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FORMATION OF ATYPICAL TUBULIN STRUCTURES IN PLANT CELLS AS A NONSPECIFIC RESPONSE TO ABIOTIC STRESS

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Abstract

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In response to abiotic and biotic stress factors the production of reactive oxygen species (ROS) in plant cells is increased and series of cascade reactions for their neutralization are being triggered. Targets of ROS in cells can be various biochemical and physiological processes, as well as cellular organelles including the cytoskeleton. Many abiotic stress factors, by provoking increase of the level of ROS in plant cells, elicit reorganization of the microtubules leading to disruption of mitosis. The reorganization of interphase and mitotic microtubule system was proposed to be an integral part of the response of cells to external stimuli. The state of microtubule system (tubulin cytoskeleton) under various abiotic stresses, including salinity, low temperatures, Al toxicity and flooding, was analysed in cells of representatives of family Solanaceae (*S. lycopersicum* L., *S. tuberosum* L. and *N. tabacum* L.) and cereals (*T. aestivum* L. and *H. vulgare* L.) by using transmission electron microscopy. Various tubular structures that were structurally different from microtubules were observed in plant cells subjected to abiotic stress. Fibrillar bundles with different densities of their constituent components, macrotubules, orderly packed paracrystalline aggregates and strands having hexagonal packing in cross sections, were observed in potato leaf mesophyll cells under low positive temperature stress, wheat root cells subjected to flooding stress, barley root cells under Al toxicity stress, as well as in root cells of tomato and tobacco under salinity stress imposed by NaCl or Na_2SO_4. The structure of these cytoskeletal elements is identical to stable atypical tubulin polymers induced by different chemical treatments. The presented data suggests that the formation of abnormal tubulin structures in plant cells is a common and non-specific response to various abiotic stress factors.

Key words: tubulin, cytoskeleton, microtubule, stress condition, ultrastructure

Introduction

The mechanism of effective protection against damaging effects of oxidative stress, caused by the formation of excessive amounts of reactive oxygen species (ROS) in plant cells is one of the topic issues of plant physiology. ROS are integral part of normal cell metabolism. They are important signaling molecules involved in regulation of many physiological processes associated with plant growth and development. In response to abiotic and biotic stress the production of ROS in plant cells is increased and series of cascade reactions for their neutralization are being triggered (Kreslavski et al., 2012). The targets for ROS in plant cells are various biochemical and physiological processes

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as well as cellular organelles (Baxter et al., 2014), including microtubules (Livanos et al., 2015). For example, many abiotic stress factors, by causing elevation of ROS level in plant cells, induce reorganization of microtubules (tubulin cytoskeleton) followed by the mitotic abnormalities (Livanos et al., 2012a; Livanos et al., 2012b). It has been suggested that reorganization of tubulin cytoskeleton is a part of the response to external stimuli and is coordinated by interactions with phospholipase D, phosphatidic acid, p38-like mitogen – activated protein kinase (p38-like MAPK) and ROS (Livanos et al., 2014). Experimental increase or decrease of the ROS level in plant cells by application of menadione and N -acetylcystein initially led to microtubule disassembly. Subsequently, abnormally shaped tubulin structures – macrotubules are formed in cells with low levels of ROS, whereas in cells with high level of ROS, paracrystalline tubulin strands are observed. (Livanos et al., 2012b). It has been suggested that when the ROS homeostasis in the cell changes, the tubulin cytoskeleton switches to another state by assembly of atypical tubulin structures (Livanos et al., 2014).

However, up to date there is no evidence that similar changes in tubulin cytoskeleton can occur under the influence of any abiotic stress factor. In this paper, using the transmission electron microscopy we analyzed the state of tubulin cytoskeleton in cells of *Solanaceae* species (*N. tabacum* L.*, S. tuberosum* L. and *S. lycopersicum* L.) and cereals (*H. vulgare* L. and *T. aestivum* L.) under various abiotic stress conditions – salinity (NaCl and Na_2SO_4), low positive temperatures, Al toxicity and flooding.

Materials and Methods

Plant materials were *in vitro* regenerated tomato (*S. lycopersicum* L.) cv. Beliy naliv, potato (*S. tuberosum* L.) cv. Skoroplodniy and tobacco (*N. tabacum* L.) cv. Samsun, wheat (*T. aestivum* L.) cv. Nemchinovskaya 24 and barley (*H. vulgare* L.) cv. Belogorskiy. Plant regenerants were *in vitro* cultivated in nutrient medium containing ½ strength MS (Murashige and Skoog, 1962), supplemented with 2% sucrose and 0.7% agar in a phytotron chamber at 22°C, with illumination of 5.5 klux and LD 16/8 h photoperiod.

For cold stress treatment potato regenerants were kept at + 8°C for 6 days under the same light intensity and photoperiod.

For salt stress treatments, tomato and tobacco regenerants were transferred to tubes containing ½ MS agar supplemented with 96.2 mM NaCl or 76.5 mM Na_2SO_4, to increase the osmotic pressure to 400 kPa and were cultivated in this medium for 8 days.

Flooding stress was imposed by submerging germinated wheat seeds in water without access to oxygen for 36 hours at 24°C.

For Al toxicity stress treatment, 3d old barley seedlings were placed in acidic medium (pH 4.0 adjusted with HCl). Aluminum treatment of 3-d-old seedlings was initiated addition of aluminum sulfate (0.74 mM $Al_2(SO_4)_3.18H_2O$), that brings into correlation with 1.5 mM Al^{3+} for 5-7 days at 21-23 °C (light:dark, 16:8 h) (Baranova et al., 2011).

Root tips of tomato, tobacco, wheat and barley, as well as the middle portion of potato leaves were fixed in 2.5% glutaraldehyde solution (Merck, Germany) in 0.1 M Sorensen's phosphate buffer (pH 7.2) supplemented with 1.5% sucrose. After washing of the fixing solution, samples were incubated for an hour in 1.0% water solution of osmium tetroxide (OsO_4) (Sigma, USA), dehydrated in rising concentrations of ethanol (30, 50, 70, 96 and 100%), propylene oxide (Fluka, Germany) and embedded in Araldite-Epon epoxy resins. Semi- and ultrathin sections were produced by using ultramicrotome LKB- V (LKB, Sweden). Ultrathin sections were contrasted with uranyl acetate and lead citrate ($((C_6H5O_7)_2Pb_3.\ 3H_2O$) according to Reynolds (Reynolds, 1963) and were analyzed using a transmission electron microscope TEM H-500 (Hitachi, Japan). The dimensions of microtubules and atypical tubulin structures were measured with the software "Cell – A" (Olympus, Japan).

Results

We have analyzed the ultrastructure of the cytoskeleton in leaf mesophyll cells of potato regenerants cultivated at normal and low positive temperature, in tomato and tobacco roots under salt stress (NaCl or Na_2SO_4), in root cells of wheat under flooding stress, and in barley exposed to Al toxicity stress.

In interphase cells of the studied plants grown at normal conditions numerous microtubules, arranged in parallel rows close to the cell wall were observed. The average outer diameter of microtubules was 24 nm. Microtubules were detected in the cortical layer of the cytoplasm in both, the longitudinal (Figure 1a, b) and transverse (Figure 1c, d) sections. Under salt stress, in addition to microtubules, some atypical cytoskeletal elements, that were not present in unstressed plants, were observed. Atypical cytoskeletal structures identified in the cells of tomato roots under salt stress imposed by 76.5 mM Na_2SO_4 are shown on Figure 1d.

We analyzed the ultrastructure of atypical cytoskeletal elements in potato leaf mesophyll cells at low positive temperature, in the roots of tomato and tobacco cells subjected to salinity stress, in wheat root cells under flooding stress as well as in barley root cells under Al toxicity stress.

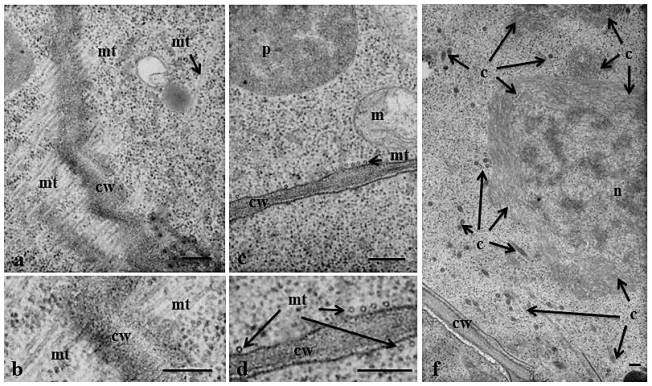

Fig. 1. Ultrastructure of tomato root cells (*Solanum lycopersicum* L.) under control conditions (a-d) and during exposure of 76.5 mM Na_2SO_4 (f); Longitudinal (a-b) and transverse (c-f) microtubule sections (indicated by arrows) located near the cell wall. f - cytoskeletal elements, structurally different from the microtubules (indicated by arrows)
***Legend*: mt - microtubules, cw - cell wall, c - atypical cytoskeletal structures, p – peroxisome, m - mitochondria, n - nucleus. Scale bar - 0.25 microns**

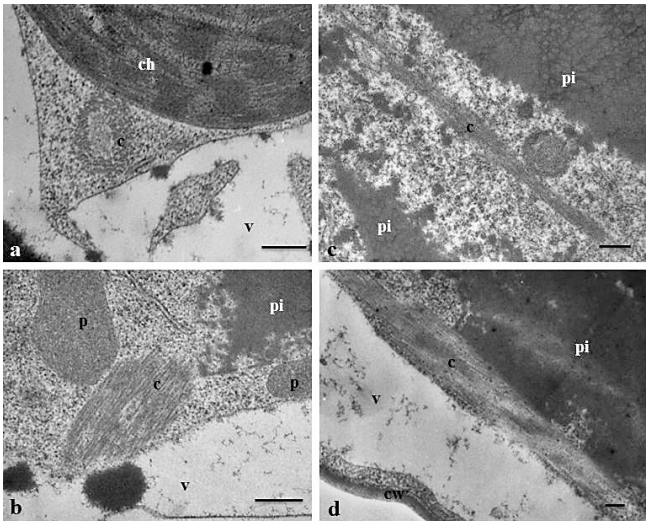

Fig. 2. Atypical cytoskeleton in leaf mesophyll cells of potato regenerants (*Solanum tuberosum* L.) after 6 days of culturing at low positive temperature; Transverse (a) and longitudinal (b-d) sections of strands formed of tubular -fibrillar structures
***Legend*: pi - protein inclusions, v - vacuole, cw - cell wall, p - the peroxisome, chl - chloroplast, c - atypical cytoskeletal structure; Scale bar - 0.25 microns**

It has been shown that exposure to low temperature leads to a complete or partial disassembly of microtubules in plant cells (Lazareva et al., 2008).

Analysis of leaf mesophyll cells from potato regenerants subjected to cold stress did not reveal any microtubules, yet structures consisting of aggregated tubulo-fibrillar components were observed in the cytoplasm. A transverse cross-section of heterogeneous in density tubulo-fibrillar aggregate is shown on Figure 2a. Fibrils of this structure are arranged more loosely in the central part than at the periphery (Figure 2a). On the longitudinal sections, the atypical elements of the cytoskeleton were represented by aggregates consisting of loosely stacked tubular components with an ordered packing (Figure 2b), elongated fibrillar strands (Figure 2c) and tubular structures with an ordered packing but with non-uniform electron density (Figure 2d).

Both, atypical components of the cytoskeleton (Figure 3 a, b, c, d) and typical microtubules (Figure 3b) were present in tomato root cells subjected to NaCl or Na_2SO_4 stress. On Figure 3c the atypical cytoskeletal elements can be seen in both, longitudinal and transverse sections of the same cell. Longitudinal section of the bundle located to the right demonstrates that it consists of a cluster of orderly packed tubular structures. In contrast to microtubules, these tubular structures either have no central lumen or its diameter is much smaller. On the transverse cross sections, the tubular structures composing the bundles have an ordered mesh package typical for tubulin paracrystals observed after colchicine treatment (Apostolakos et al., 1990; Galatis and Apostolakos, 1991; Karagiannidou et al., 1995; Utrilla et al., 1989). Longitudinal bundles with different thickness and packing density of the constituent structures are shown on Figure 3a, d. In addition to the typical microtubules, tubular structures with larger diameter (about 55 nm) were identified in salt (Na_2SO_4) stressed cells. Those are most probably macrotubules, which are another type of abnormal tubulin structures (Figure 3b). The outer diameter of macrotubules we observed matched to that previously reported for macrotubules in colchicine treated cells (Lazareva et al., 2003).

Under salt stress in the cytoplasm of tobacco root cells, strands or bundles of various density, consisting of tubular structures were observed in both longitudinal (Figure 4a, b, f) and transverse (Figure 4c) sections. In the presence of NaCl, long strands with layered structure composed from wavy tubular elements were identified (Figure 4a). On Fig. 4b and d respectively, a tightly packed bundle of tubular elements and a short bundle with loosely packed tubular elements are shown. Transverse cross section demonstrates that the tubular structures of the strands show hexagonal packaging, characteristic

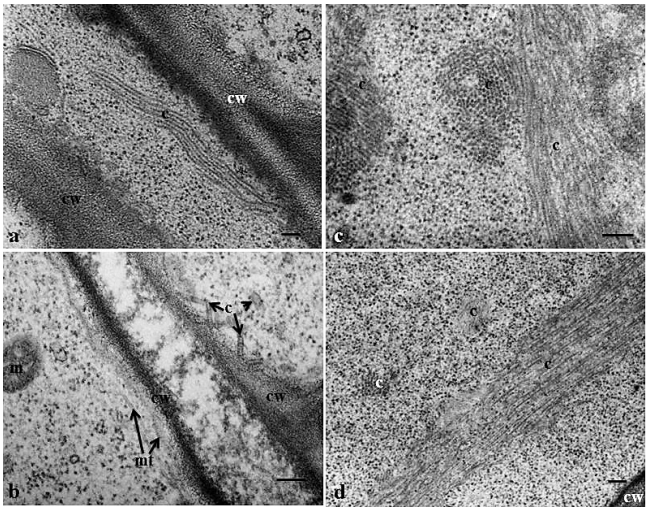

Fig. 3. Atypical cytoskeleton in root cells of tomato (*Solanum lycopersicum* L.) in 96.2 mM NaCl (a) and 76.5 mM of Na_2SO_4 (g-b); Longitudinal (a, c) and transverse (d) sections of tubular-fibrillar strands. b - micro and macrotubules located in neighboring cells (indicated by arrows)

***Legend*: cw - cell wall, m - mitochondria, mt - microtubules, c - atypical cytoskeletal structure;**
Scale bar - 0.25 microns

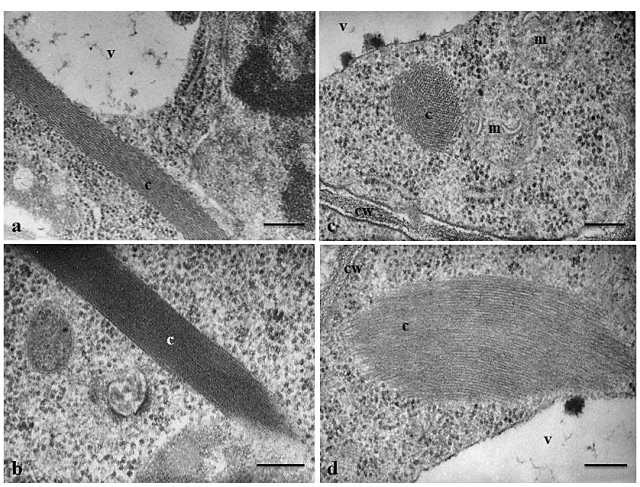

Fig. 4. Atypical cytoskeleton in root cells of tobacco (*Nicotiana tabacum* L.) in 96.2 mM NaCl (a) and 76.5 mM of Na_2SO_4 (b-d); a, b, d - longitudinal sections of strands with different packing density of tubular components; c - the strand cross-section with a typical crystal package form of its components

***Legend*: v - vacuole, cw - cell wall, m - mitochondria, c - atypical cytoskeletal structure;**
Scale bar - 0.25 microns

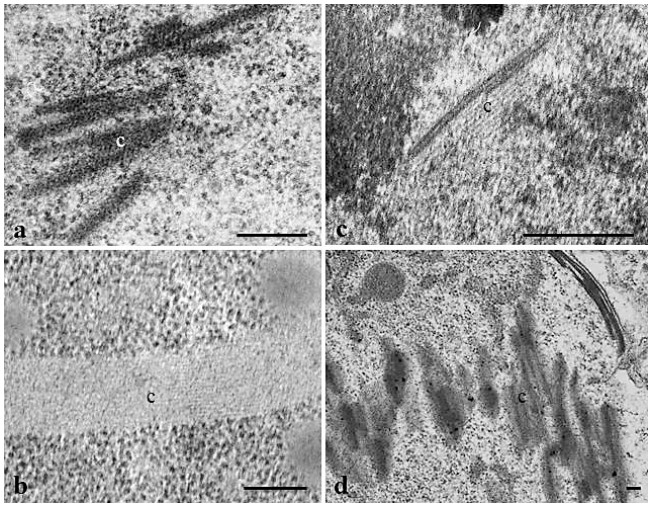

Fig. 5. Atypical cytoskeleton of wheat root cells (*Triticum aestivum* L.) under flooding stress; a, b, c, d – sections of strands with different density packing of tubular components

***Legend*: c - atypical cytoskeletal structure;**
Scale bar - 0.25 microns

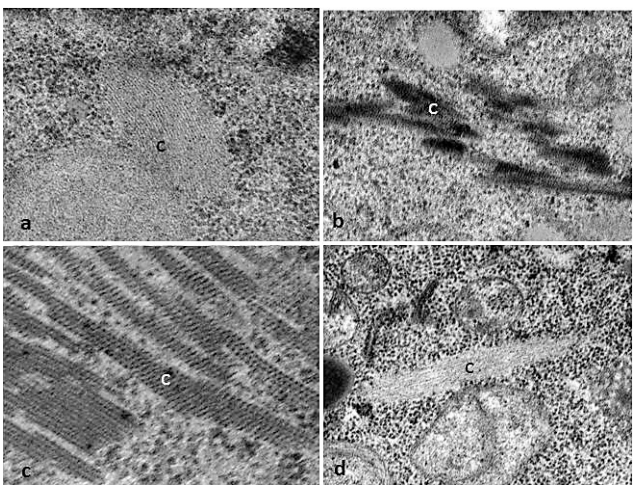

Fig. 6. Atypical cytoskeleton in root cells of barley (*Hordeum vulgare* L.) exposed to Al^{3+} pH 4.0; a, b, c, d - with sections of strands with different packing density of tubular components

***Legend*: m - mitochondria, c - atypical cytoskeletal structure;**
Scale bar - 0.25 microns

for previously described tubulin paracrystals (Apostolakos et al., 1990; Bennett and Smith 1979; Galatis and Apostolakos, 1991; Karagiannidou et al., 1995; Masurovsky and Horwitz, 1989; Starling, 1976; Utrilla et al., 1989). Identical structures were found in root cells of cereals including wheat under

flooding stress (Figure 5) and germinating barley seedlings under aluminum toxicity stress (Figure 6).

Discussion

Homeostasis of ROS plays important role in the regulation of physiological processes in plants. The disruption of ROS homeostasis under adverse environmental conditions launches a series of cascade reactions that can either neutralize ROS or cause irreversible damages and cell death. It has been suggested that in plant response to stress, accompanied by disruption of ROS homeostasis, remodeling of tubulin cytoskeleton plays important role. This process, involves the recruitment of mitogen – activated protein kinase via MAPK signaling cascade as well as regulatory mechanisms that act at both transcriptional and post-transcriptional level (Kreslavski et al., 2012; Baxter et al., 2014).

Formation of atypical tubulin structures (macrotubules and paracrystalline tubulin strands) have been previously observed in root cells of wheat and Arabidopsis under ROS imbalance induced by menadione and N -acetylcystein (Livanos et al., 2014). We suppose that the atypical cytoskeletal structures we have observed in the cells of *Solanaceae* representatives under stress are a tubulin paracrystals.

It has been shown previously, that colchicine treatment leads to formation of atypical tubulin polymers in plant cells. Thus, short term colchicine treatment causes disassembly of cortical and mitotic microtubules, while the prolonged exposure leads to the formation of tubulin structures having heterogeneous ultrastructural organization (Bennett and Smith, 1979; Deysson, 1975). Although these structures are composed of tubulin, they are not organized as microtubules (Apostolakos et al., 1990; Galatis et al., 1991; Karagiannidou et al., 1995; Utrilla et al., 1989) Some of the tubulin structures induced in plants by colchicine treatment (Apostolakos et al., 1990; Bennett and Smith, 1979) are identical to tubulin paracrystals formed in animal cells after treatment with the alkaloids, such as vinblastine and vincristine, produced by plants of the genus *Vinca* (Bensch and Malawista, 1969; Manfredi and Horwitz, 1984; Masurovsky and Horwitz, 1989; Na and Timasheff, 1982; Starling, 1976; Takanari et al., 1990; Takanari et al., 1994). Treatment of *Allium cepa* L. roots with colchicine leads to formation of long tubulin strands in the cortical layer of the cytoplasm only in K-mitotic cells (Utrilla et al., 1989) in root meristem of *Vigna sinensis* L. similar structures were identified only in interphase cells (Apostolakos et al., 1990).

The role and significance of colchicine-induced atypical tubulin structures in the plant cells is not clear. It has been proposed that during recovery period after colchicine treatment, the cells use tubulin deposited in the form of paracrystals as stocks for assembly of normal microtubules (Karagiannidou et al., 1995). Since tubulin paracrystals and macrotubules in wheat cells have been identified during hyperosmotic stress, the authors suggested that the assembly of atypical tubulin-containing structures may be a non-specific reaction and/or a basic response to various damaging factors (Komis et al., 2002). However, the mechanisms of formation of atypical tubulin structures under stress are not known. It is worth noting that under acclimation of plants to stress factors such as cold, the expression of tubulin genes is changing (Christov et al., 2008), that, in turn, might influence the organization of tubulin cytoskeleton.

Depolymerization of cortical microtubules, followed by a new assembly of microtubules and their organization in a stable system was shown in *Arabidopsis* interphase cells subjected to salinity stress. According to the authors of the study, this transient disassembly is related to improvement of plant salt tolerance (Wang et al., 2007; Wang et al., 2011). However, the data obtained using immunocytochemical staining of tubulin in cells, do not answer the question as to what kind of structure is formed after the depolymerization of microtubules. Tubulin paracrystals that are not microtubules have been are recognized not only by using antibodies to tubulin but also to protein MAP65 (Panteris et al., 2010) and gamma-tubulin (Panteris et al., 2000). The data presented here suggests that exposure to various stress conditions (salinity and low positive temperatures) may induce complete and/or partial depolymerization of microtubules, followed by assembly of atypical tubulin polymers. Elucidation of the role of these structures in the plant cell reaction to various abiotic stress factors requires further studies.

Conclusion

In this study the state of microtubule system (tubulin cytoskeleton) under abiotic stresses, including salinity, low temperatures, Al toxicity and flooding, was analysed in cells of representatives of *Solanaceae* (*S. lycopersicum* L., *S. tuberosum* L. and *N. tabacum* L.) and *Gramineae* (*T. aestivum* L. and *H. vulgare* L.) families by using transmission electron microscopy. Various tubular structures that were structurally different from microtubules were observed in plant cells subjected to abiotic stress. These data suggests that exposure to abiotic stresses (salinity, low positive temperatures, flooding and Al toxicity stress) may induce complete and/or partial depolymerization of microtubules, followed by assembly of atypical tubulin polymers. Further studies on the mechanisms of formation of atypical tubulin

structures and determination of their role in the plant cell reaction to various abiotic stress factors are necessitated.

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