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 VBI-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* — VBI-0.

Abbreviations: cMTs, cortical microtubules; DIC, differential interference contrast; DMSO, dimethylsulphoxide; EGTA, ethylene glycol-bis(beta-aminoethyl ether)-*N*,*N*,*N′*,*N′*-tetraacetic acid; FDA, fluorescein diacetate; FITC, fluorescein isothiocyanate; MSB, microtubule-stabilising buffer; MTs, microtubules; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid).

Introduction

Aluminum (Al) ions released from soils under low pH conditions have been recognised as a major factor limiting plant growth in acid soils. The molecular base 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 (Sasaki et al. 1997b, Blancaflor et al. 1998). The root tip with actively dividing and elongating cells seems to be 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 Al^{3+} ions enter and bind to the apoplast (Horst 1995), and change the properties of the plasma membrane (Wagatsuma et al. 1995). Most of the apoplastic Al is bound to the pectin 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 (Horst et al. 1999). Moreover, the greatest proportion of cytosolic 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 Martin 1988).

Al-induced effects on root growth have been attributed to putative interactions between Al ions and the cytoskeleton, since Al^{3+} ions were shown to affect microtubular polymerisation 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 (Sasaki et al. 1997b). Similarly, the cytoskeleton of maize roots was reorganised and stabilised 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 stabilised depending on cell position and the time of exposure to Al (Sivaguru et al. 1999a).

Generally, these cytoskeletal changes caused by Al^{3+} 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 homogenous 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, Ikegawa et al.
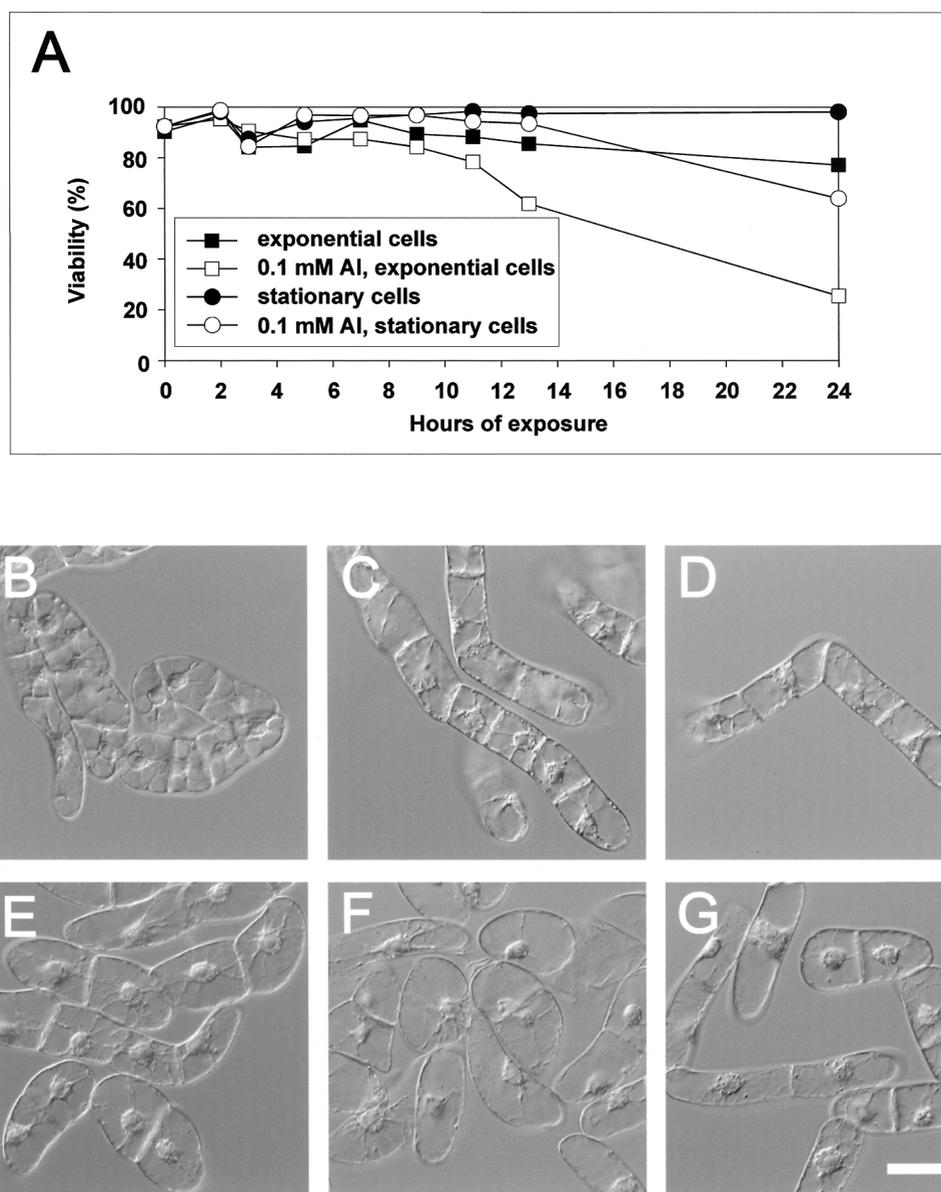
Fig. 1 Viability and phenotype of tobacco cell line during the exposure to Al. (A) Viability of exponential and stationary cells during 24 h of exposure to 0.1 mM $AlCl_3$, pH = 4.5 in 3% sucrose. Both controls were grown in 3% sucrose, pH = 4.5. (B, C, D) Phenotype of exponential cells after 11 h of exposure to full (MS) medium (pH = 5.8), 3% sucrose (pH = 4.5) and 0.1 mM $AlCl_3$ in 3% sucrose (pH = 4.5), respectively. (E, F, G) 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 $AlCl_3$ 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, Vitorello and Haug 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 (Sivaguru et al. 1999b). The physiological base of this difference 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 (Opatrný and Opatrná 1976) 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.
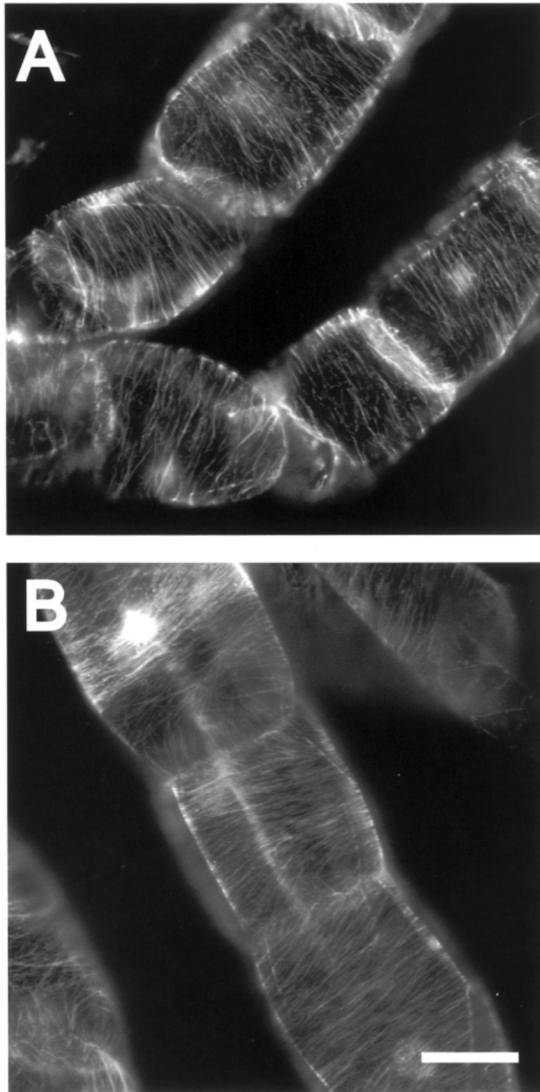
complex CCT). In contrast, prolonged time of exposure to Al ions resulted in extensive disorganisation of cMTs and decreased levels of α-tubulin, tyrosinated α-tubulin, and CCTε.

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 cultures. Actively dividing cells that formed multi-cellular files 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 files 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 minimal medium without Al, the viability 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 conspicuously, when 100 μM of AlCl$_3$ were added to the minimal medium (Fig. 1A). This decrease initiated earlier (from 11 h after the addition of Al) and was more pronounced in exponential cells as compared to stationary cells. But till 10 h after the addition of Al, the cells were observed to remain perfectly viable maintaining morphology and intracellular structure (Fig. 1D, G).

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).

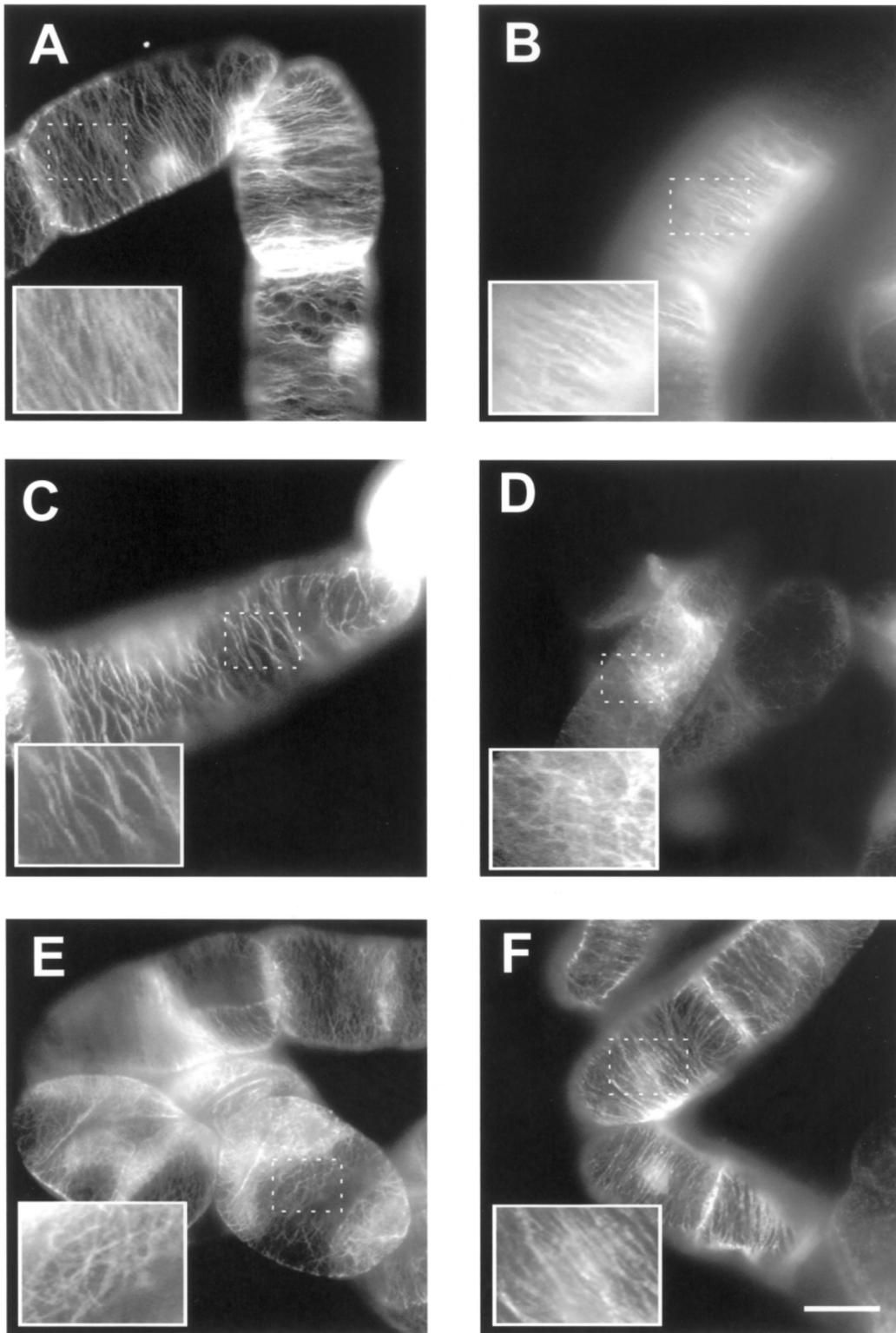Later, from about the sixth h of exposure, cMTs became

Fig. 2 MTs of exponential VBI-0 cells after the exposure to Al. (A) Transversally oriented cortical MTs in control cells grown in full medium. (B) Cortical MTs in cells treated with 0.1 mM AlCl$_3$ in 3% sucrose for 2 h. Cells were stained with anti-α-tubulin antibody and FITC-conjugated secondary antibody. Bar, 40 μm.

1997b), the distribution of post-translationally modified tubulins (Smertenko et al. 1997a), and the co-localisation of MTs with HSP90 (Petrášek et al. 1998) or elements of the tubulin-folding chaperone CCT (Nick et al. 2000) have been characterised 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 CCTε (a subunit of the tubulin-folding chaperonin

Fig. 3 MTs in the exponential BY-2 cells after the exposure to Al. (A) Cells grown in the full medium. (B, D and E) Cells grown in 0.1 mM AlCl$_3$ for 1, 6 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, 20 μm.

A

Percentage of number of MT bundles per 100 μm of lenght

B

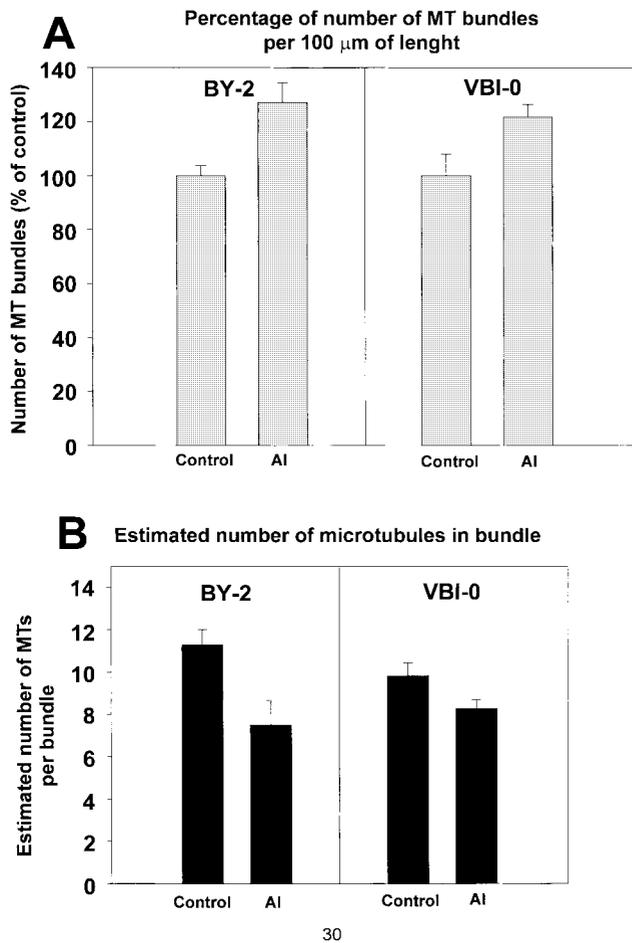**Estimated number of microtubules in bundle**

30

Fig. 4 Response of cortical MTs in BY-2 and VBI-0 cells grown for 1 h in 3% sucrose, pH = 4.5 (controls) and in 3% sucrose, 0.1 mM AlCl$_3$, pH = 4.5 (Al). Frequency of microtubular bundles in the cortical region per unit of length was evaluated. The values obtained in control cells were set to 100%. (B) Estimated number of individual MTs per bundle. For details, see Material and Methods. Error bars represent coefficients on the confidence level 95%.

progressively disoriented in Al-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 Al (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 Al. Within 15 min of treatment, microtubular bundles were found to be

affected in both control and Al-treated cells (Fig. 5). However, in the Al-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 Al

To understand the Al-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 Al-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 Al 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 Al.

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

The increase in the number of MT bundles in response to Al 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 Al and gradually returned to the levels observed in the controls cultivated on minimal medium without Al.

Discussion

The inhibition of root growth by Al ions seems to be based mainly upon the toxicity of Al to cell division and elongation in the root tip (reviewed in Delhaize and Ryan 1995 and Kochian 1995). The cytoskeleton has been shown to be affected in the roots treated with Al (Grabski and Schindler 1995, Sasaki et al. 1997a), and this effect occurs even after short exposure to Al (Blancaflor 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 Al 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 cytologically well-defined plant cell culture BY-2 and VBI-0 were used as an experimental material for evaluation of role of cytoskeleton in Al toxicity.

Because the complex growth media make it difficult to predict what Al-species occur in what effective concentration, we used a minimal medium at low pH such that Al^{3+} ions were the prevalent species. Consistent with previous results for VBI-
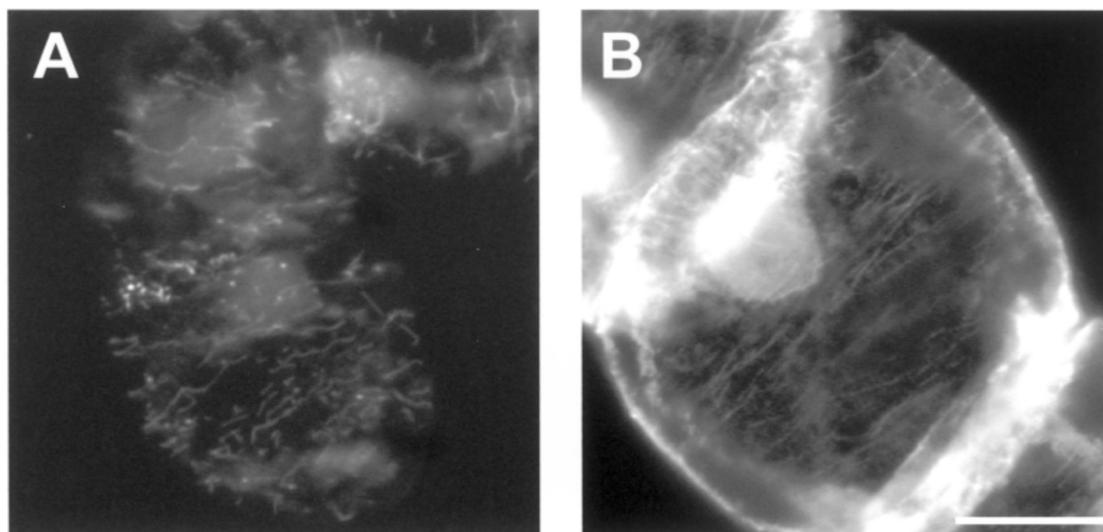
Fig. 5 MTs in exponential BY-2 cells treated with 1.5 μM oryzalin after 1 h of exposure to the Al-toxic medium. (A) Control cells grown in full medium treated with 1.5 μM oryzalin for 15 min. (B) Cells treated with 0.1 mM AlCl$_3$ for 1 h and subsequently with 1.5 μM oryzalin for 15 min. Cells were stained with anti-α-tubulin antibody and FITC-conjugated secondary antibody. Bar, 20 μm.

0 (Zazímalová 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 Al ions 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 (Vitorello and Haug 1999). The reduced sensitivity of stationary cells might by related to their reduced secretory and metabolic activity.

The microtubular response was rapid (Fig. 2, 3) and preceded the decrease of Al on viability by several hours (Fig. 1). Studies on Al uptake in cell cultures (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 but not the only cellular structure influenced by Al. The actual cause for cell death has remained obscure so far.

The microtubular response to Al during the first hours of exposure (Fig. 2, 3) involved an increase in the number of MT 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 esti-

mated 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 the same cell line (Sonobe et al. 2001). This congruence justifies the conclusion that Al 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 depletions 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 Al (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 (Morejohn et al. 1987). However, the Al-induced additional MTs remain sensitive to oryzalin (Fig. 5). A reduced turnover of tubulin subunits is expected to decrease the tyrosination of α-tubulin. The enzyme responsible for detyrosination 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 detyrosination. The level of tyrosination does not decrease; it increases significantly (Fig. 6). It is therefore more likely that the additional MTs formed in response to Al originate from elevated tubulin synthesis rather than increased stability of MTs.
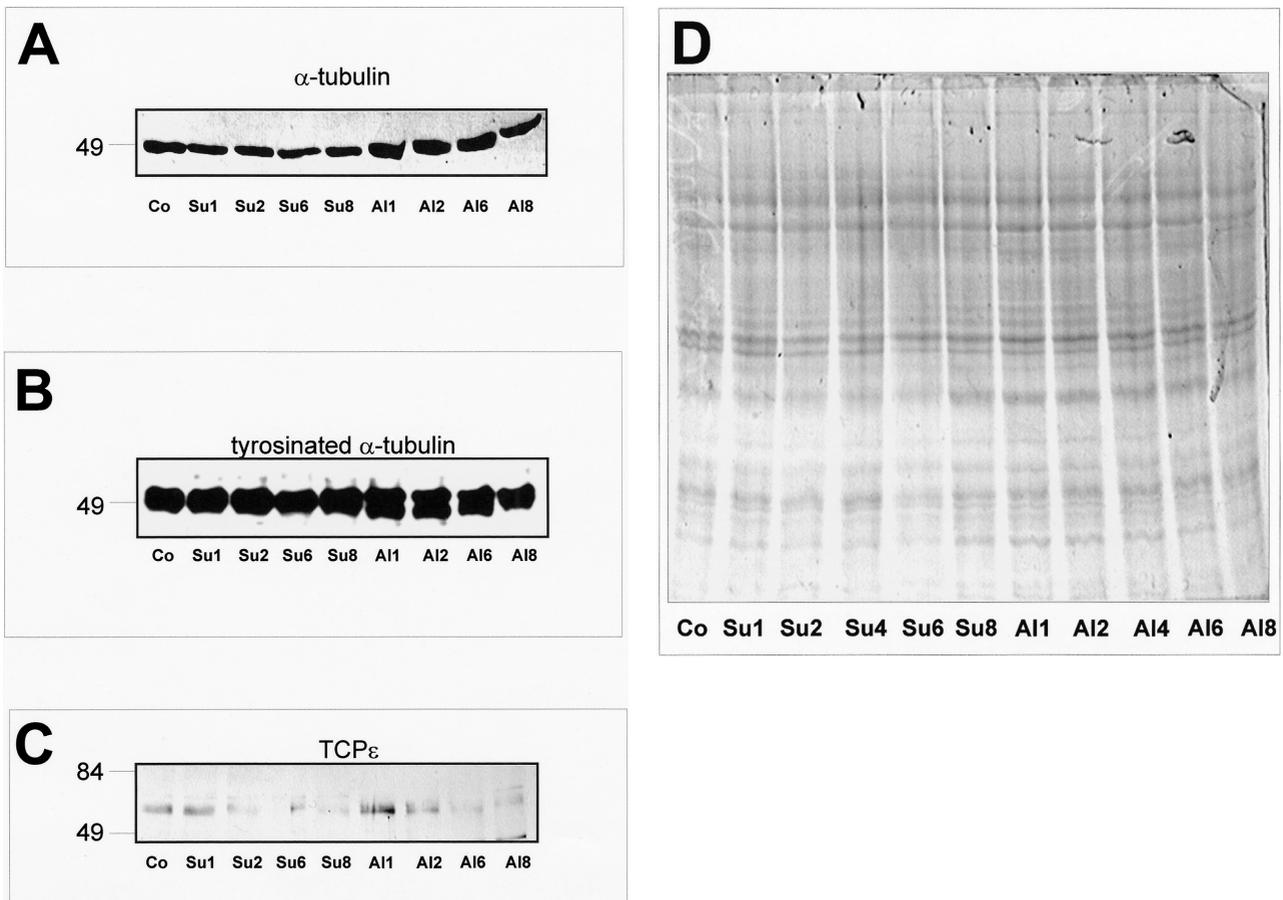
Fig. 6 Changes in the tubulin and CCTε content in the total protein extract of the BY-2 cells. (A,B,C) Immunoblots of the total protein extracts from cells exposed to 0.1 mM AlCl$_3$ in 3% sucrose, pH = 4.5 (Al), or 3% sucrose, pH = 4.5 (Su), compared with the control BY-2 cells grown in the full medium (Co). The numbers indicate the exposure time (hours). (D) Coomassie stained proteins separated by SDS-PAGE on 10% acrylamide gel after the determination of protein concentrations.

An additional mechanism might be responsible for the additional MT-bundles acting in BY-2 cells. The quantification of MT thickness (Fig. 4B) allowed estimation of the number of individual MTs per bundle. The congruence 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 Al reduces the extent of cMT-bundling. It is therefore possible that Al 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 extensive disorientation and depletion of fine MTs occurred. These changes represented the second phase of the cellular response to Al 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 (Sasaki et al. 1997b, Blancaflor et al. 1998) and maize after 6 h of exposure (Sivaguru et al. 1999a). Since the deposition of newly synthesized cellulose microfibrils into the cell wall is a microtubular-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 Al.

This two-phase pattern of the microtubular response to Al 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 base for the increased number of MTs in response to Al remains to be elucidated. The data presented in this study, however, rule out certain mechanisms. Al was discussed to compete with magnesium ions for the GTP-binding site of tubulin (Macdonald 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 (Bayley 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 synthesis (as seen in the elevated level of α-tubulin) and folding (as seen in the elevated level of CCTε) by Al-triggered signaling. The additional tubulin dimers would then be assembled into new MTs. This either means that Al induces additional sites of nucleation or that MT nucleation is not limited by nucleation sites, but by the level of available tubulin dimers. These mechanisms seem to be supported by a reduced number of individual MTs comprised into one bundle (Fig. 4B). This effect could arise from an interaction of Al with factors that influence organisation of MTs in the cortical region. MT-associated proteins that bundle MTs (Chan et al. 1999, Marc et al. 1996, Durso and Cyr 1994, Vantard et al. 1991) or microtubule-associated proteins that connect MTs with the plasma membrane (Sonobe 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 Al stress. Upon prolonged exposure to Al, the proper organisation 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 to understand the signals that regulate the synthesis of new tubulin subunits and the role of different tubulin isotypes in response to Al stress.

Materials and Methods

Plant material

The tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow) cell line (Nagata et al. 1992) was cultured in Murashige-Skoog medium (Murashige and Skoog 1962) supplemented with 1 μM of 2,4-dichlorophenoxyacetic acid (2,4-D). Every 7 d 1.5 ml of cells were transferred to 30 ml of the fresh medium and cultured on a horizontal shaker at 25°C in the dark.

The tobacco VBI-0 (*Nicotiana tabacum* L. cv. Virginia Bright Italia) cell line (Opatrný and Opatrná 1976) was cultured on 3% agar (Sigma, Steinheim, Germany) in modified Heller's medium (Heller 1953) supplemented with 5 μM of 1-naphtylacetic acid (NAA) and 5 μM of 2,4-D as a stock callus culture. To obtain a suspension culture, an inoculum was suspended in liquid Heller's medium with 5 μM of NAA and 5 μM of 2,4D and cultured on a horizontal shaker at 25°C in the dark. The subculture interval was 14 d and the inoculation density was 5×10^4 cells ml^{-1}.

Treatment of cells with Al

The cells in exponential (3-day-old) or stationary (5-day-old) phases of the culture were washed in a solution of 3% sucrose in a Nalgene bottle-top filter for 5 min and re-suspended in 3% sucrose supplemented with various concentrations of $AlCl_3$ (diluted from a 1 M stock solution) at pH 4.5. The final density was 5.10^4 cells ml^{-1}. The pH was adjusted immediately before the experiments.

Treatment of cells with oryzalin

Surflan (40.4% oryzalin, Elanco products Co., U.S.A.) was added directly to a final concentration of 1.5 μM of oryzalin.

Quantification of cell viability

The viability of cells was assessed using fluorescein diacetate (FDA). Aliquots of 40 μl of a 0.2% w/v stock solution of FDA in acetone were diluted in 7 ml of culture medium and at least 500 cells were counted in each sample immediately after the addition of FDA.

Indirect immunofluorescence

MTs were visualised as described in Wick et al. (1981) with modifications described in Mizuno (1992). Briefly, fixation of cells in 3.7% PFA (Serva) in MT stabilisation buffer (MSB: 50 mM PIPES, 2 mM EGTA, 2 mM $MgSO_4$, pH 6.9) for 50 min was followed by fixation in 3.7% PFA and 1% Triton X-100 in MSB for 20 min. After treatment with an enzyme solution (1% macerozyme and 0.2% pectinase) for 7 min, the cells were attached to coverslips coated with poly-L-lysine (Sigma, Steinheim, Germany) and treated with 1% Triton X-100 for 20 min. Subsequently, unspecific binding was blocked with 0.5% bovine serum albumin (BSA, Fluka, Steinheim, Germany) and incubated with a monoclonal mouse antibody against α-tubulin (TU-01, Viklický et al. 1982) for 45 min, diluted 1 : 500 in PBS. After washing with PBS, a secondary FITC-conjugated anti-mouse antibody (Sigma, Steinheim, Germany) diluted to 1 : 128 in PBS was applied for 1 h. The cells were washed with PBS and stained with 0.1 μg ml^{-1} Hoechst 33258 (bisbenzimide, 2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5′-bi-1H-benzimidazole trihydrochloride pentahydrate; Sigma, Steinheim, Germany) in PBS. The specimens were washed in PBS and embedded in Mowiol (Polysciences, Warrington, PA, U.S.A.) solution. All procedures were performed at room temperature (25°C).

Microscopy and image processing

The cells were observed using an epifluorescence microscope (Olympus Provis AX 70) equipped for Nomarski differential interference contrast (DIC). For observation of Hoechst fluorescence the filter set with excitation at 330–385 and a 420 nm barrier filter was used. For observation of FITC fluorescence the filter set with excitation at 450–480 nm and 515 nm barrier filter was used. Pictures were collected and processed using the image analysis Lucia G/F system (Laboratory Imaging Prague, Czech Republic).

Evaluation of number of microtubules in the cortical region

Visualization by immunofluorescence detects and distinguishes bundles of MTs rather than individual MTs. In order to quantify the response of MTs to Al, we used 10 interphase cells with transversal MT arrays that had been labeled by immunofluorescence. Microtubular bundles in the cortical region were scanned under identical settings of CCD integrating camera and the image analysis system Lucia G/F. The images were converted to TIFF files and analyzed using the Scion Image analysis software (Scion Corporation, Frederick, MD, U.S.A.). Averaged density profiles of fluorescence were sampled for probing lines of 100 pixels standard length and 4 pixel width, whereby the line was oriented perpendicular to the measured MT array (i.e. parallel to the long axis of the cell). The averaging suppressed stochastic background and produced peaks that were approximately linear and symmetrical, which is a prerequisite for the thickness measurements described below. The profiles were plotted as measured density against pixel position and the number of MTs could then be easily counted as number of negative peaks in this graph. From these numbers, the MT frequency per 100 μm could be calculated using a calibration standard. To evaluate the thickness of MT bundles, the first derivative of the density profile was plotted against pixel position and was subtracted by the absolute value of this derivative. The result of this algorithm plotted against pixel position will produce a non-zero negative value at the transition from an interspace between two MTs to the flank of a MT, whereas it will be zero at the transition from the center of the MT to the next interspace or within the interspace. Under conditions of linearly changing and approximately symmetric density values, the dis-

tances between the zero values of this graph estimate the average thickness of the MT bundles in pixels, which could then be recalculated into thickness in μm. Electron-microscopical studies of bundles of cortical MTs that had been isolated from BY-2 cells (Sonobe et al. 2001) show that the individual MTs measure 25 nm in diameter and that the spacing between two individual MTs amounts roughly to 30 nm. Using these values, it is possible to express the measured thickness of a bundle in terms of estimated number of individual MTs per bundle (see Fig. 4).

Electrophoresis and immunoblotting

The Al-treated and the control cells were harvested by filtration through nylon mesh and homogenized in liquid nitrogen using mortar and pestle. The frozen powder was then mixed with an equal volume of extraction buffer (50 mM Tris-HCl, pH 6.8; 2% SDS; 36% w/v urea; 30% v/v glycerol; 5% v/v mercaptoethanol; 0.5% w/v Bromphenol Blue), vortexed for 1 min, boiled for 3 min, and centrifuged at 13,000 rpm and 4°C for 5 min. The supernatant was transferred into a new tube and re-centrifuged at 13,000 rpm and 4°C for 5 min. The resulting supernatant was defined as total protein extract and stored at –20°C until use. The total protein concentration was determined directly after staining with amidoblack (Popov et al. 1975). After boiling for 3 min, samples were separated by SDS-PAGE on 10% acrylamide gels transferred to nylon membranes and probed for specific proteins as described in detail in Nick et al. (2000). The mouse monoclonal anti-α-tubulin N356 (Amersham-Pharmacia, Freiburg, Germany) was used at 1 : 4,000 dilution, the mouse monoclonal anti-α-tyrosine tubulin TUB-1A2 (Sigma, Neu-Ulm, Germany) at 1 : 800 dilution, and the rabbit polyclonal anti-CCTε (Ehmann et al. 1993) at 1 : 300 dilution were used for the Western blot analysis. Polyclonal antisera against mouse IgG and rabbit IgG conjugated to horseradish peroxidase (both Sigma, Neu-Ulm, Germany) were used to visualise the primary antibodies by bioluminescence as described in Nick et al. (2000).

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