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Aluminum Induces Changes in the Orientation of Microtubules and the Division Plane in Root Meristem of Zea Mays

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Aluminum (Al) induces agricultural problems limiting crop productivity in acid soils. Since Al causes morphological changes in roots, and because microtubules (MTs) play important roles in determination of tissue morphology, we investigated whether Al affects the arrangement of MTs in maize root meristem using immunolocalization techniques. When seedling roots were treated with 50 μM Al, the orientations of MTs were dramatically altered in a population of cells located in the protoderm and the two outer layers of cortex: interphase cortical MT arrays lost their normal transverse organization and became random or longitudinal; the preprophase band of MTs, mitotic spindle, and phragmoplast developed at planes 90° rotated compared to their counterparts in controls. These changes in MT orientation resulted in the change of the division plane from transverse to longitudinal, producing daughter cells positioned side by side instead of above and below. The rotation of the otherwise normal MT arrays and the division plane in Al-treated roots indicates that Al interferes with the normal polarity sensing mechanism, which may contribute to the reduced axial growth of the Al-treated roots.

Keywords: Aluminum, cell division, microtubule, polarity, root tip

INTRODUCTION

Aluminum (Al) is the most abundant metal in the earth's crust, comprising about 8% of its mass. As soils become acidic by acid precipitation and application of acidic fertilizers for agriculture, phytotoxic forms of Al are solubilized into the soil solution to levels that affect plant growth. Thus, Al is the growth limiting factor in acid soils (Foy, 1988). In humans, Al has also been implicated as an etiological agent in the pathogenesis of various neurodegenerative disorders such as Alzheimer's disease (Doll, 1993). The most easily recognized symptom of Al toxicity in plants is the inhibition of root growth (Taylor, 1991), which results in inefficient absorption of nutrients and water. The root apex is the primary site of Al-induced root growth inhibition (Rincon and Gonzales, 1992; Ryan *et al.*, 1993), but the type of the cell in the tissue affected and the mechanism of the inhibition are not clearly understood.

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The toxicity of Al may be a consequence of adverse effects on a variety of cellular pathways (Johnson and Jackson, 1964; Foy *et al.*, 1978; Huang and Kochian, 1992; Ryan *et al.*, 1993; Jones and Kochian, 1995). Al-injured roots show many morphological changes, such as tip swelling and lateral root deformation (Foy *et al.*, 1978), suggesting an Al-induced alteration in the cytoskeleton. Supporting this possibility, Al has been shown to induce a rapid and dramatic increase in the rigidity of the actin network in suspension-cultured soybean cells (Grabski and Schindler, 1995), to strongly promote tubulin assembly into microtubules *in vitro* (Macdonald *et al.*, 1987), and to disrupt the stability of MT in elongating cells of wheat root (Sasaki *et al.*, 1997). However, there has been no report yet of Al-induced alterations of microtubules in the root meristem, the major site of Al toxicity. Thus, we investigated whether the arrangement of microtubules is influenced by Al in maize root meristem. Our results show that Al changes the orientations of MTs and division plane, and suggest an inhibition by Al of cell polarity signal as the mechanism of the changes.

MATERIALS AND METHODS

Plant Material

Maize (*Zea mays* L. cv. Golden Cross Bantam) seeds were sterilized for 15 min with 0.5% calcium hypochlorite, washed for 15 min and soaked for 10 h in deionized water. They were germinated on damp filter paper for 3 d at 22°C.

Growth Conditions

Germinated seeds with straight 1- to 2-cm primary roots were cultivated hydroponically. The seeds were placed on a plastic mesh which was floated on 1.2 L of aerated nutrient solution containing 200 μM $CaCl_2$ solution (pH 4.5). After 10-14 h, seedlings were transferred to the chamber containing nutrient solution (200 μM $CaCl_2$, 100 μM KCl, pH 4.5±50 μM $AlCl_3$) and incubated with constant aeration.

Fixation and Embedding

After Al treatment, apical segments (3~5 mm) of primary roots were excised and fixed with 4% paraformaldehyde, 10% DMSO and 0.1% Triton X-100 in 5 mM EGTA/50 mM phosphate buffer for 1.5 h at room temperature. They were then rinsed in phosphate buffer four times for 1 h. Root segments were infiltrated with mixtures of 20% sucrose in PBS plus O.C.T. compound (Tissue-Tek, Elkhart, USA) made up in the proportions of 2:1, 1:1 and 1:2 (v/v) for successive steps which lasted 2 h each, then with absolute O.C.T. compound for 8 h. Root segments prepared in this way were frozen onto cryo-disks (Reichert-Jung, Germany) and stored at −20°C.

Indirect Immunofluorescence Microscopy

Longitudinal sections of 10~15 μm thickness were cut using a microtome (2800 Frigocut, Reichert-Jung, Germany) at −20°C. Median sections were mounted on slides coated with poly-L-lysine. Sections were treated for 15 min with 1% cellulase solution containing 0.4 M mannitol, 1% Triton X-100, and 0.3 mM PMSF, all of which were made up in phosphate buffer. Following a 15 min rinse, the sections were extracted with 1% Triton X-100 in phosphate buffer for 20 min. After another 15 min rinse, they were incubated with mouse monoclonal antibody raised against chick brain tubulin (Amersham, Buckinghamshire, UK) diluted 1:50 in PBS for 1.5 h at 37°C. After this rinse with PBS, the sections were stained with FITC-conjugated anti-mouse IgG raised in goat (Sigma Chemical Co, St. Louis, MO, USA), diluted 1:200 in PBS for 1 h at 37°C. Nuclear DNA was counterstained with Hoechst 33258 (0.5 mg L^{-1}) for 5 min. After rinsing in PBS, the sections were finally mounted with anti-fade mountant containing 90% glycerin, 10% PBS (pH 8.9 adjusted with $NaHCO_3$) and 0.1% *p*-phenylenediamine.

Fluorescence was examined using a fluorescence microscope (Nikon Optiphot-2) equipped with filter blocks of a narrow band pass (excitation 465-495, barrier Ba515-555). Photographs of fluorescent images were taken on Tmax 400 film using a photographic attachment (Nikon Microflex UFX-DX).

Measurement of Root Growth

After 10~14 h of growing the germinated seeds, the roots of individual maize seedlings were placed in a narrow chamber containing nutrient solution (200 μM $CaCl_2$, 100 μM KCl, pH 4.5±50 μM $AlCl_3$) and incubated with constant aeration. Root growth was determined using a video-camera and a monitor connected to a dissecting microscope (magnification X70) over a period of 4 h by measuring the distance between the tip of the root and a reference spot which was marked with water-proof ink 1 mm distal from the tip at the beginning of Al treatment. Hoechst staining of dividing cells confirmed that the mark remained in the meristematic zone during the duration of the experiment.

RESULTS

Effect of Al on the Arrangement of MTs throughout the Cell Cycle of Root Meristematic Cells

Root protodermal cells of maize have a very flat rectangular shape, but their interphase cortical MTs were transversely oriented to the root axis (Fig. 1A) as have been reported (Baluska *et al.*, 1992). This is unlike many other types of plant cells which align their cortical MTs transverse to the long axis of the cell during interphase (Hush and Overall, 1996). In maize root cells approaching their mitotic stage, the interphase array of cortical MTs is replaced by a band of MTs, the preprophase band (PPB) was localized transversely to the root axis at the site bisecting the flat cells (Fig. 1B).

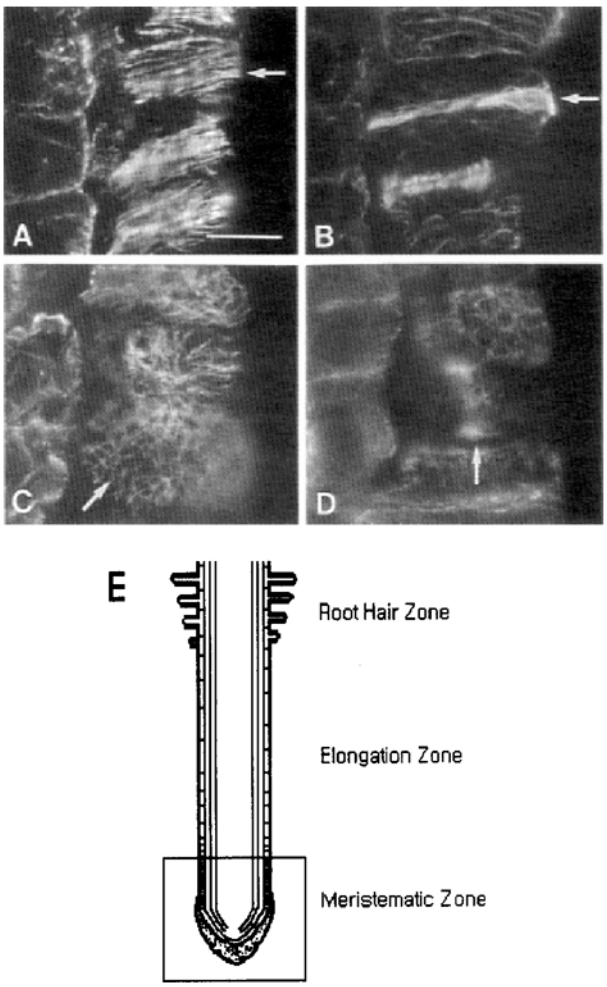

Fig. 1. Interphase arrays of MTs in protodermal cells of the meristematic zone of maize roots 4 h after the onset of Al treatment. In control cells, interphase cortical MTs (A) and PPB (B) were aligned transversely to the root axis. On the other hand, in Al-treated cells, interphase MTs were random (C) and PPB was altered to parallel orientation to the root axis (D). Theses changes were observed only in the 3 outermost layers of the maize root (E) meristem (encased in a rectangle), thus only the 3 layers are shown in this and all other figures. Bar represents 10 μm and applies to all figures.

Treatment of seedlings with 50 μM Al for 1~4 h induced marked changes in MT orientation at the root apex. Some protodermal cells showed interphase MTs arranged randomly (Fig. 1C) instead of the normal transverse orientation (Fig. 1A). The PPB MTs of protodermal cells were rarely observed. However, when labeled, they were also altered to be longitudinal, thus being parallel to the root axis (Fig. 1D). In outer layers of root cortex, in addition to cells showing transversely-oriented MT arrays as those in control samples (Fig. 2A), there were cells

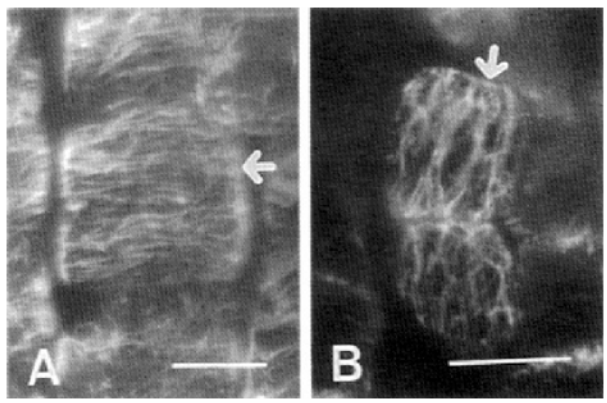

Fig. 2. Interphase arrays of MTs in outer cortical cells of the meristematic zone of maize roots 4 h after the onset of Al treatment. In control cells, the orientation of interphase MTs was transverse (A), but in Al-treated cells it was longitudinal (B). Bars represent 10 μm.

with interphase MTs aligned longitudinally (Fig. 2B).

During the mitosis of control cells, the spindle is formed with its axis parallel to the root axis (Fig. 3A), and the orientation of chromosomes in the same cell, observed using double staining with Hoechst dye, was transverse to the root axis (Fig. 3B). At the end of mitosis the phragmoplast of control cells were organized transverse to the root axis (Fig. 3C) and the separated chromosomes were positioned above and below of each other (Fig. 3D).

Al induced changes in the arrangement of MTs during mitosis as well. The mitotic spindle of which axis became transverse to the root axis was observed only in Al-treated cells (Fig. 3E); i.e. metaphase spindle was rotated by 90°. The orientation of metaphase plate in these cells was parallel to the root axis (Fig. 3F). The rotation of mitotic MTs was also apparent in the phragmoplast; it was parallel to the root axis (Fig. 3G) with telophase DNAs positioned side by side (Fig. 3H). Thus, when cell division was completed, daughter cells were produced side by side (Fig. 4, A and B).

Interestingly, the changes in the arrangement of various MTs throughout the cell cycle of root meristematic cells were confined to the protoderm and the two outermost layers of cortex (Fig. 1E) and were not observed in any other layers of the root tissue.

Time-dependent Occurrence of Al-induced Longitudinal Division

Cells in the protodermal layer were chosen to

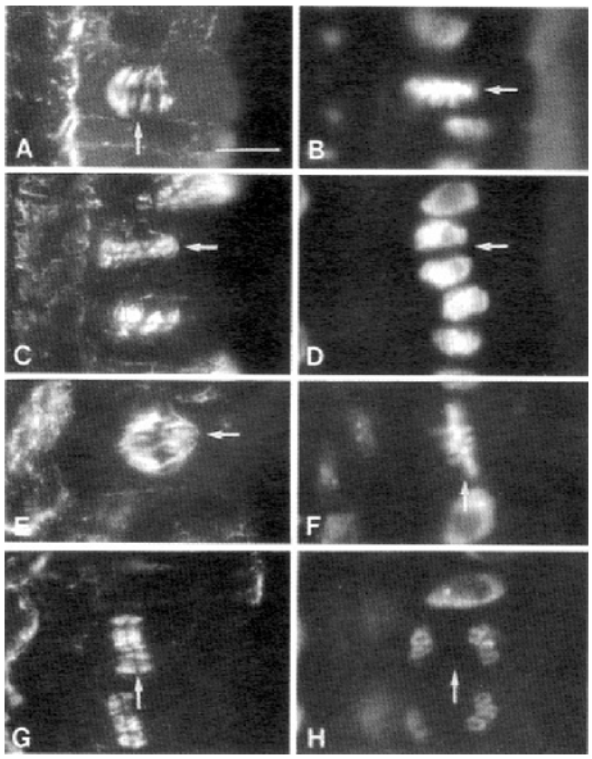

Fig. 3. Organization of mitotic MTs and DNAs in protodermal cells of control (A, B, C, D) and Al-treated (E, F, G, H) maize roots. Metaphase spindle (A, E) and phragmoplast (C, G) at telophase are indicated with arrows. Note that the axis of metaphase spindle was changed from parallel to transverse orientation to the root axis (A, E). Hoechst staining of DNA in the same cells showed the rotation of the plane of chromosome alignment during metaphase (B, F). Phragmoplast was rotated by 90° after Al treatment (C, G). Hoechst staining of the same cells shown in C and G (D, H) revealed different positioning of the translocated DNA in control and Al-treated protoderms. Maize roots were treated with 50 μM Al for 4 h. Bar indicates 10 μm and applies to all figures.

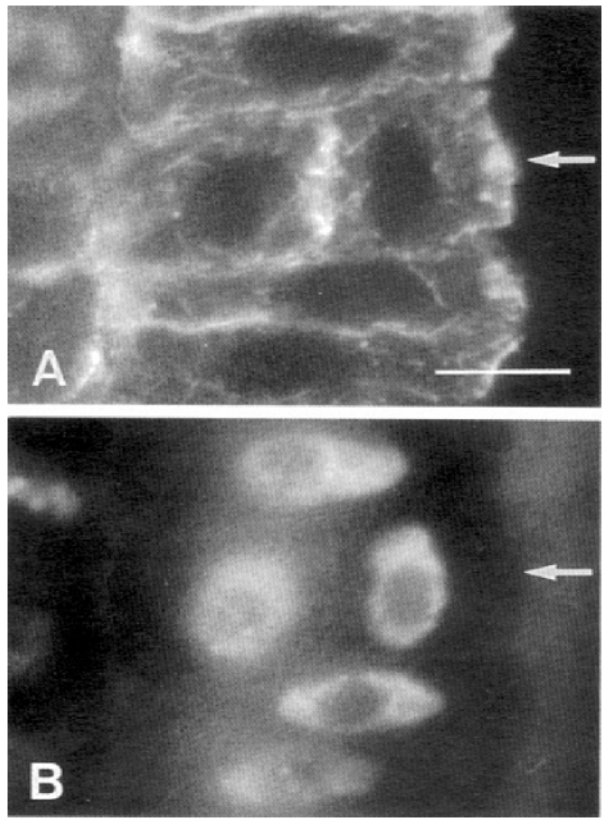

Fig. 4. Daughter cells positioned side by side after mitosis in the protoderm of root exposed to 50 μM Al for 4 h. Both immunostaining of MTs (A) and Hoechst staining of nucleic acids (B) of the same cells showed juxtaposed daughter cells. Bar indicates 10 μm and applies to both A and B.

quantify the percentage of cells that finished longitudinal division and those with 90°-rotated orientations of PPB and mitotic MTs. Daughter cell pairs produced side by side were 0.2±0.3 (Average±SE), 4.3±0.7 and 6.3±1.4% of total protodermal cells at

Table 1. Time-course of Al-induced longitudinal division in protodermal cells of maize root. Cells in the outermost layer of a median section of root meristem from the tip to the farthest dividing cell were included in the count. Means±SE of 10 independent experiments are shown

Time (h)	Total # of protodermal cells		# of dividing cells		% of longitudinally dividing cells in Al-treated protoderm[a]
	Control	+Al	Control	+Al	
1	231.1±6.1	236.3±4.7	10.4±0.5	10.8±0.5	0.8±0.8
2	226.7±3.9	234.0±5.6	11.9±0.6	11.2±0.7	6.6±2.5
3	242.4±6.8	233.7±8.0	12.9±0.8	12.3±0.6	14.5±2.2
4	227.4±9.6	197.9±7.6	11.0±0.9	7.8±0.7	45.2±4.1
5	234.4±9.5	141.3±8.3	12.5±0.5	2.1±0.5	80.8±7.2

[a] $\frac{\#\text{ of longitudinally dividing cells}}{\#\text{ of total dividing cells}} \times 100$

3, 4 and 5 h after Al treatment, respectively, showing the progressive induction of longitudinal cell division by Al. Cells with rotated orientations of MTs, the longitudinal PPB, transverse mitotic spindle or longitudinal phragmoplast, were noticeable as early as 1 h after the onset of Al treatment, and comprised up to 45.2% and 80.8% of the total dividing cells in the protoderm after 4 and 5 h of Al treatment, respectively (Table 1). Total division rate also dropped, but the change lagged behind that of the division plane; it was apparent at h 4 of Al treatment. The division rate decreased so much in Al-treated root meristem that at h 5 dividing cells were only rarely observed.

Effect of Al on the Growth of Maize Root Meristem

Longitudinal cell division by Al would be predicted to affect root growth in the meristem. Although inhibition of root growth by Al has been reported (Van *et al.*, 1994; Jones and Kochian, 1995; Sasaki *et al.*, 1996), existing results reflect mainly the effects of Al on the elongation of the cells in the elongation zone and not on the meristem of the root. Therefore, we investigated the effect of Al on the growth of the meristematic region of maize root. The growth of the meristematic region proceeded slowly (about 8 μm/h) compared to that of the elongation zone (about 70 μm/h). When roots were exposed to Al, the reduction in the growth of the meristematic region became apparent after 2 h of treatment, and the extent of the growth was 73% of the control after 4 h of Al treatment (Table 2).

DISCUSSION

Al-induced Longitudinal Division

The plane of cell division has been reported to change during normal developmental processes (Lang Selker and Green, 1984; Hush and Overall, 1996) as well as upon wounding or mechanical stress (Hardham and McCully, 1982; Lintilhac and Vesecky, 1984; Hush *et al.*, 1990), although the mechanism of the regulation remains unknown. We show in this paper that rotation of the division plane occurs in response to Al, a common environmental stress, in root meristematic cells.

Cells in maize root protoderm are very flat in their shape. However, they are programmed to divide transversely and thus maintain axial growth of the root. The mechanism by which these extremely flat cells achieve transverse division is not understood, although studies in other organisms suggest the influence of the previous division plane or cell wall asymmetry on the determination of the plane of new division (Rhyu and Knoblich, 1995; Quatrano and Shaw, 1997). In case of asymmetric division, the plane of division is determined by intrinsic marks of dividing cells or by extrinsic influences (Way *et al.*, 1994; Rhyu and Knoblich, 1995; Quatrano and Shaw, 1997). Analogous determinants or marks for the transverse division may exist in protodermal cells, and Al may obscure their signals. In the absence of integral guidance required for transverse division, the shape of cells may govern the plane of division as proposed previously (Barlow and Adam, 1989; Oud and Nanninga, 1992), i.e., tall cells tend to divide transversely, while flat cells divide longitudinally.

Actin filaments may be important for the Al-induced change of the division plane since they play critical roles in establishing cell polarity (Bouget *et al.*, 1996), and their physical properties have been reported to change in response to Al (Grabski and Schindler, 1995). Moreover, actin antagonists cause abnormal orientations of division planes (Palevitz and Hepler, 1974; Cho and Wick, 1990), supporting the roles of actin filaments in determination of division plane. Al may also interact with some components of cell wall (Van *et al.*, 1994; Sasaki *et al.*, 1996), interfering with the normal function of cell wall in relaying positional information (Quatrano and Shaw, 1997).

Table 2. Effect of Al on the growth of meristematic region of maize roots. Average±SE (n=5, unit=μm) of increase in the distance from the root tip to the dot marked at 1 mm from the tip at t=0 is presented. Three other separate experiments gave similar results

Time (h)	Control	Al-treated
0	0	0
2	15.0±1.7	6.4±2.4
4	25.1±2.7	18.2±0.5

Al-induced Changes in MT Orientation

Our study shows that organizations of all four distinct MT arrays which appear sequentially during the cell cycle of plant cells are affected by Al. Since the temporal and spatial formation of MTs in plant cells are governed by microtubule organizing centers

(MTOCs) (Cyr and Palevitz, 1995) as in other eukaryotes, changes of MT alignment in Al-treated roots may be resulted from changes in the location of MTOCs. It is remarkable that each configuration of MT arrays, except the random interphase MTs of protodermal cells, appears normal except that the planes of MT arrays are shifted 90° to those of control cells. This observation indicates that functions of MTOCs as entities that initiate and organize MTs are still fulfilled, but the position of MTOCs is now rotated. Then, what controls the sites for MTOCs? Recently, several genes are identified for their roles in establishing cell polarity (Rhyu and Knoblich, 1995). Al may disrupt expression of the genes or function of the gene products involved in positional sensing, thus obscure the direction of polar axis and change the cell-specific location of MTOCs. Polarity sensing mechanism is a new aspect of plant morphogenesis which is affected by Al, to the best of our knowledge.

Mitotic spindles of which axis become transverse under Al stress could be deviated from their normal sites due to malfunction of actin filaments. Al affects the physical aspect of actin filaments (Grabski and Schindler, 1995), and induces accumulation of mRNAs of fimbrin-like protein, an actin bundling protein (Cruz-Ortega *et al.*, 1997). It is intriguing that fimbrin is required for actin organization and proper cell division in yeast (Adams *et al.*, 1991). In addition, changes in cellular Ca^{2+} by Al (Huang and Kochian, 1992; Ryan *et al.*, 1993; Jones and Kochian, 1995; Huang *et al.*, 1996) may also disturb actin organization by affecting activities of actin binding proteins such as gelsolin, and in turn the orientation of MTs. Since MTs are also sensitive to Ca^{2+}, MT alignment may be directly altered by Al-inhibition of Ca^{2+} homeostasis.

Longitudinal Cell Division as a Possible Cause of Al-induced Inhibition of Root Growth

Al-induced longitudinal cell division may contribute to the root growth retardation found in Al-treated seedlings, because longitudinal division prevents insertion of new cells in a cell file. We envisage two arguments against this possibility. First, since in some cases observed during leaf development, the division pattern did not alter the overall leaf morphology (reviewed in Smith, 1996), it may be argued likewise that the reduction in cell number may be compensated by changes in the length of the individual cells. However, it is unlikely, since the cells in the elongation zone of the Al-treated roots are flatter than their counterparts in control (Sasaki *et al.*, 1996). Therefore, the changes we observed in the meristem is most likely to have a direct influence on the growth of the root as a whole organ.

Second, since cells with altered division planes are confined to the protoderm and the two outermost layers of cortex, which appears to be caused by the difference in Al concentration which is higher in the outer layers (Eeckhaoudt *et al.*, 1992), it may be argued that longitudinal division in only these three cell files is insufficient to inhibit growth of the whole organ. However, in the case of young shoots and coleoptiles, the outer epidermis forms the growth-limiting tissue of the organ (Went and Thimann, 1937), i.e. these cell layers on the outside have a high wall tension relative to inner tissues, and inner tissues are constricted in intact organs by the peripheral cell layers. Therefore, it is possible that the difference in length of Al-treated and control root tips derives, at least in part, from the Al-induced rotation of the division plane in the three external cell files and the consequent restraint on global root tip growth.

Factors other than the rotated division plane may also contribute to the observed reduction in the growth of maize root meristem by Al treatment (Table 2): reduced division rate and possible reduction in elongation of the individual meristematic cells. Al-induced decrease in the division rate has been reported in root meristem of several plant species (Clarkson, 1965; Horst *et al.*, 1983; Tepper *et al.*, 1989). However, since the growth of the root meristem is reduced as early as 2 h after the onset of the treatment, before the division rate begins to drop (Table 1), the reduction in division rate cannot be an important reason for the reduced growth at the early stage of Al-treatment. In contrast, Al-induced rotation of the division plane is shown from h 1 of the treatment, which suggests its possible role in initial reduction of root tip growth by Al. Since the elongation of the cells of the elongation zone is reduced in response to Al (Sasaki *et al.*, 1996), Al may also inhibit the elongation of individual meristematic cells, which would contribute to the initial reduction in the growth of meristem.

Ethylene also induces root swelling and retardation of growth (Baskin and Williamson, 1992; Ecker, 1995), symptoms typical to Al-treated roots. Therefore we tested if ethylene may also induce longitudinal division in root apex, but treatment for 4 h with 1 mM ACC, an ethylene precursor, did not

rotate the division plane (data not shown). Therefore, ethylene is not involved in the mechanism of rotation of cell division by Al.

In conclusion, throughout the cell cycle of root meristematic cells, Al causes rearrangement of MTs to directions which support longitudinal cell division, and this rotation of the otherwise normal MT arrays suggests interference by Al of the polarity control mechanism. Our results also suggest that the Al-induced longitudinal cell division may contribute to the Al-induced reduction in axial growth of root.

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