus represents sites of actin nucleation and polymerization. The appearance of the thin and branched filament connecting the dots and rods (beads-on-string structures, or bright dots at the junctions of V-shaped filaments) later during the recovery period is presumably the outcome of AF assembly from the initiation sites. The structure and function of actin filament nucleation centres are only beginning to be understood in plants (reviewed in Ventard & Blanchard 2003). It seems likely that the Arp2/3 complex protein, which plays a key role in de novo AF polymerization in protozoa, fungi, and animals (Machesky & Gould 1999), is also involved in AF nucleation in plants, since mutations Arp2 and Arp3 genes affect actin organization and cell shape in Arabidopsis thaliana (Maturo et al. 2003). Moreover, the transient, branched AFs shown here resemble those formed by the Arp2/3 complex (Svitkina & Borisy 1999). It remains to be established whether the transient dots and rods in our experiments do indeed co-localize with components of the Arp2/3 complex.

Some cells formed a dense actin network without any dot stained within 30 s after transfer to control temperature. Different modes of actin re-polymerization were probably connected with the severity of the preceding cold-induced AFs de-polymerization. In cells in which actin depolymerization was complete, re-assembly required de novo formation of new actin filament by actin-nucleation sites. In cells that retained some remnants of AFs, re-assembly could presumably occur by means of addition of new monomers to existing filament ends. In the latter case, no transient actin structures were formed and an actin network would be detectable immediately.

Similarly to that of AFs, the polymerization of Mts was a very rapid process. Within 1 min after transfer to control temperature, disoriented Mts appeared in the cortical cytoplasm, although their transverse position was not established until 1 h. Later on, obviously newly polymerized Mts after cold-induced de-polymerization, as well as their subsequent rearrangement into transverse orientation during warming, have also been reported for Clon- rium ehnobargii (Hogestatt 1986). The author suggested that MT nucleation and ordering were governed by two independent mechanisms. Our data support this hypothe- sis. We showed that, simultaneously with the polymerization of cortical Mts, another set of Mts elongated from the perinuclear region towards the cortical cytoplasm within 1 min of recovery. The most massive polymerization on the nuclear surface was observed in cells at late tele- phase/pair G1 phase. This observation is consistent with previous reports that the nuclear envelope plays an important role in the polymerization of Mts (Stoppin et al. 1994), especially in this phase of the cell cycle (Nagata & Hanetsu 1993).

An overview of our observations in the changes in the organization of AFs and Mts during cold treatment and subsequent recovery is depicted in Fig. 8, together with a proposed course of reorganization of the actin and micro- tubular cytoskeleton.

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Intranuclear accumulation of plant tubulin in response to low temperature

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Running head: Nuclear accumulation of plant tubulin
Summary

Concurrently with cold-induced disintegration of microtubular structures in the cytoplasm, gradual tubulin accumulation was observed in a progressively growing proportion of interphase nuclei in tobacco BY-2 cells. This intranuclear tubulin disappeared upon re-warming. Simultaneously, new microtubules rapidly emerged from the nuclear periphery and reconstituted new cortical arrays, as was shown by immunofluorescence. A rapid exclusion of tubulin from the nucleus during re-warming was also observed in vivo in cells expressing GFP-tubulin. Nuclei were purified from cells that expressed GFP fused to an ER-retention signal (BY-2-mGFP5-ER), and GFP was used as a diagnostic marker to confirm that the nuclear fraction was not contaminated by nuclear envelope proteins. These purified, GFP-free nuclei contained tubulin when isolated from cold-treated cells, whereas control nuclei were void of tubulin. Furthermore, highly conserved putative nuclear export sequences were identified in tubulin sequences. These results led us to interpret the accumulation of tubulin in interphase nuclei, as well as its rapid nuclear export, in the context of ancient intranuclear tubulin function during the cell cycle progression.

Keywords
BY-2, microtubules, nucleus, plant cell, tubulin

Abbreviations
NLS nuclear localization sequence; NES nuclear export sequence

Introduction

The microtubular cytoskeleton plays a dual role during the progression of the cell cycle. In interphase, microtubules are exclusively found in the cytoplasm, where they participate in intracellular transport, distribution of organelles and maintenance of cell shape. Depending on the phase of the cell cycle, microtubules are organized in different structures – cortical microtubular arrays at G1, cortical and endoplasmic microtubules at S and G2 phase, preprophase band of microtubules at late G2 phase – being, however, always confined to the cytoplasm and separated from the karyoplasm. When the nuclear envelope disintegrates at the onset of mitosis, the organized scaffold of microtubules disappears. The division spindle is established and the major role of microtubules is to ensure an equal distribution of genetic material between the two daughter cells. After the completion of nuclear division and reformation of the nuclear envelope, a new cytoplasmic interphase array of microtubules is reestablished (Hasezawa and Kumagai 2002).

The microtubular structures listed above differ not only with respect to their timing, but also with respect to their nucleation. In contrast to animal and plant cells, some protists, fungi and algae perform a so-called closed mitosis, where nuclear division takes place within an intact nuclear envelope (for review see Heath 1980). In cells with closed mitosis, the entry of tubulin and microtubule-nucleating components into the nucleus at the onset of mitosis, as well as their exclusion at the end, must be under strict cell cycle control. For example, in Saccharomyces cerevisiae, microtubules of the mitotic spindle are organized inside the nucleus by a specialized structure, the spindle pole body (SPB) (Pereira et al. 1998). It has been shown that the SPB assembles in the cytoplasm and is transported in a cell cycle-dependent manner into the nucleus via the nuclear localization sequence (NLS) of Spc98p protein (Pereira et al. 1998). Interestingly, α- and β-tubulin, the basic components of the mitotic spindle, are not found in the nucleus of cells with closed mitosis during interphase. Therefore, in organisms with closed mitosis, tubulin has to be transported into and out of the nucleus. Indeed, it has been shown in Aspergillus that tubulin enters the nucleus immediately before the onset of closed mitosis and is quickly excluded at the end (Ovechkina et al. 2003). Although the molecular mechanisms of nuclear tubulin transport remain to be elucidated, these results suggest that the movement of tubulin through the nuclear envelope during the cell cycle is an active and highly regulated process.

The mitotic spindle microtubules of animals, higher plants (Embryobionta) and charophycean green algae (Charophyceae) interact with chromosomes after the nuclear envelope has disintegrated at the onset of mitosis (Heath 1980). Consequently, directed movement of tubulin through the nuclear envelope is not required for formation of the mitotic spindle. After nuclear envelope disintegration, the microtubular spindle can exploit
the pool of cytoplasmic tubulin that is no longer withdrawn by compartmentalization. The spindle microtubules are nucleated from centrosomes in animal and algal cells (Wiese and Zheng 1999), but from rather diffuse microtubule-organizing centers (MTOCs) in the acentriolar cells of higher plants (Baskin and Cande 1990; Shimamura et al. 2004). In addition, the kinetochores of both animal and plant cells are endowed with a microtubule nucleating activity (Cande 1990). γ-tubulin, a minus-end nucleator of microtubule assembly, is an indispensable component of both centrosomes and plant MTOCs (Pereira and Schiebel’ 1997; Stoppin-Mellet et al. 2000).

In plant cells, the association of γ-tubulin with the prospective kinetochore sites already occurs during the G2 phase and thus precedes the disintegration of the nuclear envelope (Binarová et al. 2000). This indicates that, like the SPB of Saccharomyces cerevisiae, the microtubule-nucleating component γ-tubulin must be actively transported through the intact nuclear envelope before mitosis. Again, neither the molecular mechanism of γ-tubulin transport into the nucleus is known nor whether other components of MTOCs persist in the nucleus during the whole cell cycle or have to be imported as well.

In our previous work, we followed the response of microtubular and actin cytoskeleton to low temperature in tobacco cells (Pokorna et al. 2004). We observed a progressive disintegration of microtubules during prolonged exposure of tobacco BY-2 cells to 0°C and a re-assembly of microtubules during subsequent recovery at 25°C. In the present work, we show by means of several methods that cold-induced disintegration of microtubules is accompanied by the entry of tubulin into the interphase nuclei and its gradual accumulation there. Upon re-warming, tubulin was quickly excluded from the nuclei, and immediately polymerized into cytoplasmic microtubules such that free tubulin became undetectable in most nuclei within a few minutes of incubation at 25°C. The accumulation of tubulin in nuclei and its exclusion from them were shown by immunofluorescence staining and also in vivo in cells that expressed GFP-α-tubulin. Furthermore, increasing levels of α-tubulin could be detected in isolated and highly purified nuclei during cold treatment. We interpret these findings first of all as evidence for a reversible permeabilization of the nuclear envelope for tubulin, possibly via altered function of nuclear pore complexes (NPCs). Further, these data indicate intranuclear tubulin-binding sites that are either unmasked or activated under certain environmental conditions. The subsequent quick exclusion of tubulin from interphase plant nuclei during re-warming could be explained by the recovery of tubulin transport between karyo- and cytoplasm and seems to be an active process. This is supported by the identification of five putative signatures for nuclear export in sequences of plant α- and β-tubulin. This transport
Materials and methods

Plant material and chemicals

The tobacco cell line BY-2 (Nicotiana tabacum L., cv. Bright Yellow 2; Nagata et al. 1992) was cultured in medium containing 4.3 g/l Murashige-Skoog salts (Sigma, St. Louis, MO, USA), 1 mg/l thiamine, 200 mg/l KH$_2$PO$_4$, 100 mg/l myo-inositol, 30 g/l sucrose and 0.2 mg/l (0.9 μM) 2,4-dichlorophenoxyacetic acid (2,4-D), pH 5.8. The transgenic BY-2 cell lines GT16, expressing GFP-a-tubulin (Kumagai et al. 2001), and BY-2-mGFP-ER, expressing mGFP5-ER (Petrášek et al. 2003), were maintained on the same medium supplemented with 100 μg/ml kanamycin and 100 μg/ml cefotaxim. Every 7 days, 1.5 ml of cells were transferred to 30 ml of fresh medium and cultured in darkness at 25°C on an orbital shaker (IKA KS501, IKA Labortechnik, Staufen, Germany; 120 rpm, orbital diameter 30 mm). All chemicals were obtained from Sigma unless stated otherwise.

Cold treatment and recovery experiments

Cells in the exponential phase of growth (3 days after inoculation) were used for all experiments. Cell suspensions in Erlenmeyer flasks were placed in a bath of ice water to maintain the temperature at 0°C and shaken on an orbital shaker at 100 rpm (IKA KS501) in darkness. For recovery experiments, flasks were taken from the ice water and the cells were immediately collected by filtering through nylon mesh (mesh diameter 20 μm). They were then re-suspended in conditioned medium at optimal temperature (25°C) that had been obtained by sterile filtration of 3-day-old BY-2 cells. Thereafter, the cells were cultivated at 25°C. Samples of cells for cytological observation or extraction of proteins were taken at specified time points during the cold treatment and subsequent recovery at normal temperature.

Observation of tubulin exclusion from nuclei in vivo

The tobacco cell line expressing GFP-tubulin was cultured at 0°C as described above. For observation under the confocal laser scanning microscope, a drop of cell suspension was collected from the flask and placed on a pre-warmed (25°C) microscopic slide. The drop of cell suspension was covered immediately with a coverslip and observed under the confocal laser scanning microscope (Leica TCS NT, Heidelberg, Germany). The optimal confocal plane through the nuclear region of a selected cell or cell file was defined and a time-lapse sequence was recorded at this confocal plane. The time-lapse scanning was recorded over 20 minutes at 1 minute intervals.

To measure the exclusion of tubulin from the nucleus and its accumulation in the cytoplasm around the nucleus during the recovery period, the first and last confocal sections from time-lapse series were analyzed. The intensity of the tubulin signal was measured with the Scion Image program (Scion Corporation, Frederick, MD, USA) using a linear probe (profile plot line width = 6) that was placed so as to cover the nuclear region, the cytoplasm around the nucleus and the vacuolar region with one probe. Intensity profiles were plotted onto graphs and the respective parts of curves were labeled as nucleus, cytoplasmin and vacuole.

Visualization of microtubules

Microtubules were visualized as described by Wick et al. (1981) with the modifications described by Mizuno (1992). Briefly, 3 ml of cell suspension (at day 3 after inoculation) were pre-fixed for 10 minutes at 25°C in 3.7% (w/v) paraformaldehyde (PFA) in microtubule stabilizing buffer (MSB), consisting of 50 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), 2 mM ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA) and 2 mM MgSO$_4$·7H$_2$O, pH 6.9, followed by fixation in 3.7% (w/v) PFA with 1% Triton X-100 in MSB for 50 minutes. In the case of cold-treated cells, the first 10 minutes of the fixation was performed in fixation solution chilled to 0°C. After digestion with an enzyme solution (1% [w/v] macerozyme and 0.1% [w/v] pectolyase) for 7 minutes at 25°C, the cells were attached to poly-L-lysine-coated coverslips and treated with 1% (w/v) Triton X-100 in MSB for 20 minutes. Subsequently, the cells were treated with 0.5% (w/v) bovine serum albumin (Fluka, Buchs, Switzerland) in phosphate-buffered saline (PBS; NaCl 8.0 g/l, KCl 0.2 g/l, KH$_2$PO$_4$ 0.158 g/l, Na$_2$HPO$_4$·12 H$_2$O 2.31 g/l) for 30 minutes and incubated with a monoclonal mouse antibody against α-tubulin (N356, Amersham Biosciences, Europe GmbH, Freiburg, Germany) for 45 minutes at 25°C (dilution 1:500 in PBS). After washing with PBS, a secondary FITC (fluorescein isothiocyanate) or TRITC (tetramethylrhodamine isothiocyanate)-conjugated anti-mouse antibody diluted, 1:80 in PBS, was applied for 1 h at 25°C. The specimens were washed in PBS, embedded in 50% glycerol supplemented with 0.1 μg/ml bisbenzimide, 2-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl) (Hoechst 33258) to stain nuclei and viewed immediately.

Microscopy and image processing

Immunolabeled samples were viewed under an epifluorescence microscope (Olympus Provis AX 70, Olympus Optical Co., Ltd., Japan) equipped with appropriate filter sets for the detection of TRITC (excitation at 510–550 nm, barrier filter at 590 nm), FITC (excitation at 450–480 nm, barrier filter at 515 nm) and Hoechst
33258 fluorescence (excitation at 330–385 nm, barrier filter at 420 nm). Objective lenses used were Plan Apo (magnification 40×, numerical aperture 0.85) or LUMPlan FL (magnification 40×, numerical aperture 0.80). The fluorescence signal was grabbed with a monochromatic integrating CCD camera (COHU 4910, Cohu, Inc., Poway, CA, USA) and Nonaraki DIC images were taken with a 3CCD color-video camera (SONY DXC-950P, Sony Corp., Tokyo, Japan). Images were digitally stored using the LUCIA image analysis software (Laboratory Imaging, Prague, Czech Republic). Optical sections were obtained with a confocal laser scanning microscope (Leica TCS NT, Heidelberg, Germany) equipped with an ArAr laser using filter sets for GFP (excitation at 488 nm, emission at 515–545 nm), FITC (excitation at 488, emission at 515–545 nm) and TRITC (excitation at 568 nm, emission at 590 nm). An objective lens Plan Apo (magnification 63×, numerical aperture 1.2) was used for all observations.

For the determination of the percentage of cells containing tubulin in nuclei during cold treatment, at least 200 cells were assessed in total for each sample. During image grabbing, the setup of the frame grabber and the intensity of the fluorescence lamp were optimized for control cells with no signal in the nucleus and all other samples were grabbed using this setup. Therefore, all obtained values are corrected to this “base line”. Cells in which the tubulin signal was stronger in nuclei, but not in nucleoli, as compared to the cytoplasm (refer to Figs. 1C, D, 2A, C’), were considered as containing tubulin in nuclei. All other cells were considered as not containing tubulin in nuclei.

Isolation of nuclei

All procedures were performed at 4°C unless stated otherwise. 100 ml of BY-2-mGFP5-3R cells in exponential phase (3 days after inoculation) were collected by filtering through a nylon mesh (mesh diameter 20 μm) and subsequently re-suspended in 10 volumes of chilled buffer A (10 mM Tris-HCl, pH 6.1; complemented with 5 mM MgCl₂, 10 mM β-mercaptoethanol, 0.15 M sucrose, 50 % [v/v] glycerol, 1 mM PMSF, 50 μM N-p-tosyl-L-phenylalanine chloromethyl ketone [TPCK] and 0.6 % [v/v] Triton X-100). Cells were homogenized in a Waring blender for 30 seconds and filtered through Miracloth (Calbiochem, Merck Biosciences Ltd., Nottingham, Great Britain). Cell material retained by the filter was collected and re-suspended in 10 volumes of buffer A, homogenized once again for 30 seconds and filtered through Miracloth. Filters were mixed and filtered through a nylon mesh (mesh diameter 20 μm). The filtrate was centrifuged at 500 g for 5 minutes at 2°C and the resulting supernatant was centrifuged again at 2000 g for 10 minutes at 2°C. An aliquot of 100 μl was collected from the supernatant and stored at –20°C as fraction I. The sediment containing nuclei was re-suspended carefully in 1 ml of cold buffer A, and again an aliquot of 100 μl was collected as fraction II. Nuclei were further purified by centrifugation through a 25 % / 50 % Percol gradient prepared in buffer A at 7000 g for 30 minutes at 2°C. The interface which contained the nuclei was collected, re-suspended in 15 ml of buffer A and centrifuged at 4000 g for 10 minutes at 2°C. Sedimented nuclei were washed with buffer A and centrifuged at 2000 g for 10 minutes at 2°C. Again, an aliquot of 100 μl was collected from the supernatant as fraction III. The sedimented nuclei (fraction IV) were assayed under the microscope and proteins were extracted and separated electrophoretically.

Protein electrophoresis and immunoblotting

Proteins from fractions I–IV were precipitated with 10% trichloroacetic acid (TCA) and diluted in denaturing sample buffer (50 mM Tris-HCl, pH 6.9, 2 % [w/v] SDS [sodium dodecyl sulphate], 36 % [w/v] urea, 30 % [w/v] glycerol, 5 % [w/v] β-mercaptoethanol, 0.5 % [w/v] bromophenol blue). The total concentration of proteins was determined after staining with amido black (Popov et al. 1975). Protein samples were vortexed, boiled for 5 minutes and separated by SDS-PAGE on 10 % [w/v] acrylamide gels. The separated proteins were transferred onto PVDF membranes by electro-blotting (SemiDry, BioRad) and probed with antibodies. For the Western-blot analyses, we used the mouse monoclonal α-tubulin antibody N356 (Amerham Biosciences) at 1:4000 dilution, the mouse monoclonal anti-tyrosinated α-tubulin antibody TUB-1A2 (Sigma) at 1:800 dilution, and the mouse monoclonal anti-GFP antibody (mixture of clone 7.1 and 12.1, Boehringer Mannheim GmbH, Mannheim, Germany) at 1:1000 dilution. After incubation with an anti-mouse horseradish peroxidase-conjugated secondary antibody (ICN Biochemicals), the proteins were visualized by means of a chemiluminescence ECL-detection kit (Amerham Biosciences) on X-ray films (Foma, Hradec Králové, Czech Republic). The PVDF membrane with the blotted proteins was stained with amido black.

Identification of potential nuclear export sequences

All reported amino acid sequences of Arabidopsis thaliana α- and β-tubulin and three known amino acid sequences of Nicotiana tabacum α-tubulin (nucleotide database at National Center for Biotechnology Information website http://www.ncbi.nlm.nih.gov/) were scanned by eye to identify nuclear export sequences that correspond to a consensus as follows: Z-Z(1-4)-J-X(2-3)-Z-X-Z, where Z = Leu (L), Ile (I), Val (V); J = Z, Met (M), Phe (F); X = any (Mirković et al. 2003).
Result

Tubulin accumulates in the nucleus during chilling

At the control temperature of 25°C, the microtubular cytoskeleton of interphase tobacco BY-2 cells was arranged in the classical pattern of transversely oriented microtubules. Tubulin was present exclusively as polymerized microtubules in the cortical cytoplasm, not in the nuclei, as observed by confocal laser scanning microscopy (Fig. 1A, A', nucleus 1A'). Cortical microtubules were clearly affected after 1 hour of chilling at 0°C (Fig. 1B) and a diffuse tubulin signal accumulated progressively around the nuclei (Fig. 1B', B'), although it was largely excluded from the karyoplasm. However, weak staining of nuclei in Fig. 1B' might reflect early stages of intranuclear tubulin accumulation. The first clear incidence of intranuclear tubulin was detected after around 4 hours of chilling (Fig. 1G). During prolonged chilling (up to 12 hours), the cortical microtubules disappeared progressively, whereas the frequency of cells with intranuclear tubulin increased steadily, reaching approximately 50% after 12 hours of chilling. Tubulin appeared to be present throughout the karyoplasm in a diffuse or very fine punctate pattern but was clearly absent from the nuclei (Fig. 1C, C' for epifluorescence microscopy, 1D for confocal laser scanning microscopy, central confocal optical section, arrow indicates position of nucleus). Cells that were presumably in mitosis at the time of the shift to 0°C exhibited depolymerized mitotic spindles, leading to a diffuse tubulin signal surrounding the spiraled chromosomes (Fig. 1E, E'). The number of cells with intranuclear tubulin increased during the 12 hours of chilling (Fig. 1G).

In order to test whether the elimination of cytoplasmic microtubules would generally result in accumulation of tubulin in nuclei, the cells were treated with 5 μM oryzalin. Microtubules were effectively removed after 11 hours of this treatment (Fig. 1F). A diffuse tubulin signal accumulated near the nuclei but clearly remained excluded from the karyoplasm as shown by double staining with Hoechst 33258 (Fig. 1F').

New microtubules are rapidly nucleated from nuclear tubulin upon re-warming

After 7 hours of cold treatment, cells retained only very few microtubules in the cortical region, but up to 25% of cells exhibited a massive tubulin signal in the nuclei (Fig. 2 A, A'). However, upon re-warming, new microtubules were initiated within a few seconds on the surface of the nuclei, and a reticular array developed around the nuclei within 10 minutes after the temperature had been raised (Fig. 2 B, B'). At the same time, the tubulin signal decreased in the nucleus. However, transient stages with newly polymerized microtubules in the cortical cytoplasm (Fig. 2C) and around the nucleus (Fig. 2C'), and a persistent intranuclear tubulin signal (Fig. 2C', C') were observed as well. Note that even such cells tubulin remained excluded from the nucleoli. The reformation of new microtubules was most pronounced at the nuclear surface of early G1 nuclei (Fig. 2D, D').

Similarly, upon re-warming, new mitotic spindle microtubules were polymerized around the spiralized chromosomes (Fig. 2 E, E'). Within 30 minutes of incubation at 25°C, the microtubular cytoskeleton had completely recovered in all surviving cells (Fig. 2 F, F') and was almost indistinguishable from that of control cells that had not been chilled by chilling (compare Fig. 1 A, A'). Conversely, the intranuclear tubulin had almost completely disappeared (Fig. 2 F, F').

Tubulin exclusion from nuclei can be followed in vivo within a few minutes of re-warming

To observe tubulin accumulation during cold treatment and its exclusion from nuclei to the cytoplasm during re-warming in vivo, we used the transgenic BY-2 cell line GT16 that expresses GFP-tubulin (Kumagai et al. 2001). Tubulin accumulated in the nuclei of a gradually increasing number of transgenic cells during cultivation at 0°C, as observed under the confocal microscope (Fig. 3A). Fluorescence intensity profiles measured within the linear probe showed that the GFP signal in cold-treated cells was more intense in the nuclear region than in the perinuclear cytoplasm (Fig. 3A). Upon re-warming, tubulin was quickly excluded from the nucleus into the cytoplasm (Fig. 3B) and after 20 minutes at 25°C, the GFP signal was stronger in the cytoplasm than the nucleus (Fig. 3B'). In control cells, the GFP-tubulin signal was detectable in the perinuclear cytoplasm as a diffuse signal. However, a much weaker GFP signal was always detectable in nuclei (Fig. 3C, c), which was possibly caused by unfused GFP that is present at a low level in transgenic cells (detected on Western blots using anti-GFP antibody, data not shown) and that can freely pass through nuclear pores (Haseloff et al. 1997). Even after 20 minutes of observation under the confocal microscope, the ratio between perinuclear, cytoplasmic and intranuclear GFP-signals did not change in the control, with the GFP signal remaining strongest in the cytoplasm (Fig. 3D, d). This suggests that the reduction in the GFP signal observed during recovery at 25°C after chilling was caused by an active exclusion of tubulin from the nucleus and not due to laser-induced bleaching of GFP during observation.

Tubulin cofractionates with nuclei from cold-treated cells

The immunofractionation and in vivo analyses showed that tubulin accumulated in the nuclei of cold-treated cells. To challenge this finding, protein extracts from isolated nuclei of the BY-2 cell line BY-2-mGFP5-ER (Petrášek et al. 2002) were analyzed by Western blotting using monoclonal antibodies recognizing α-tubulin
in general, and tyrosinated α-tubulin in particular (for details refer to Wieser et al. 2002). Whereas tubulin could not be detected in control nuclei, it was clearly present in nuclei from cold-treated cells (Fig. 4A, B). The nuclear signal of α-tubulin and its tyrosinated form increased progressively with the duration of chilling (Fig. 4A, B, compare 8 and 12 hours), in agreement with the increased frequency of tubulin-containing nuclei found in the immunofluorescence study (Fig. 1G). Fig. 4C shows an amido black stain of proteins separated by SDS-PAGE and blotted in parallel under the same conditions.

Furthermore, we tested whether the tubulin signal found in extracts from cold-treated nuclei was possibly caused by contamination with non-nuclear, but sedimentable, proteins. The transgenic cell line BY-2-mGFP5-ER used in this experiment constitutively expresses mGFP5 fused at the N-terminus to the signal peptide from Arabidopsis thaliana basic chitinase and at the C-terminus to the ER-retention signal (the HDEL peptide) (Petrášek et al. 2003). This so-called mGFP5-ER fusion protein has been previously shown to localize to the endoplasmic reticulum (Haseo et al. 1997 and Fig. 5A) and thus it can serve as a marker for perinuclear membrane structures. Nuclei were isolated from BY-2-mGFP5-ER cells that had been cultivated under standard conditions, and were examined under the epifluorescence microscope. No GFP signal was found in these isolated nuclei (Fig. 5B, C). When different fractions from the isolation procedure were tested by Western-blot analysis using an anti-GFP antibody, the GFP signal was clearly present in the first supernatant, but absent in fraction IV that contained the purified nuclei, irrespective of whether they had been isolated from control or from cold-treated cells (Fig. 5D). Nevertheless, nuclei isolated from cold-treated cells by the same protocol contained abundant tubulin (Fig. 4A, B).

Both tubulin molecules contain putative nuclear export sequences

Tubulins lack at least the canonical nuclear import signals (data not shown). We screened various sequences of plant α- and β-tubulins for nuclear export sequences (NES). We identified two putative NES in the α-tubulins and 3 in the β-tubulins (Table 1). Moreover, according to our preliminary studies, the potential NES motifs 2a and 1–3β are also well conserved between plant and animal tubulins, whereas the NES 1α seems to be plant-specific (data not shown).

Discussion

We observed that tubulin accumulated in interphase nuclei during the response to prolonged chilling that progressively eliminated cortical microtubules, and that this phenomenon was fully reversible when the temperature was restored to 25°C. Intranuclear accumulation of tubulin during chilling and its exclusion during re-warming was shown by immunofluorescence in situ, as well as in vivo using transgenic cell lines expressing GFP-α-tubulin. Another transgenic cell line that expresses a GFP-marker for endoplasmatic reticulum and nuclear envelope (mGFP5-ER) was used for the detection of tubulin in isolated nuclei. Nuclear preparations that were void of cytoplasmic contamination (as evident from the complete removal of the GFP-signal), nevertheless contained tubulin, but only when the nuclei had been prepared from cold-treated cells. No tubulin signal was detected in nuclear preparations from cells that had not been exposed to low temperature.

Hitherto, reports of intranuclear tubulin have been scarce. This indicates that the separation of interphase and mitotic microtubular functions is correlated to a strict compartmentalization of tubulin. Intranuclear tubulin has been reported for the mouse cell line SV3T3 (Menko and Tan 1980). More recently, non-assembled αβ-tubulin heterodimers have been detected in cultured rat kidney mesangial cells (Wallis et al. 1999). The β1 isoform was present exclusively in nuclei and could be reversibly removed from the nuclei by vinblastine treatment (Wallis-Bass et al. 2003). The authors suggested that nuclear tubulin constitutes a pool of dynamic tubulin that might assist the rapid cell proliferation characteristic of cancer cells. In plant cells, intranuclear microtubule-like structures have been documented in Aesculus hippocastanum L. using electron microscopy (Barnett 1991). However, the function of these intranuclear microtubules remained enigmatic. In our study, the tubulin was apparently not present in the form of microtubules, but as non-assembled tubulin-heterodimers.

Although intranuclear tubulin seems to be a fairly exotic phenomenon in animal and plant cells, it is quite common in primitive eukaryotes with closed mitosis, where the division spindle develops within the karyoplasm surrounded by a nuclear envelope. This type of mitosis is frequently found in certain protists, fungi and algae (for review see Heath 1980). There is a mounting body of evidence that tubulin is imported into the nucleus along with components of MTOCs just prior to the onset of mitosis (Aspergillus nidulans, Ovechkin et al. 2003; spindle-pole body of Saccharomyces cerevisiae, Pereira et al. 1998). The precise timing of this nuclear import with respect to the cell cycle suggests that, in these organisms, the presence and absence in the nucleus of cytoskeletal components that participate in mitosis is carefully regulated and represents an important checkpoint within the cell cycle. In animal and plant cells, the disassembly of the nuclear envelope that results in an
immediate and perfect exposure of chromatin to tubulin (open mitosis) has evolved during evolution as a more effective mechanism. However, even under conditions of open mitosis, some components of the spindle-regulation machinery are imported into the nucleus prior to mitosis. For instance, γ-tubulin already appears in the nucleus of *Vicia faba* in the G2 phase (Binavová et al. 2000). It is important to note that the antagonistic process – active exclusion of αβ-tubulin heterodimer from the nucleus – must occur at the transition from M to G1, when the daughter nuclei become enclosed by a new nuclear membrane, not only in cells with closed mitosis, but also in those with open mitosis. Moreover, independently of the respective type of mitosis, all interphase cells must efficiently exclude αβ-tubulin from the nucleus or prevent its entry, because it is not found in interphase nuclei. These facts suggest that, in spite of substantial differences between open and closed mitosis, some mechanisms, such as the import of cytoskeletal components regulating polymerization of microtubules at the beginning of mitosis and export of tubulin at the end, must be preserved in all organisms in order to ensure proper cell cycle progression.

It remains to be asked how tubulin is actually kept out of the interphase nucleus. We show in the present work that this mechanism is impaired during chilling at 0°C. Transport between the cytoplasm and the nucleus is mediated by NPCs residing in the nuclear envelope. Transport of molecules through the nuclear pores can be either non-selective (i.e. passive) or selective (i.e. active, mediated by numerous protein transporters). Passive diffusion is limited by the exclusion size of the NPC, which is approximately 50 kDa (for review see Talcott and Moore 1999). Although molecules of up to 60 kDa are in principle allowed to passively diffuse through NPC, the diffusion rate is very low for molecules over 50 kDa (Rose et al. 2004). Therefore, it is unlikely that αβ-tubulin heterodimers (110 kDa) can pass the pores without specific interaction with the nuclear transport machinery. The massive increase in the pool of free tubulin dimers in the cytoplasm as a consequence of cold-induced elimination of assembled microtubules (Pokorná et al. 2004) might simply overload highly selective transport through the nuclear envelope such that tubulin "leaks" into the nucleus. However, an excess of free tubulin dimers generated by other means (oryzalin treatment, inhibiting the assembly of dimers into microtubules) does not produce such an entry of tubulin into the nucleus. This suggests that intranuclear tubulin is specific to the cold response. Since low temperature affects biological membranes (Kacperska 1999; Orsar et al. 2000), a possible explanation would be that cold-induced membrane injuries allow a slow leakage of tubulin into the karyoplasm. However, passive leakage of tubulin into the karyoplasm occurring as a consequence of membrane damage cannot explain its striking accumulation in the karyoplasm resulting in a clear concentration gradient in response to chilling. The accumulation of intranuclear tubulin coupled with a much lower concentration in the surrounding cytoplasm suggests that it binds to an unknown intranuclear lattice. The affinity to this lattice seems to be rather strong since tubulin remained attached even during the isolation of nuclei.

The entry of tubulin into the nucleus seems to be perfectly reversible when the cells are re-warmed, because it is not detectable in nuclei within minutes of recovery, as evident from immunofluorescence microscopy results as well as from live cells expressing GFP. Since this process is so rapid, it is unlikely that tubulin disappearance reflects degradation; the half-life of tubulin has been shown to be cell-cycle dependent, varying between 1 and 15 hours in *Physarum* (Daicomann and Wright 1989). Even degradation of regulatory proteins (enzymes) usually takes much longer. A specific mechanism that excludes tubulin from the karyoplasm and is strongly (but reversibly) inhibited by low temperature must be involved in the recovery processes. Rapid and complete exclusion of tubulin during the first minutes of re-warming is unlikely to be caused by simple diffusion, since the temperature dependency of diffusion is relatively low. Moreover, we observed that the intranuclear tubulin is rich in tyrosinated α-tubulin indicating that this has not been incorporated into stable microtubules and thus might represent a highly dynamic reservoir for the assembly of new microtubules (Wiesler et al. 2002).

The rapid recovery of tubulin exclusion from the nucleus demonstrates that the membranes of the nuclear envelope are not damaged or perturbed irreversibly. The target of chilling seems rather to be the NPCs and indicates that import and/or export of tubulin must be somehow regulated. Tubulin lacks at least the canonical NLS (Ovechkina et al. 2003 and unpublished results). It is clear, however, that the standard pathways of protein import must be complemented by additional import mechanisms, since several proteins lacking an obvious NLS can enter the nucleus in both plants and animal cells (Evans et al. 2004). Interestingly, the intranuclear tubulin in cultured rat kidney mesangial cells reported by Walls et al. (1999) entered nuclei only if the cells underwent mitosis (Walls-Bass et al. 2001). The authors suggested that tubulin was trapped by nuclear factors at the end of mitosis rather than being actually transported through the nuclear envelope. However, we searched for putative NES in tubulin molecules and identified 5 in the α- and β-tubulins. Though the functionality of these nuclear export signals must be confirmed experimentally, their existence is consistent with a model where tubulin is exported through the nuclear pores by means of the conventional nuclear-transport machinery, most of the components of which, such as importins, exportins, Ran, RanGAP and others (for review see Dasso 2002), have also been identified in higher plants (Smith and Raikhel 1999; Merkle and Nagy 1997; Pay et al. 2002).

We interpret the presence of tubulin in the nuclei of cold-treated cells in the following way: Tubulin is exclusively cytoplasmic during interphase, probably because its presence in the interphase nucleus could induce
serious disorders of cell-cycle regulation. We hypothesize that the integrity of the nuclear membrane during interphase, when nuclear pores control the transport of molecules, is the main mechanism that ensures the sequestration of tubulin to the cytoplasm during interphase. The interaction of tubulin with chromatin is crucial during mitosis. For closed mitosis, tubulin is imported into the nucleus by an unknown mechanism, whereas for open mitosis, no tubulin transport through the nuclear membrane is required since the nuclear envelope breaks down at the onset of mitosis. However, when daughter nuclei are formed at the end of mitosis, new nuclear envelopes have to be reestablished and the pool of tubulin molecules from the disintegrated spindle has to be removed from the karyoplasm. At this stage, tubulin is excluded from nuclei, possibly utilizing the NES export signals conserved in all tubulins. The integrity of the nuclear envelope and the active transport through the nuclear pores are impaired during cold treatment, resulting in intranuclear accumulation of tubulin. In our future work, we would like to verify the functionality of the different NES signatures in tubulin and to identify the corresponding binding partners of the nuclear transport machinery in both the cyto- and the karyoplasm.

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Figure legends

Figure 1 α-tubulin accumulates in the nuclei during the response to chilling (0°C). Microtubules (A, A', B, B', C, D, E, F) were visualized by immunofluorescence using a monoclonal anti-α-tubulin antibody and a TRITC-conjugated secondary antibody, and DNA was stained with Hoechst 33258 (A'', B'', C'', E'', F'') at 0°C (A'--A''), 1°C (B--B''), 1°C (C', E', F') and 12 hours (D) of chilling, and after 11 hours of treatment with 5 μM oryzalin (F, F''). Cells were observed by conventional epifluorescence microscopy (B--C, E, F') and confocal laser scanning microscopy (A--A'', D, F and F''). A--A'', B and B'' show two focal planes, one cortical (A, B) and one central (A'', A'', B' and B''). D shows a central confocal optical section, the arrow indicates position of the nucleus. E and E' show a residual mitotic spindle around spiraled metaphase chromosomes. F and F' show four merged optical sections (Z step size 1 μm) through central regions of cells. Bar, 20 μm. (G) Frequency of cells with intranuclear tubulin during cold treatment. Average values, n=200 cells per sample.

Figure 2 Intranuclear α-tubulin disappears simultaneously with the reconstitution of cortical microtubules during re-warming. Tobacco BY-2 cells were subjected to chilling for 7 hours at 0°C and subsequently returned to 25°C. Microtubule images are shown in A, B, C--C'', D, E, F, F'. DNA images in A', B', D', E', F'. For details of staining refer to Figure 1. Interphase nuclei after 7 hours at 0°C (A, A') and after 2°C (C--C''), 10°C (B, B'') and 30 minutes (F, F', F'') of recovery. Cells in early G1 (D, D') and a reconstituted mitotic spindle (E, E') are shown after 5 minutes of recovery. Cells were observed by conventional epifluorescence microscopy (A--B', D--F'). Sequential images were recorded by confocal laser scanning microscopy from a Z-stack taken after 10 hours chilling at 0°C and subsequent re-warming at 25°C for 2 minutes (C--C''). Partial recovery of microtubules can be seen in the cortical region as well as on the nuclear surface (optical sections Nos. 1 and 7). However, intranuclear tubulin is still detectable at this stage of recovery (optical sections No. 19 and 28). Bar, 10 μm.

Figure 3 Exclusion of tubulin from nuclei during recovery in vivo. (A, B) Tobacco cells expressing GFP-tubulin were cultivated at 0°C for 8 hours and then subsequently at 25°C for 20 minutes. (A) Confocal optical section in the nuclear region of cells cultivated for 8 hours at 0°C. (B) The same confocal optical section of the same cell after 20 minutes of recovery at 25°C. The intensity of the GFP signal was maximal in nuclei of cold-treated cells (a) suggesting that tubulin accumulated in the nuclei during cold treatment. Tubulin was excluded from the nuclei during recovery at 25°C and accumulated in the surrounding cytoplasm (b). (C, D) A control cell expressing GFP-tubulin kept at 25°C throughout the experiment. Confocal optical section in the nuclear region (C) at time 0, and after 20 minutes (D). The intensity of the GFP signal was maximal in the cytoplasm surrounding nuclei (c), and this distribution of GFP fluorescence did not change during the 20 minutes of observation (d, compare with c). Vac = vacuole, cyt = cytoplasm, nuc = nucleus.

Figure 4 Detection of tubulin in protein extracts from isolated nuclei. (A, B) Immunoblot stained with antibodies directed against α-tubulin (A) and tyrosinated α-tubulin (B). (C) Amido black stain of proteins separated by SDS PAGE and blotted in parallel under the same conditions. 15 μg of total protein were loaded per lane.

Figure 5 Isolated nuclei are free from non-nuclear contamination. (A) The tobacco cell line BY-2-mGFP5-ER expressing mGFP5 fused at the N-terminus to the signal peptide from Arabidopsis thaliana basic chitinase and at the C-terminus to the ER-retention signal HDEL. A projection of eight optical sections (Z step size 0.6 μm) through a central part of cell is shown. DIC image (B) and GFP signal (C) of a nucleus isolated from a cell expressing mGFP5-ER maintained under control conditions. (D) Immunoblot detection of GFP protein in fractions collected during the isolation of nuclei. Fraction I: Supernatant from the first centrifugation at 2000 g. Fraction II: Nuclei before loading onto the Percoll gradient. Fraction III: Supernatant from the last centrifugation at 2000 g. Fraction IV: Sediment from the last centrifugation at 2000 g (purified nuclei). 10 μg of total protein were loaded per lane. Note that all GFP remained in fraction I. Bar, 10 μm.
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Summary

This PhD is based on four published papers, whose common aim is the investigation of the role of microtubules in the response of plant cells to abiotic stress. Two abiotic stresses were studied that represent important limiting factors for crop productivity on acid soils that comprise 40% of arable land in the world (in case of aluminum toxicity) and areas of the temperate climate zones (in case of cold stress). In published papers, the dynamics of plant microtubules during stress response was studied, with special interest in the most early phases of stress influence. It has been suggested that considering very rapid changes of the microtubular cytoskeleton during early phases of the stress response, microtubules could play a role in the transduction of signals triggered by the stress of Al ions and low temperature. Recent results available in the literature confirm this hypothesis. Therefore, signalization during both stresses seems to have one common important characteristic: interphase cortical microtubules as elements mediating transduction of signals from the plasma membrane and as modulators of Ca^{2+} channels or regulators of downstream effects at the same time.

Further, we have reported about the incidence of cold-induced plant tubulin in the interphase nucleus. The discovery of cold-induced nuclear tubulin enabled us to build a hypothesis that the transport of tubulin through the nuclear envelope occurs during the cell cycle that might be functionally relevant for the dynamic changes in the nuclear envelope at the beginning and the end of the mitosis. The dynamics of plant tubulin during the cell cycle progression, the role of potential NESes and the potential role of nuclear tubulin in signalization will be further studied, with special emphasis on in vivo visualization techniques.