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Kondiční a vývojová remodelace rostlinného cytoskeletu

HABILITAČNÍ PRÁCE

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Conditional and developmental remodeling of the plant cytoskeleton

HABILITATION THESIS

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Acknowledgments

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Contents

1. Unique features of plant cells

Plant cell structure is unique in many anatomical and functional aspects compared to cells of other eukaryotes. Apart from the existence of the plant unique plastidial energy factories (chloroplasts; Jensen and Leister, 2014), the most distinctive feature of the plant cell is the restricting cell wall which surrounds the plasma membrane and confines the cell in a rigid matrix. The presence of the cell wall has been an evolutionary trigger of functional divergence of plant cells, using sometimes conserved components present in all eukaryotes. Cell growth for example is not so much based on metabolism and biosynthesis. Rather it relies on a hydraulic mechanism of vacuolar water accumulation, the building of a substantial turgor pressure as the driving force of cell expansion and the orientation of cell wall cellulose microfibrils as a growth directionality determinant (reviewed in Komis et al., 2015a).

Cell tissue patterning and formation of distinct non-parenchymatous cell types are processes which are again adapted to the presence of the cell wall. By comparison to mammalian cells for example, organ development and differentiation does not depend on cell migration, but rather on the coordinated response of multicellular assemblies to tensional forces (e.g., Uyttewaal et al., 2012) and the formation of unique cytoskeletal arrays that predetermine polarity of cell division and cell division plane orientation (CDP; Lipka et al., 2015).

The mass coordinated responses of plant cells within multicellular tissues, can be mediated by intercellular communication via plasmodesmata which are tubular channels traversing the cell wall and establishing protoplasmic continuity between adjacent cells through the intercellular extension of endoplasmic reticulum (ER) elements (Brunkard and Zambryski, 2017). However multicellularity in plants can be also achieved by paracrine hormonal signaling through the appropriate polar arrangement of membrane localized hormone receptors and hormone transporters. Such controlled cellular influx and efflux of hormones like indole-3-acetic acid (IAA; auxin) have been elaborated in shoot apical meristem growth and phyllotaxis establishment (reviewed in Sassi and Vernoux, 2013) but also in the establishment of primary and secondary root growth and architecture (Liu et al., 2017). Again the existence of the cell wall appears to be significant for the polar localization of hormone effectors such as the PIN1 auxin transporter (reviewed in Sassi and Vernoux, 2013).

2. Scope of the thesis

The present thesis aims to position the plant cytoskeleton at the interphase between the plant cell and the outside world. To emphasize on the dynamic nature of this widespread component of the cell architecture and to reason its participation to extracellular signal perception, decoding and response. Integration of plant cytoskeletal remodeling with signal transduction mechanisms occurs during the early stages of plant reaction and adaptation to its external environment and especially under transient unfavorable conditions. However, several signal transduction mechanisms are associated with physiological processes of plant growth, differentiation and finally development where the plant cytoskeleton plays key roles. Therefor the present thesis is divided in two major parts. In the first part

the integration of signaling and cytoskeletal remodeling will be addressed in the conditional context and in the second part its developmental implications will be given.

3. General overview of the plant cytoskeleton

3.1. Introduction

The plant cytoskeleton comprises of microtubules and actin microfilaments, both of which are filamentous polymeric structures with unique intracellular distribution in diverse plant cell types (Hepler et al., 2013). Microtubules are dynamic polymers of the αβ-tubulin dimer and exhibit an end-wise stochastic behavior of growth and shrinkage which depends on the GTPase activity of tubulin (Desai and Mitchison, 1997). As previously reviewed, plant genomes harbor the most expansive families of both α- and β-tubulin subunits (Komis et al., 2015a) signifying the importance of both gene families in plant growth and development. The transcriptional diversity of the tubulin superfamily is further expanded at the post-translational level through modifications that alter microtubule dynamics or modulate their interactions with microtubule associated proteins (reviewed in Komis et al., 2015a; Yu et al., 2015).

The dynamic behavior of microtubules, their subcellular organization and their conditional or developmental remodeling depend on the auto-organizational capacity of large microtubule populations (Tabony et al., 1994) and its modification by a pleiade of proteins which can be classified as microtubule crosslinkers (Hamada, 2014), microtubule end binding proteins (Hamada, 2014), microtubule severing proteins (Luptovčiak et al., 2017), microtubule motor proteins (Nebenführ and Dixit, 2018) and modulators of microtubule dynamic behavior. As will be explained in the coming sections these interactions are controllable and can be manipulated by signaling on and off switches.

The extensive subcellular occupancy of microtubules allows them to fulfil numerous roles in plant cell growth (Smith, 2005), differentiation (Landrein and Hamant, 2013) and plant cell division (Masoud et al., 2013; Rasmussen et al., 2013). Since the early identification of microtubules in the cortical cytoplasm of root epidermal cells of different plants, their connection to the plasma membrane and their positional relationship with the overlying cellulose microfibrils of the primary cell wall was noted (Ledbetter and Porter, 1963). Thereon, cortical microtubules have been considered indispensable partners of the so-called cell wall – plasma membrane – cortical cytoskeleton continuum (Baluska et al., 2003). The primary connection of cortical microtubules to cellulose microfibril deposition, relates to the provision of microtubular tracks for the movement of cellulose synthesizing protein complexes (CESAs; reviewed in Komis et al., 2015a).

Microtubules in this case provide docking sites for vesicles harboring CESA complexes and guide their movement which is caused by the expelled cellulose microfibril (reviewed in Komis et al., 2015a). Therefor the patterns of cortical microtubule organization reflect to the patterns of cellulose microfibrils in the innermost layer of the primary cell wall. In this way organization of cortical microtubules is not only related to cell growth directionality of diffusely growing cells but also

predisposes the unique cell wall architecture of specialized cell types like tracheary elements, leaf or cotyledon pavement cells and stomatal guard cells to name some few (e.g., **Fig. 1**).

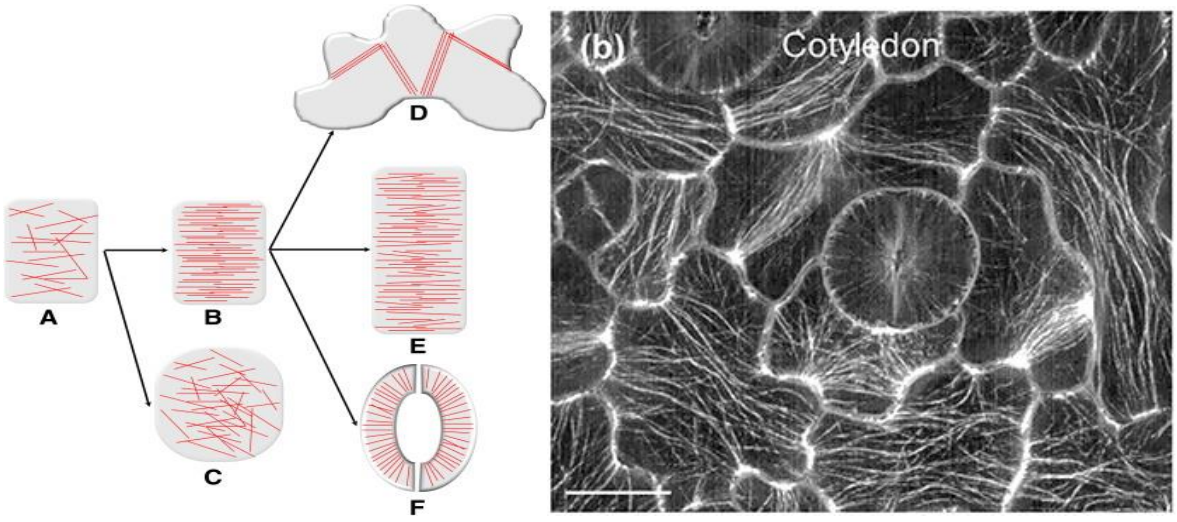

Fig. 1. A graphic model and an example of how microtubule organization is related to cell growth and cell differentiation. Specific patterns of cortical microtubule organization in the cotyledon epidermis of *Arabidopsis thaliana* seedlings stably expressing a green fluorescent protein of the TUBULIN ALPHA 6 protein (GFP-TUA6; From Komis et al., 2015a, 2017).

However, cortical microtubules are also a hub for extracellular signal perception as will be explained in later sections. In this way, cortical microtubules exhibit prompt changes in their organization, integrity and dynamics after appropriate extracellular stimulation and in this way they adapt and participate to short term cellular responses to environmental challenges when these are perceived as perturbations of the cell surface (Nick, 2013).

In the developmental context, cortical microtubules through the formation of the preprophase microtubule band (PPB) are implicated in cell division plane orientation (CDP; Rasmussen et al., 2013). PPB is mostly related to the robustness of mitotic spindle positioning (Schaefer et al., 2017; Komis et al., 2017) and to the positional control of the centrifugally expanding cytokinetic phragmoplast (Lipka et al., 2015) as will be explained more thoroughly later. On the other hand both the mitotic spindle and the phragmoplast are microtubule-based assemblies that underlie chromosome segregation and cell plate formation during mitosis and cytokinesis respectively (Yamada and Goshima, 2017). The controlled formation of the mitotic spindle and the phragmoplast are essential for the faithful partitioning of genetic material to the resulting daughter cells. However the positional control of both structures is of key importance to global developmental processes of plants in the absence of cell motility by regulating symmetry of cell division (Rasmussen et al., 2011).

Actin filaments are also dynamic cytoskeletal polymers comprising by the monomeric protein G-actin. The Arabidopsis genome contains 8 actin isoforms which like tubulin may also be subjected to

posttranslational modifications (McCurdy et al., 2001). Actin filaments exhibit partially different organization than microtubules, although in particular instances they colocalize with microtubules in the cortical cytoplasm and the PPB, the mitotic spindle and the phragmoplast. Mitotic actin systems may serve as negative markers of the cortical division site since they are absent from the site (cortical division zone) previously occupied by the PPB during mitosis (Panteris, 2008). Other mitotic actin systems function as membrane – microtubule connections during mitotic spindle positioning, or phragmoplast pivoting during cytokinesis and cell plate deposition using kinesin or myosin motors with dual binding affinities against microtubules and actin microfilaments (Higaki et al., 2008; Klotz and Nick, 2012; Wu and Bezanilla, 2014; Sun et al., 2018).

Together with myosin molecules, plant actin filaments mediate active, large scale intracellular movements as evident from cytoplasmic streaming and most notably the positive or negative phototactic movements of plastids under different intensities of light irradiance (Wada, 2013). A large population of actin microfilaments occupies submembrane domains in the cortical cytoplasm and is implicated to cell morphogenetic processes often in collaboration with microtubules (Mathur and Hulskamp, 2002). In this case actin systems guide the organization of other extensive cortical structures such as elements of the endoplasmic reticulum (ER; Brandizzi and Wasteneys, 2013; Griffing et al., 2017).

3.2. Conditional rearrangements of microtubule arrays in plants

3.2.1. Overview

Cortical microtubules are blueprinting the patterns of cellulose deposition in the cell wall and hence direct cell growth directionality and cell morphogenesis (Li et al., 2015). However, the occurrence of microtubules in the cell cortex and their participation in the cell wall – plasma membrane – cytoskeleton physical continuum, positions them at the interphase between the extracellular and the intracellular environment. Therefor cortical microtubules can be directly targeted by extracellular stimuli perceived by the cell surface and respond accordingly either by reorganization, by effects on their structural integrity and/or by regulation of their dynamic properties and interactions with other molecules. In this respect, earlier studies have shown that the organization, dynamics and integrity of microtubules can be affected by exposure to extracellular salinity (Shoji et al., 2006; Wang et al., 2011; Dou et al., 2018; Angelini et al., 2018), light conditions (Lindeboom et al., 2013; Ma et al., 2018; Nakamura et al., 2018) and temperature extremes (Müller et al., 2007) among others. However the most thoroughly examined condition that triggers cortical microtubule remodeling is the exposure of plant cells to hyperosmotic solutions causing plasmolysis (e.g., Lang-Pauluzzi and Gunning, 2000; Komis et al., 2001 **Included Publication 1**, Komis et al., 2002a **Included Publication 2**; Lang et al., 2014).

Plasmolysis occurs under hyperosmotic conditions, through the retraction of the protoplast and the partial or complete detachment of the plasma membrane from the overlying cell wall. Depending on the rate of osmotic water efflux, the nature of the osmoticum (ionic vs non-ionic and small or large molecular size) and certain characteristics of the cell type examined (e.g., homogeneity and density

of cell wall – plasma membrane attachments and degree of vacuolation), plasmolysis can be concave or convex (**Fig. 2**). Membrane detachment from the cell wall can be potentially injurious, leading to membrane rupture, leakage of cytoplasmic material and eventually necrosis. Moreover, water efflux will affect cytoplasmic organization since it is leading to vacuolar shrinkage and fragmentation and condensation of the cytosol with adverse effects on cytoplasmic solute concentration and eventually on enzyme activities and patterns of macromolecular interactions through mechanisms of molecular crowding (Bhattacharya et al., 2013).

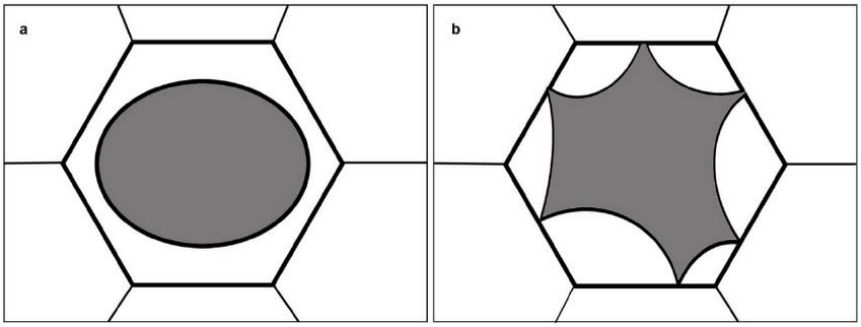

Fig. 2. Oversimplified schematic depiction of the two major plasmolytic forms. Convex (a) and concave (b). Adapted from Lang et al., 2014.

As will be explained in the following sections, mechanical and chemical stresses associated with the process of plasmolysis evoke among others rapid and global cytoskeletal rearrangements which may lead to short term cell survival and long term adaptation in persistent hyperosmotic conditions.

3.2.2. Microtubule reorganization in plasmolyzed cells of *Chlorophyton comosum* leaf

The first experiments of hyperosmotic stress were conducted on meristematic cells of the spider plant (*Chlorophyton comosum*). In contrast to other meristematic systems (see later), meristematic cells of *C. comosum* exhibit a considerable degree of vacuolation, suggesting that they would massively respond to the intense hyperosmotic conditions chosen (1 M mannitol, 30 min; Komis et al., 2001 **included Publication 1**). Indeed most of such cells plasmolysed in a concave manner suggestive of abrupt and extensive protoplast retraction.

Following immunofluorescence detection of tubulin, preexisting microtubule systems such as cortical microtubules, mitotic spindles and cytokinetic phragmoplasts were disintegrated/reorganized at a variable extend. In interphase cells, cortical microtubules were completely disassembled and instead fluorescing spots were observed. Transmission electron microscopy (TEM) of such plasmolyzed cells, showed the occurrence of extensive masses of paracrystalline material (**Fig. 3**).

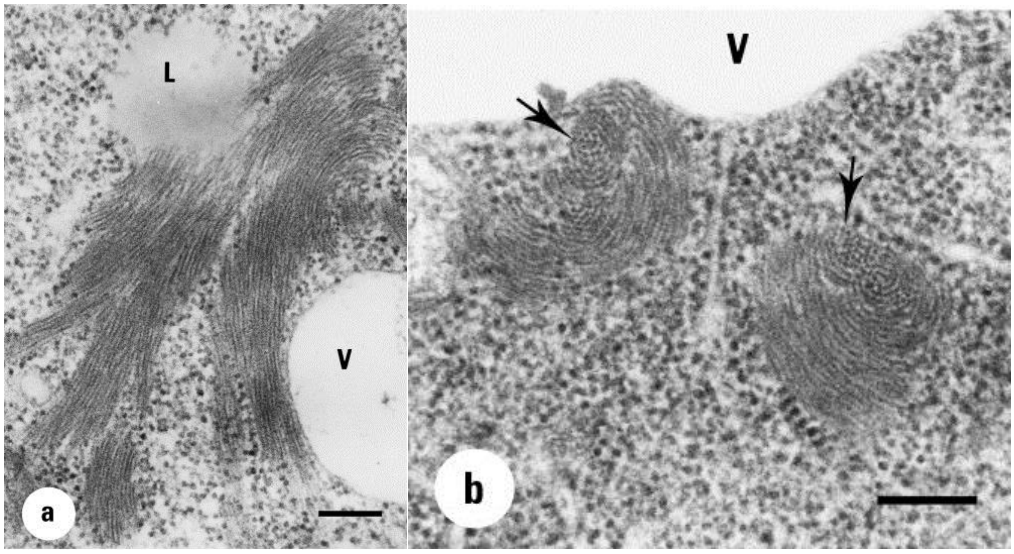

Fig. 3. Grazing (a) or transverse (b) sections of paracrystalline material observed in plasmolyzed leaf cells of *C. comosum* with TEM documentation (From Komis et al., 2001 **included Publication 1**).

This material loosened when plasmolyzed cells were treated with 5 μM of oryzalin, a microtubule depolymerizing agent, suggesting that they comprise of tubulin, self-associating in an aberrant manner. Therefor the fluorescing spots observed in immunolabeled cells most likely correspond to the paracrystalline material observed in TEM.

In plasmolyzed mitotic cells, chromosomes or separated sister chromatid groups coalesce into very dense chromatin masses as visualized by DAPI fluorescence. Moreover they exhibited abnormal mitotic spindles which were considerably more elongated and sharply focused at the pole regions compared to control dividing cells. TEM examination of plasmolyzed mitotic cells corroborated the high degree of chromatin condensation and revealed that these aberrant mitotic spindles consist of tightly packed microtubules with a significantly bigger outer diameter (termed macrotubules with ca. 32 nm diameter compared to the 25 nm of typical, 13-protofilament microtubules; Desai and Mitchison, 1997), while such microtubules were frequently seen embedded into the dense chromatin masses (**Fig. 4**). Moreover, tubulin-based paracrystalline material was again found, this time embedded in the dense chromatin masses (**Fig. 4**).

Likewise, cytokinetic cells examined with TEM, showed the formation of atypical phragmoplasts again consisting of macrotubules, unable to support cell plate deposition. In both mitotic and cytokinetic plasmolyzed cells, TEM documented the occurrence of tubulin paracrystals concomitantly to macrotubules. These paracrystals were morphologically identical to those observed in interphase cells. Of particular interest was the identification of tubulin paracrystals embedded within the extremely condensed chromatin masses (**Fig. 4**).

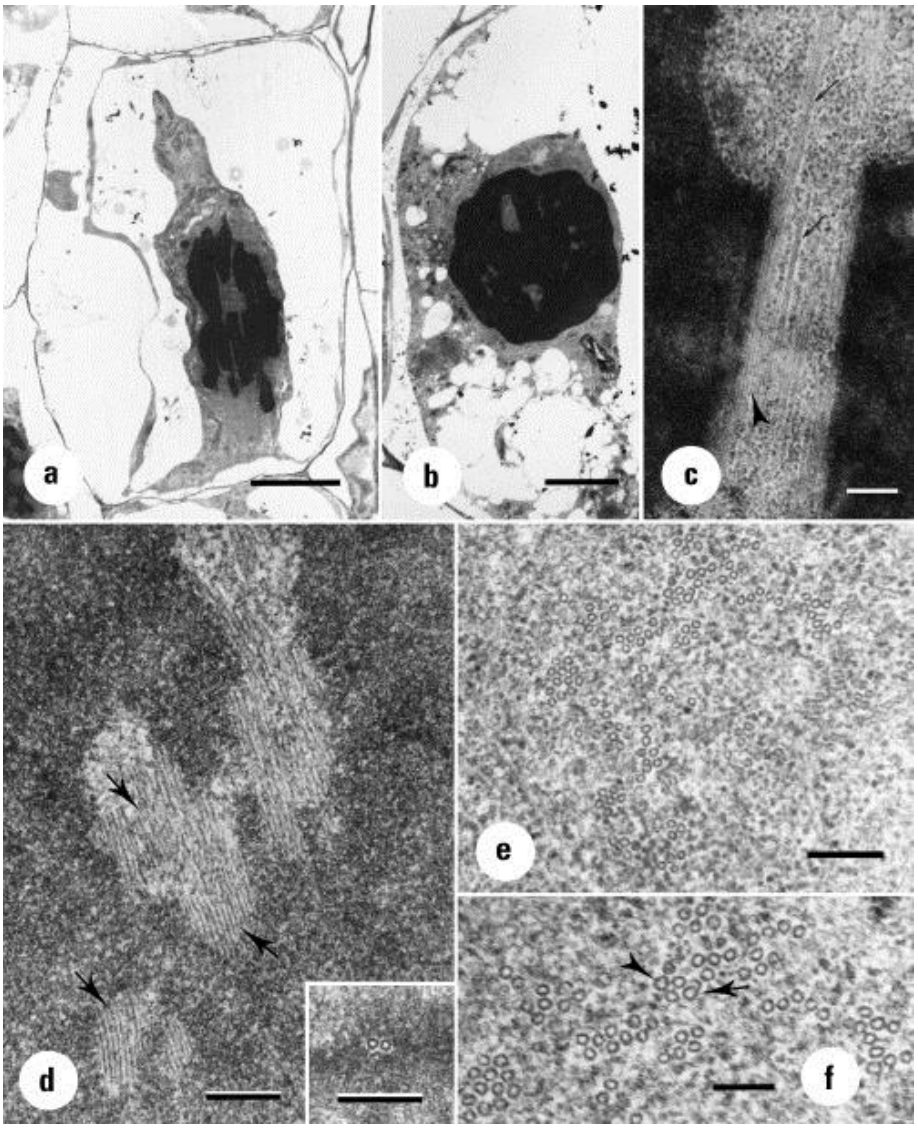

Fig. 4. Chromatin and microtubule configurations in plasmolysed mitotic cells of *C. comosum*. (a, b) Extreme chromatin condensation and chromosome fusion in plasmolyzed anaphase (a) and prophase (b) cells. (c) Macrotubule bundles traversing dense chromatin areas. (d) Paracrystal and macrotubules (inset) embedded in dense mitotic chromatin masses. (e,f) Arrangement and crosslinking between hyperosmotically-induced macrotubule-based kinetochore fibers (From Komis et al., 2001 **Included Publication 1)**.

As noted before, the paracrystalline material is readily disassembled by short oryzalin treatment while upon the removal of the hyperosmotic solution (during deplasmolysis) it is rapidly replaced by typical microtubules. This observation suggests that hyperosmotically-induced tubulin paracrystals may serve as a reservoir of tubulin for the reinstatement of typical microtubules during the recovery when hyperosmotic conditions are reversed. It is notable that oryzalin-treated plasmolyzed cells are not resuming during the replacement of the hyperosmotic by isosmotic medium and they are massively necrotized. This suggests that the hyperosmotically-induced accumulation of tubulin in atypical paracrystals is essential for cell viability during subsequent deplasmolysis. This is consistent with the fact that soluble αβ-tubulin dimers are quite labile in aqueous environments and thus tend to

accumulate to a polymer phase to escape denaturation and subsequent degradation. Normally, αβ-tubulin dimers should polymerize within the microtubule pool.

However, crowding conditions within the condensed cytoplasm of hyperosmotically-treated cells, may promote the formation of aberrant polymer phases such as the paracrystals observed by allowing interactions of low affinity sites (Bhattacharya et al., 2013). Similar diverse polymer structures of αβ-tubulin dimers have been observed in vitro after incubation of purified tubulin in the presence of zinc cations (Larsson et al., 1976), but may be also observed in cells after treatment with Vinca alkaloids (Starling, 1976).

3.2.3. Hyperosmotically-induced remodeling of the microtubule cytoskeleton in Triticum turgidum root-tip cells

The same experimental approach of hyperosmotic treatment was followed in the case of root-tip cells of *Triticum turgidum* which in contrast to *C. comosum* meristematic cells are characterized by minimal vacuolar occupation. Being cytoplasm-rich, *T. turgidum* meristematic cells were expected to respond more slowly and less extensively to the extreme hyperosmotic conditions chosen (again 1 M mannitol for 30 min). Indeed in their vast majority such cells exhibited convex plasmolysis patterns, indicative of slower, more uniform and less extensive protoplast retraction (Komis et al., 2002a **included Publication 2**).

Immunofluorescent studies of tubulin distribution in plasmolyzed root-tips of *T. turgidum* showed the occurrence of extensive cortical and endoplasmic, perinuclear polymeric arrays in interphase cells (**Fig. 5**). Especially cortical arrays seemed to follow patterns of organization similar to those of cortical microtubules of control cells. TEM observations of such cells showed that these cortical or endoplasmic microtubules are atypical resembling the macrotubules observed in *C. comosum* plasmolyzed cells bearing an average outer diameter of approximately 35 nm (**Fig. 5**). Tubulin paracrystals of similar characteristics to those of plasmolyzed *C. comosum* leaf cells, were also found in hyperosmotically-treated root cells of *T. turgidum* but they were not so much prevalent (Komis et al., 2002a **included Publication 2**).

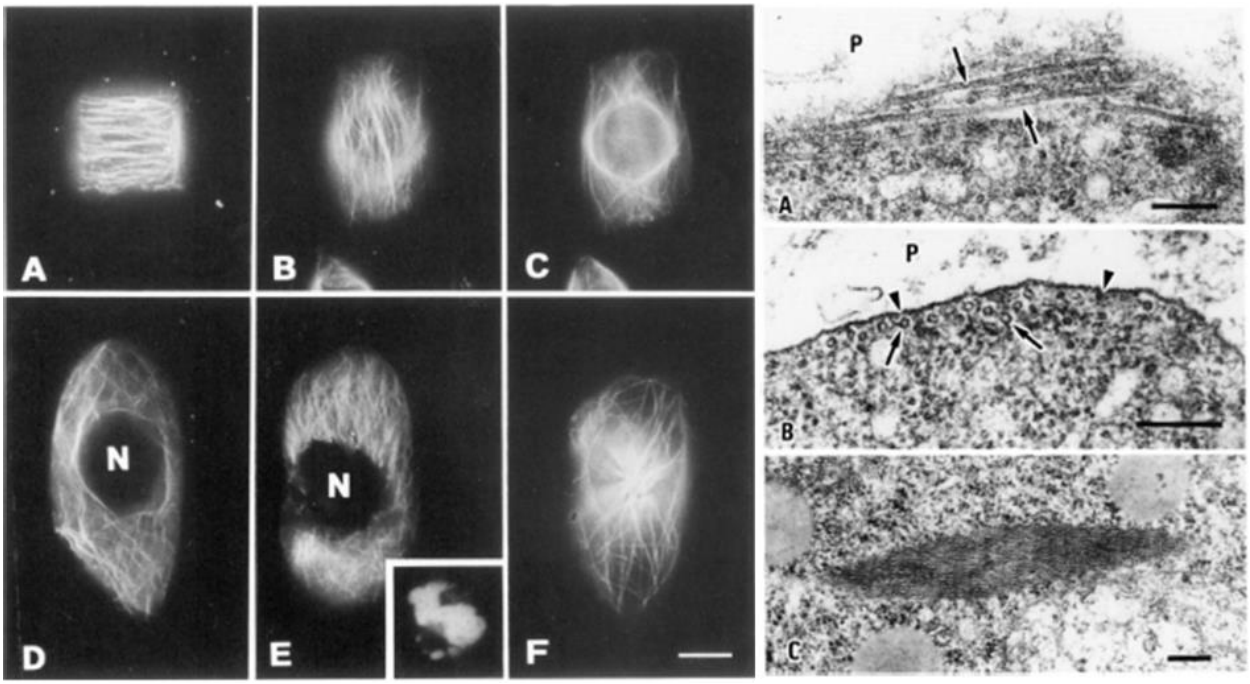

Fig. 5. Immunofluorescence visualization of microtubule organization in plasmolyzed root cells of *T. turgidum* (A-F, left panel) and TEM documentation of their structure (A-C, right panel). Immunofluorescence imaging shows the abundance and enrichment of cortical (A, B, left panel) and endoplasmic (C-F, left panel) linear structures resembling cortical microtubules. TEM and subsequent quantitative measurements showed that these structures are tubulin macrotubules (A,B right panel) while rarely paracrystals (C, right panel) may also be observed (From Komis et al., 2002a **Included Publication 2**).

In dividing root cells of *T. turgidum* tubulin macrotubules assemble into aberrant and highly elongated mitotic and cytokinetic apparatuses resulting in the disturbance of cell division and cytokinesis. In all cases, as also was the case in *C. comosum*, chromatin and chromosomes become extremely dense as evident by DAPI staining and fluorescence imaging. Likewise, putative tubulin paracrystals were occasionally observed in plasmolyzed mitotic and cytokinetic root tip cells of *T. turgidum*, but only rarely (Komis et al., 2002a **included Publication 2**).

Quantitative assessment of the overall fluorescent intensity of tubulin immunolabeled cells, showed that the amount of polymeric tubulin in plasmolyzed cells exceeded that of control cells. When the hyperosmotic treatment of root-tip cells is prolonged to 2–8 h typical cortical microtubules are reinstated and the turgidity of the root recovers without noticeable change of the volume of the plasmolyzed protoplast. This discrepancy can be explained by the mass deposition of cell wall material in the periplasmic space, i.e., the space between the cell wall and the retracted protoplast. Apart from the recovery of cortical microtubules, chromatin recovers as well assuming its typical appearance while the cells are able to undergo cell divisions under such hyperosmotic conditions (Komis et al., 2002a **included Publication 2**).

Oryzalin and colchicine treatment induced macrotubule disintegration and a significant reduction of protoplast volume in every plasmolyzed cell type compared to non-treated plasmolyzed cells,

whereas cytochalasin B had only minor effects restricted to differentiated and highly vacuolated cells. Moreover oryzalin treatment compromised the viability of plasmolyzed cells. These results suggest that microtubule destruction by hyperosmotic stress, and their replacement by tubulin macrotubules and putative tubulin paracrystals is a common feature among angiosperms and that macrotubules are involved in the mechanism of protoplast volume regulation.

3.2.4. The role of microtubules in the hyperosmotic response of plant cells

As evident from the previous sections, cortical microtubules are rapidly and massively responsive to the exposure of plant organs to hyperosmotic solutions. The response can be broken down to distinct steps including the disassembly of preexisting microtubule systems and the reassembly of liberated soluble tubulin to aberrant structures such as macrotubules and tubulin paracrystals. Both systems represent structurally conserved tubulin reservoirs between *C. comosum* leaf cells and *T. turgidum* root-tip cells, that may be used for the timely reinstatement of conventional microtubule arrays once the hyperosmotic conditions are reserved. Soluble αβ-tubulin dimers are notoriously labile when kept unassembled (https://puresoluble.com/storing-and-handling-cryopreserved-tubulin/) therefor their preservation in polymeric pools preserves their assembly-competitiveness which is necessary for the recovery of typical microtubule systems once hyperosmotic conditions are reversed. At large the aberrant tubulin assemblies observed in plasmolyzed cells are non-functional. However, cortical tubulin macrotubules formed in plasmolyzed, non-dividing *T. turgidum* root-tip cells seem to support long-term adaptive mechanisms to hyperosmolarity since they support cell survival and progressive resumption of root girth and physiological functions under such conditions.

Later studies showed that similar experimental conditioning of plant systems with ionic or non-ionic hyperosmotic stress, evoke microtubule remodeling with physiological consequences regarding cell survival and adaptation. In the genetically tractable plant *Arabidopsis thaliana*, hyperosmotic stress conditions are frequently linked with salt stress via the experimental application of high concentrations of NaCl. Studies in Arabidopsis are benefited from the possibility to study appropriate mutants and dissect the consequences of hyperosmotic stress at molecular details. Moreover hyperosmotic stress responses of the cytoskeleton may be studied in living plants that express an appropriate marker for microtubules or actin based on the green fluorescent protein, (GFP; e.g., Lang et al., 2014) its spectral variants or other inherently fluorescent proteins. Such markers may include GFP-tubulin fusions (like GFP-TUA6; Shaw et al., 2003) or GFP fusions of heterologous domains/proteins. For example a commonly used microtubule marker is a GFP fusion of the microtubule binding domain (MBD) of the mammalian, non-neuronal microtubule associated protein 4 (MAP4; Marc et al., 1998).

Such studies in living plants revealed the detrimental effects of extracellular hyperosmolarity on cortical microtubule integrity (e.g., Lang et al., 2014; Cheng et al., 2017) which can be partially attributed to α-tubulin phosphorylation (Ban et al., 2013). Further studies on pfd3 and pfd5 mutants denoted the role of tubulin (and actin) folding by members of the PREFOLDIN family which are regulating the levels of assembly competent tubulin dimers under conditions of osmotic stress (Rodriguez-Milla and Salinas, 2009). Survival of plants under conditions of high salinity, requires

microtubule remodeling (Shoji et al., 2006; Wang et al., 2007). Under salt stress, microtubule stability requires the bundling activity of the microtubule crosslinking protein MAP65-1, which is promoted by salt-induced phosphatidic acid production (Zhang et al., 2012). In cases when salt stress induces disintegration of cortical microtubules, this was found to be related with the salt-induced proteasomal degradation of the microtubule associated protein SPIRAL1 (Wang et al., 2011).

3.3. Volume regulatory properties of actin microfilaments

3.3.1. Overview

The exposure of cells to anisosmotic conditions leads to passive water movements to or from the cytoplasm. In the case of hyperosmotic stress, extracellular hyperosmolarity leads to water efflux, whereas hypotonic conditions cause water influx. In both cases, such water movements inevitably lead to changes in the cellular volume which in extremis may compromise plasma membrane integrity and alter intracellular architecture. In animal cells, the exposure to hyperosmolarity triggers a regulatory mechanism termed regulatory volume increase (RVI; Hoffmann et al., 2009) while in the opposite situation, hypotonicity triggers a similar mechanism called regulatory volume decrease (RVD; Hoffmann et al., 2009). Both RVD and RVI mechanisms operate at a time scale of minutes and are compensatory rather than adaptive.

In this sense, such mechanisms provide the means for preventing rupture of the plasma membrane and cell death while allowing for adaptive metabolic mechanisms to come in effect. Such mid- to long-term mechanisms involve for example the biosynthesis and intracellular accumulation of osmoactive compounds which will counteract extracellular hyperosmolarity in the case of hyperosmotic stress (Hoffmann et al., 2009). Plant cells are encased within a dense and rigid extracellular matrix (cell wall), which can resist cell volume expansion during hypotonic exposure, therefor hypoosmotic stress has not been studied.

On the contrary, the exposure of plant cells to hyperosmotic conditions has been extensively addressed, since under extreme situations it induces protoplast retraction and volume reduction in a process called plasmolysis. During this process, massive water efflux occurs from the vacuole which becomes reduced and eventually, the protoplast follows. Thus the plasma membrane becomes partially or completely detached from the overlying cell wall. Since the cell wall and the plasma membrane are physically linked, plasma membrane detachment may hamper its integrity and lead to leakage of cytoplasmic material and uncontrolled cell death.

Therefor plasmolysis under severe hyperosmotic conditions, poses issues of mechanical stress and membrane integrity that need to be promptly addressed at the short-term, before any osmoadaptive mechanism can be put in effect for the long term survival.

3.3.2. Volume regulatory remodeling of the actin cytoskeleton in Chlorophyton comosum leaf cells

In order to examine the possible mechanisms of survival of plasmolyzed cells and to address the possible role of the actin cytoskeleton, actin filament (AF) organization was studied during the plasmolytic cycle in leaf cells of *C. comosum*. The plasmolytic cycle consists by the induction of plasmolysis until the protoplast volume is stabilized, followed by deplasmolysis in isotonic medium (**Fig. 6**). During the hyperosmotic exposure of the plasmolytic cycle, leaf cells of C. comosum undergo protoplast volume reduction up to a certain extent, without further shrinkage. Protoplast volume stabilization under hyperosmotic conditions occurs quite rapidly (at latest after 15 min after hyperosmotic exposure). During deplasmolysis, the protoplast responds with a lag, while its expansion proceeds fast but in a controlled manner allowing its volume reinstatement within a time slightly exceeding 30 min after exposure to the isotonic medium.

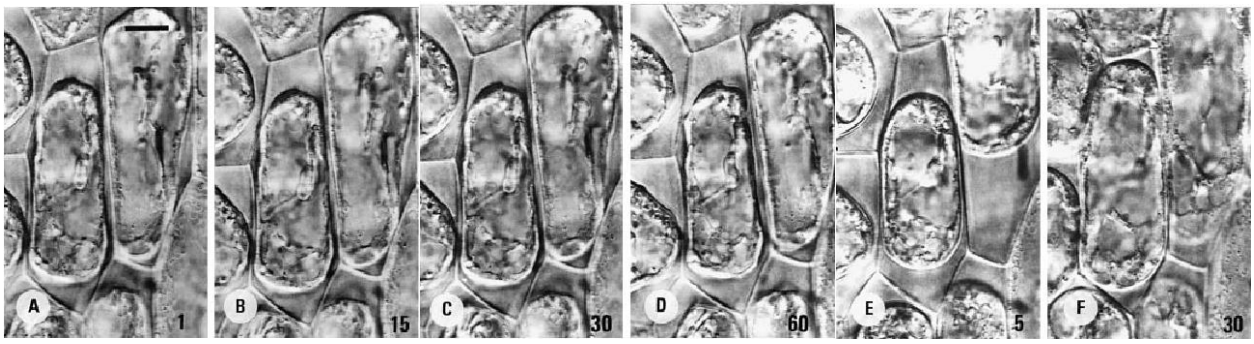

Fig. 6. Differential interference contrast documentation of the plasmolytic cycle of epidermal leaf cells of *C. comosum*. (A-D) Plasmolysis in 1 M mannitol for 60 min. (E,F) Deplasmolysis in isotonic medium for 30 min (From Komis et al., 2002b **Included Publication 3**).

In most leaf cells of *C. comosum*, the hyperosmotic treatment induced convex or concave plasmolysis and large scale remodeling of the AF cytoskeleton. In control cells AFs form distinct arrays of fine or bundled AFs either at the cortical cytoplasm or in the endoplasm most prominently traversing transvacuolar cytoplasmic strands. In plasmolyzed cells, fine cortical systems disappeared and instead AFs formed thick bundles tightly lining the plasma membrane most prominently at the boundaries between detached and attached plasma membrane areas. Spatial reorganization of the actin cytoskeleton after hyperosmotic stress, was concomitant to a significant increase in the overall AF content compared with the control cells. The endoplasmic AF bundles were mainly found in the perinuclear cytoplasm and on the tonoplast surface. AFs also traversed some of the Hechtian strands (**Fig. 7**).

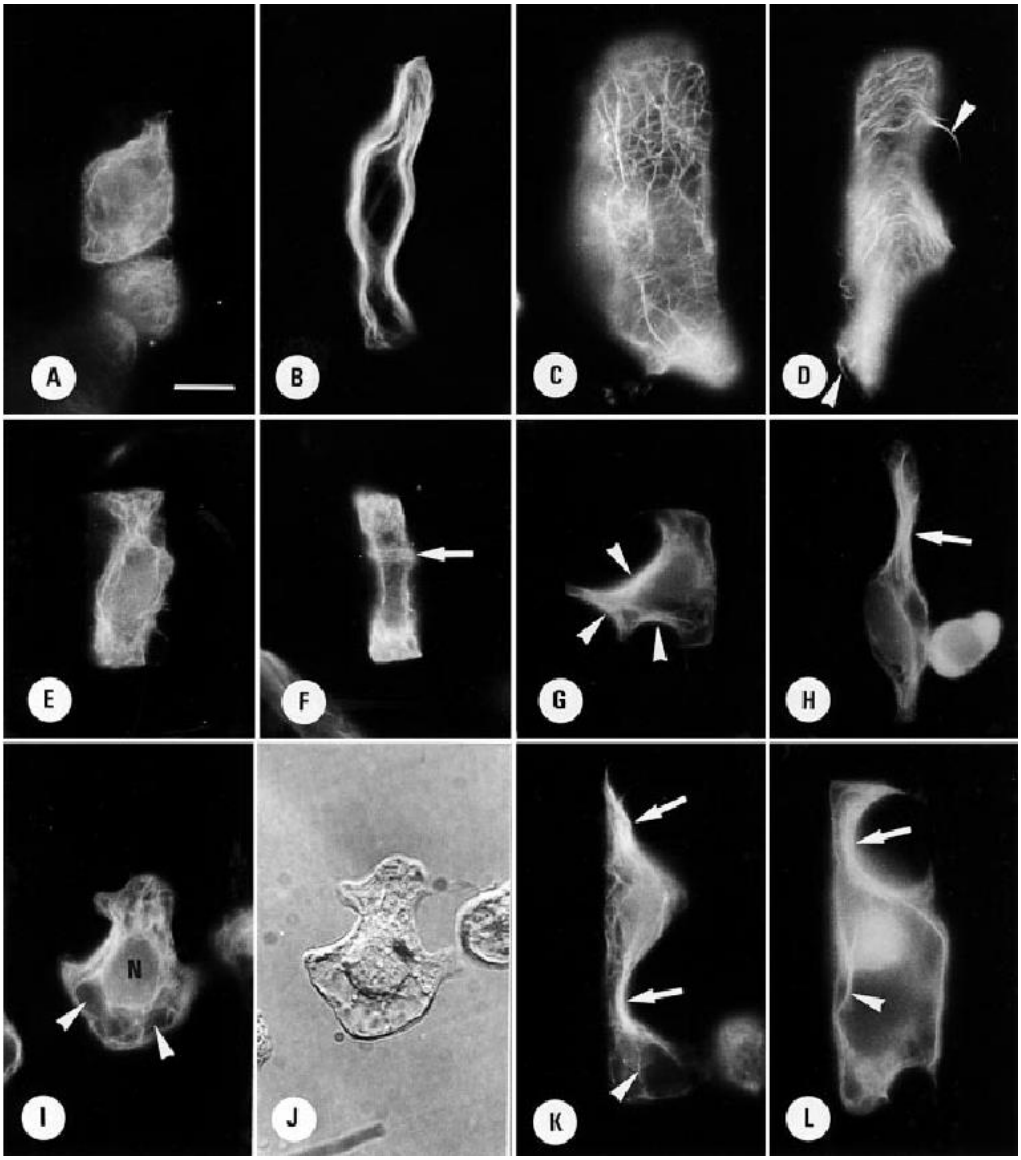

Fig. 7. AF organization in various leaf cells of *C. comosum* plasmolysed for 30 min in 1 M aqueous mannitol solution after fluorescent phalloidin staining and epifluorescent imaging. (A-F) Variants of plasmolysed meristematic (A,E,F) or differentiated (B-D, G,H) cells with excessive AFs. Of particular notice is the enrichment of AFs under retracted plasma membrane areas (G-L) which is a predominant feature of the actin response to extracellular hyperosmolarity (From Komis et al., 2002b **Included Publication 3**).

AF disorganization after cytochalasin B (CB) treatment induced dramatic changes in the pattern of plasmolysis, which lasted for a longer time and led to a greater decrease of the protoplast volume compared to the untreated cells. In many of the above cells the protoplasts assumed an 'amoeboid' form and were often subdivided into sub-protoplasts suggestive of the severity of plasmolysis after disruption of AFs (**Fig. 8**).

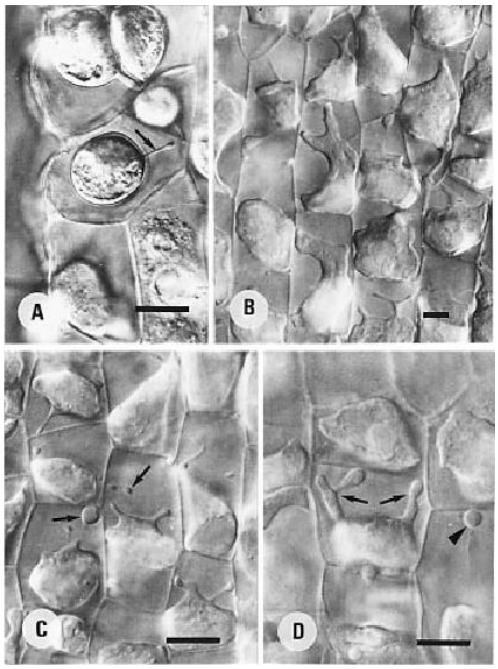

Fig. 8. CB-treated plasmolysed ECs under DIC optics. (A) In this cell the protoplast is completely detached from the cell wall. Arrow points to a protoplasmic extension. (B, C) In these cell groups, the plasmolysed protoplasts have assumed an `amoeboid' form. Arrows in C mark `vesicular elements'. (D) `Amoeboid' plasmolysed protoplast exhibiting discrete protoplasmic protrusions (arrows). Arrowhead shows a `vesicular element' (From Komis et al., 2002b **Included Publication 3**).

Soon after the removal of the plasmolytic solution both CB-treated and untreated cells were deplasmolysed, while the AF cytoskeleton gradually reassumed the organization observed in the control cells. The findings of this study revealed for the first time in angiosperm cells that plasmolysis triggers an extensive reorganization of the AF cytoskeleton, which is involved in the regulation of protoplast shape and volume.

When mammalian cells are exposed to hyperosmotic conditions they undergo a physiological cell volume increase, which in the long term is supported by the metabolic accumulation of osmolytes (Hoffmann et al., 2009). At the onset of the hyperosmotic exposure and before any visible sign of cell volume increase, the hyperosmotically-induced cell shrinkage induces the extensive remodeling of the cortical actin cytoskeleton. In such cells the cortical actin cytoskeleton is contractile pending on the interaction of actin microfilaments with myosin II motors and under conditions of either hypotonic or hypertonic stress, contractility of the submembranous actin cytoskeleton exerts a mechanoprotective role to the plasma membrane.

Membrane stress is a major issue during plasmolysis since detachment of the plasma membrane is abrupt, partial and potentially injurious. Since previously we established the protective role of actin microfilament reorganization in hyperosmotically-treated leaf cells of *C. comosum*, we extended this study to the possible functions of myosins in the process. Therefor plasmolysis was carried out in the presence of myosin inhibitors. The purpose of these experiments was to document actin organization, the course of the plasmolytic cycle and the viability of plasmolysed cells after myosin inhibition.

Although inconclusive the preliminary published results (Komis et al., 2003 **Included Publication 4**), showed that myosin inhibition prevents actin remodeling but not to the extend to compromise cell viability during the plasmolytic cycle.

3.3.3. The role of actin microfilaments in the hyperosmotic response of plant cells

Subsequent studies on the hyperosmotic response of the actin cytoskeleton, showed that AF remodeling is indispensable for cell viability under such conditions (Shi et al., 2011; Liu et al., 2013). When such studies are carried out in living cells of *Arabidopsis thaliana* expressing an appropriate molecular marker for AFs, allow the time lapse imaging of the AF response (Shi et al., 2011; Lang et al., 2014; Cheng et al., 2017). Through plasmolysis experiments in such material, our original hypothesis on the importance of AFs in plasma membrane – cell wall attachments was proven in living cells (Yu et al., 2018).

The role of actin in the immediate hyperosmotic stress response of plant cells has not been disclosed so far. Evidently, the remodeling of submembrane actin arrays has a mechanoprotective role against plasma membrane tearing under such conditions, but from more extensive studies, actin may participate to early osmoregulatory regulation via the activation of transmembrane ion channels (Hoffmann et al., 2009). However this possibility has to be yet explored in the case of plants.

3.4. Integration of signaling pathways with conditional microtubule remodeling

Our previous surveys on the effects of plasmolysis on microtubule organization and particularly in the root of *T. turgidum* brought to light an unexpected mechanism whereby microtubule reorganization is fundamental for cell viability and recovery within hyperosmotic conditions. Hyperosmotic stress-induced cortical microtubule reorganization occurs rather rapidly as shown in both fixed and immunolabeled as well as in live cells (Komis et al., 2002a **Included Publication 2**; Lang et al., 2014) and it relates well to prompt signaling responses evoked under such conditions (Ban et al., 2013). For this reason we explored the implications of known conditional signaling pathways to microtubule remodeling. Pathways with similar time patterns of activation after hyperosmotic treatment include mitogen activated protein kinases (MAPKs; e.g., Munnik and Meijer, 2001) and phospholipase D and C species, which are responsible for the generation of bioactive lipids and among others for the regulation of cytoskeletal proteins (Pleskot et al., 2013).

3.4.1. Mitogen activated protein kinases

Mitogen activated protein kinases (MAPKs) are serine/threonine kinases which participate in three-tiered signal transduction cascades which mediate rapid responses of eukaryotes to abiotic or biotic extracellular stimuli (**Fig. 9**) and thus are essential components of early defense mechanisms against abiotic stresses and of innate immunity (**Fig. 9**). Practically MAPK cascades underlie all described plant responses to physiological or developmental stimuli and this is the reason that MAPKKK, MAPKK and MAPK components of the module are so largely expanded (reviewed in Komis et al.,

2018a). The basic characteristics of plant MAPK modules are redundancy, cross-talk and networking. Many conditional or developmental roles of MAPK modules are exerted redundantly by different components. For example, stomatal ontogenesis in *Arabidopsis thaliana* that will be explained later requires the redundant function of four different MAPKKs namely MKK4, MKK5, MKK7 and MKK9 and two different but redundant MAPKs namely MPK3 and MPK6, all of which are under the control of one single MAPKKK, namely YODA (Bergmann et al., 2004; Wang et al., 2007b). During cross-talk, different pathways originating from different inputs or concluding to different outputs have shared components. For example MPK4 mediates responses to oxidative stress, but it is also required for cytokinetic progression (reviewed in Komis et al., 2018a). Cross-talk is frequently undesirable and for this reason MAPK modules with shared components but dedicated specificities are insulated by means of scaffold proteins (e.g., Cheng et al., 2015).

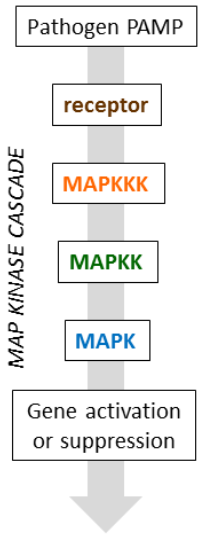

Fig. 9. Schematic depiction of MAPK involvement in innate immunity mechanisms as an example of their role in extracellular signal perception and transduction. After signal perception by some membrane receptor it is sequentially transduced from the MAPKKK, to the MAPKK and finally to the MAPK. The active MAPK phosphorylates and regulates a number of substrates including transcription factors leading to global transcription remodeling (From Pitzschke et al., 2009).

The activation of MAPKs requires the simultaneous phosphorylation of a threonine and a tyrosine residue that exist in a motif TXY within their activation loop and occurs downstream of a dual specificity MAPKK (i.e., with simultaneous Ser/Thr and Tyr phosphorylating activities), which is in turn activated by a MAPKKK (reviewed in Komis et al., 2018). Signal perception of extracellular stimuli by transmembrane receptors may be directed to MAPK modules directly or indirectly by other protein kinases (e.g., Kim et al., 2012), heterotrimeric GTPases (e.g., Cheng et al., 2015) or secondary signaling components such as bioactive phospholipids (e.g., Yu et al., 2010). The mid- to long-term goal of the activation of MAPK modules is transcriptional transactivation via the appropriate targeting of appropriate transcription factors, but in the meantime, active MAPKs may be involved to earlier cellular responses including the phosphorylation and regulation of cytoskeletal and other cytoplasmic proteins (reviewed in Šamajová et al., 2013; Komis et al., 2018).

Originally MAPKs were isolated as protein kinase activities associated with microtubule associated proteins in mammalian cells (Runge et al., 1981), but later it was realized that they are constituents of various signaling cascades pertinent to extracellular signal perception and transduction. In plants, MAPK cascades have been deciphered in numerous abiotic stress responses including the exposure to anisosmotic conditions, salinity, drought, temperature extremes and oxidative stress (reviewed in Smékalová et al., 2014) but they are also intimately associated with innate immune responses and plant reactions during their hazardous or beneficial interactions with other organisms including bacteria, fungi, nematodes and insects (Pitzschke et al., 2009).

In order to understand the involvement of MAPKs in the early steps of the plant hyperosmotic response, we followed the dose- and time- dependent activation of dually phosphorylated MAPKs in *T. turgidum* roots by means of Western blot analysis (Komis et al., 2004 **Included Publication 5**). Both the time and the dose-dependence of activated MAPK accumulation corresponded to the hyperosmotically-induced microtubule rearrangements that we reported previously (Komis et al., 2002b **Included Publication 3**). However the connection of MAPK signaling to the hyperosmotically-induced was better understood when plasmolysis was carried out in the presence of MAPK inhibitors. In this case, the inhibitor used, caused extensive reduction of the protoplast volume and severely compromised the hyperosmotically-induced cortical microtubule reorganization.

3.4.2. Phospholipases C

Phospholipases C (PLCs) and D (PLDs) are signaling enzymes which are hydrolyzing membrane lipids to produce other bioactive compounds. Phospholipase C, hydrolyzes the lipid 4,5-diphosphate phosphatidyl inositol (PIP_2), producing the water soluble 3,4,5-inositol triphosphate ($InsP_3$ or IP_3) and the hydrophobic diacylglycerol (DAG; **Fig. 10**). DAG remains embedded in the plasma membrane while $InsP_3$ freely diffuses in the cytoplasm (**Fig. 10**).

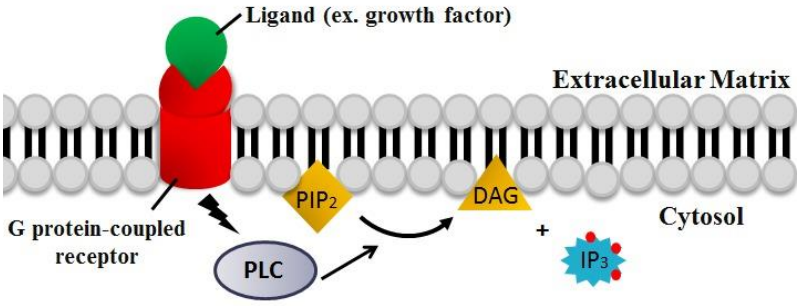

Fig. 10. Schematic depiction of PLC activation by extracellular stimuli via transmembrane G-protein coupled receptors, showing the substrate (PIP_2) and the products ($InsP_3$ and DAG; From https://en.wikipedia.org/wiki/Phospholipase_C).

PIP_2, $InsP_3$ and DAG are all considered to be secondary signaling mediators and underlie significant processes in signal transduction. PIP_2 can be used for the specific targeting of proteins that are recruited to PIP_2-rich areas of the plasma membrane and this is of particular importance for the

regulation of cortical cytoskeleton (Zhang et al., 2012). Such proteins contain special lipid binding motifs. The classical role of the (water soluble) $InsP_3$ is to mobilize calcium stores which are located in elements of rough endoplasmic reticulum (ER), via specific binding to $InsP_3$-receptors with selective Ca^{2+} channel activity (Berridge and Irvine, 1984; Putney, 1987). Finally DAG can interact with other proteins and modify their activity as it is routinely demonstrated by the DAG-mediated activation of the Ser/Thr protein kinase C (Berridge, 1984). The function of PLCs can be pharmacologically manipulated with more or less specific inhibitors such as U73122 and neomycin (see Komis et al., 2008 **Included Publication 6** and references therein).

The activation of PLC and the metabolic changes of its substrates and products are well correlated with plant stress responses and are regarded as components of the early transduction machinery (Hong et al., 2016). This together with the knowledge that many cytoskeletal proteins are regulated via interactions with PIP_2, prompted us to study possible functional relations between hyperosmotically-triggered PLC activation and microtubule reorganization in *T. turgidum* roots. PLC activation was addressed by appropriate enzymatic assay based on the rates of hydrolysis of ^3H-labeled PIP_2 and scintillation analysis of the concentration of the resulted tritiated $InsP_3$ (Komis et al., 2008 **Included Publication 6**). In this case we found that PLC activity was enhanced after the hyperosmotic treatment. Depolymerization of microtubules by oryzalin treatment also induced PLC activity in the absence of osmotic stress, while taxol stabilization of microtubules reduced PLC activity to control levels (Komis et al., 2008 **Included Publication 6**) providing evidence that microtubule integrity is somewhat related to PLC activity. The reverse possibility that PLC activity may be related to hyperosmotically-induced microtubule remodeling and to cell volume regulation was followed by applying hyperosmotic conditions in the presence of the PLC inhibitors U73122 and neomycin. In this case we found that both microtubule reorganization and the extend of plasmolysis were modified by such treatments (Komis et al., 2008 **Included Publication 6**).

The above study showed a reciprocal relation between hyperosmotically induced PLC activity and the response of cortical microtubules in plasmolysed root cells of *T. turgidum*. It seems that microtubule depolymerization at the very onset of plasmolysis is required for activation of PLC, while the activity of the latter is required for the proper formation of tubulin macrotubules that occurs slightly later.

3.4.3. Phospholipases D

Phospholipases D are enzymes which catalyze the transphosphatidylation of water to phosphatidic acid (PA) species using phosphatidylcholine (PC) as a donor (**Fig. 11**). In plants PLD species had been routinely associated with plant stress responses. More importantly PLD species were shown to interact with cortical microtubules (e.g., Gardiner et al., 2001; Angelini et al., 2018) while experimental manipulation of PLD activity using primary alcohols as alternative substrates for transphosphatidylation (**Fig. 11**) were shown to promote reorganization of cortical microtubules (Dhonukshe et al., 2003).

The production of PA may also implicate PLC signaling, since PA may be produced by phosphorylation of DAG by DAG kinases (Van Blitterswijk and Houssa, 2000). If this is the case, DAGKs may be specifically inhibited with the inhibitor R59022 (see Komis et al., 2008 **Included Publication 6** and references therein).

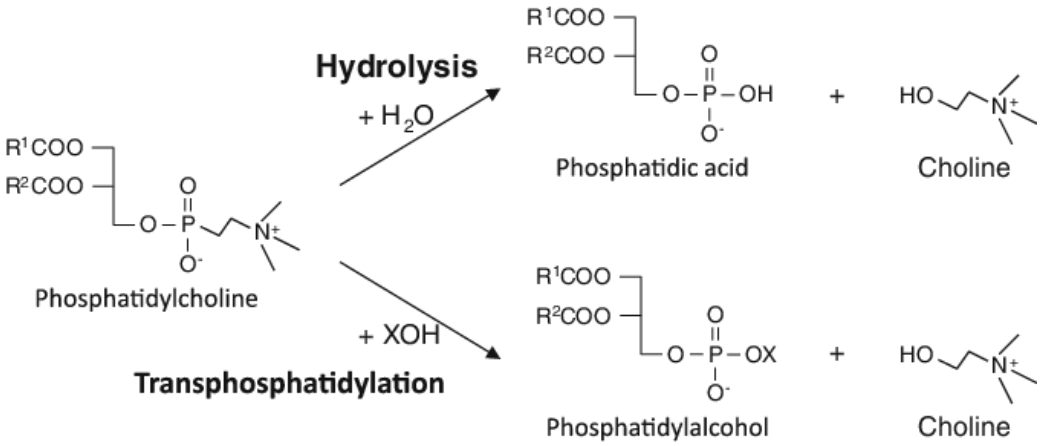

Fig. 11. PLD mediated hydrolysis of phosphatidylcholine and transphosphatidylation of water to PA species. By addition of primary alcohols, PLD activity is preferentially diverted to phosphatidylalcohol species (From Nakazawa et al., 2011).

For the above reasons we studied if and how the activation of PLD species may relate to cortical microtubule rearrangements under hyperosmotic conditions. The experimental approach was built around the application of butanol-1 as an alternative PLD substrate, or N-acylated ethanolamine (NAE) as a specific inhibitor (Motes et al., 2005; Nakazawa et al., 2011). The effects of these compounds on cortical microtubule organization were tested in both control and hyperosmotic conditions while also addressing their impact on cell viability and the extend of plasmolysis under hyperosmotic conditions (Komis et al., 2006 **Included Publication 7**).

The effects of butanol-1 on hyperosmotic stress-induced reorganization of cortical microtubule, showed that it compromised the formation of tubulin macrotubules and instead induced very large tubular structures and paracrystals which were positively labeled with tubulin antibodies (**Fig. 12**). Very frequently, fluorescent polymer masses of tubulin were localized within nuclei of butanol-1-treated plasmolyzed root cells. Notably the inactive isomers butanol-2 and tert-butanol had no effect on either cortical microtubules of control non-plasmolyzed cells or on the hyperosmotically-induced formation of cortical macrotubules (Komis et al., 2006 **Included Publication 7**).

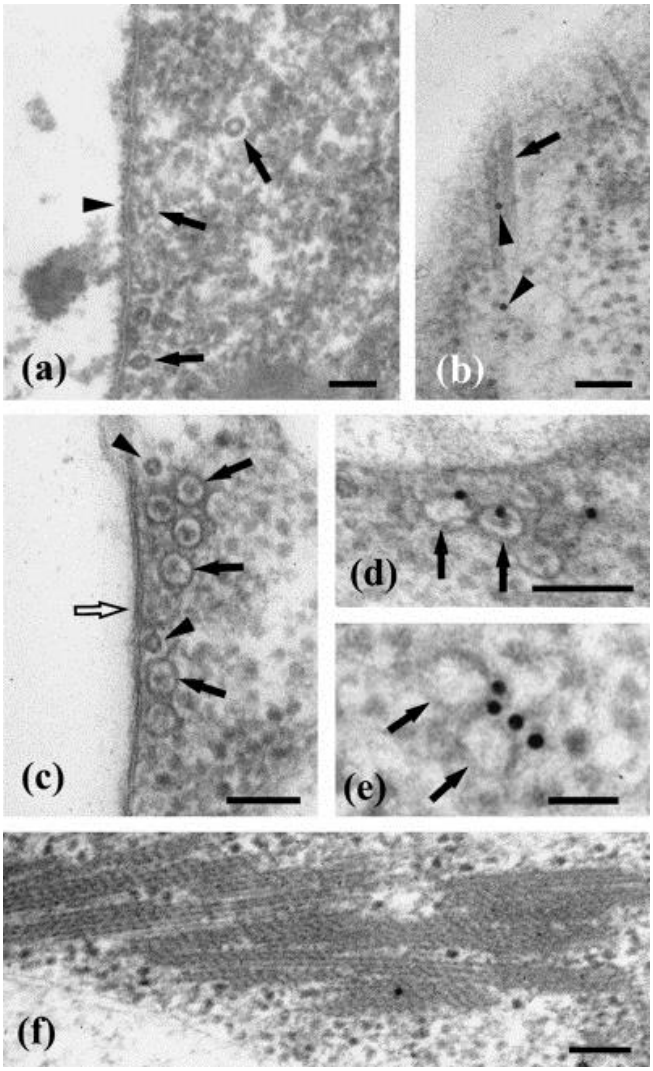

Fig. 12. Overview of the effects of butanol-1 on hyperosmotic stress-induced microtubule rearrangements in *T. turgidum* root cells as visualized by conventional TEM or after immunogold localization to tubulin. (a,b) Tubulin macrotubules in plasmolysed cells after conventional TEM (a) or immunogold (b) localization. (c-e) Aberrant butanol-1 induced tubular macromolecular assemblies of tubulin as visualized by conventional TEM (c) or after immunogold localization of tubulin (d,e). From Komis et al., 2008 **Included Publication 7**.

In physiological terms, butanol-1 caused dramatic reduction of the plasmolysed protoplast volume but did not compromise viability of plasmolysed cells in the short term (**Fig. 13**) however more extensive cell necrosis was documented at longer treatment periods (**Fig. 13**). On the other hand application of the more specific inhibitor NAE promoted extensive cell death in plasmolyzed cells, even at the early stages of the hyperosmotic treatment (**Fig. 13**).

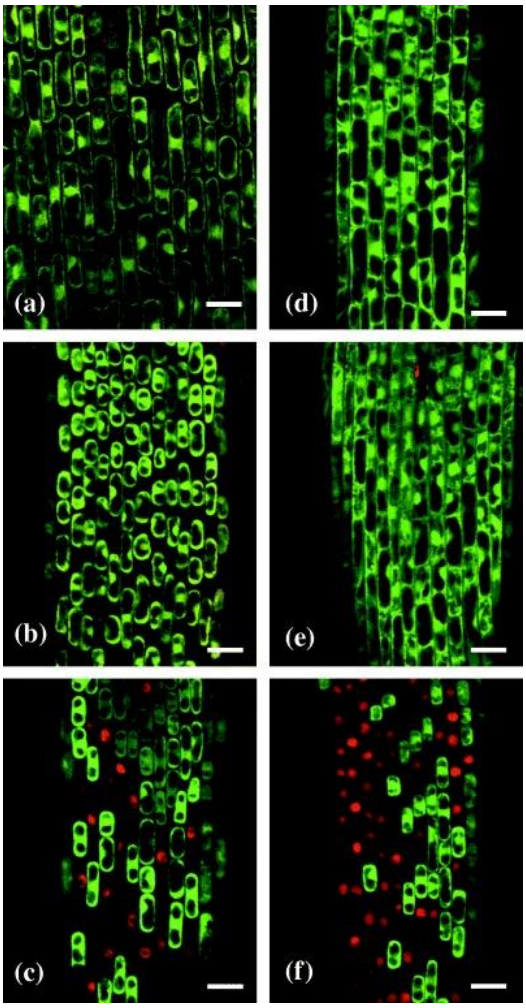

Fig. 13. Assessment of cell viability after plasmolysis in the presence or absence of butanol-1 or NAE by dual labeling with propidium iodide and fluorescein diacetate and confocal laser scanning microscopy (CLSM). (a) Control plasmolysed cells showing overall viability. (b,c) gradual compromise of the viability of cells in butanol-1-supplemented hyperosmotic medium after 30 min (b) or 2 hrs (c) of treatment. (d) non-plasmolysed cells remain viable after butanol-1 treatment. (e,f) NAE treatment does not affect cell viability in non-plasmolyzed cells (e) but induces massive cell death under hyperosmotic conditions (f). From Komis et al., 2008 **Included Publication 7**.

Western blot analyses of root extracts from experimental treatments of hyperosmotic stress in the presence or absence of butanol-1 showed that most likely PLD activity and PA production are somewhat related to the hyperosmotically-induced activation of MAPK species previously described (Komis et al., 2004 **Included Publication 5**; Komis et al., 2006 **Included Publication 7**).

3.4.4. PLD, PA and the microtubule cytoskeleton

Few later studies corroborated the importance of PLD activity in hyperosmotically-induced microtubule remodeling and cell viability under such conditions. As mentioned earlier, PLD-mediated PA production underlies enhanced association of MAP65-1 with microtubules and potentiates their bundling under conditions of high salinity (Zhang et al., 2012). Functional interactions of PLD activity and cortical microtubule organization are also related to more

physiological processes such as cell growth and differentiation (Zhang et al., 2017) and also to the integration of calcium signaling and microtubules during the ABA-induced stomatal closure (Jiang et al., 2014).

4. Microtubule organization and dynamics in plant growth and development

4.1. Introduction

As already mentioned, microtubules underlie all the major aspects of plant growth and development mostly by blueprinting the pattern of cellulose microfibril deposition in the overlying cell wall. Therefor cell growth and cell differentiation are intimately associated by cortical microtubule organization. Cortical microtubules are immediately responsive to extracellular cues such as light which tend to reprogram directionality of cell growth (Lindeboom et al., 2013) or developmental decisions which mediate the shift of cell growth directionality in response of hormonal signals (e.g., Chen et al., 2014). Therefore, cell growth is adjustable through signal transduction pathways that translate extracellular stimuli to directional control of cell growth and it is a question of whether cortical microtubules can be signaling targets in the process. For this reason, we studied microtubule organization in signaling mutants of *Arabidopsis thaliana* focusing on those related to MAPK signaling. The selection criterion was the growth phenotype of such mutants, in details that will be explained in the following sections.

4.2. A defined MAPK pathway regulates cortical microtubule organization in *Arabidopsis thaliana*

Arabidopsis double mutants of the ANP (Arabidopsis homologues of Nucleus and Phragmoplast localized protein kinase; e.g., *anp2anp3*) family of MAPKKKs exhibit robust cytokinetic phenotypes with incomplete formation of cell plates and the formation of multinucleated or polyploid cells (Krysan et al., 2002). These phenotypes are reminiscent to cytokinetic defects of the homologous Nucleus and Phragmoplast localized protein Kinase 1 MAPKKK (NPK1) of *Nicotiana tabacum* which were the first of their kind to be described (Nishihama et al., 2001). The NPK1 was studied in suspension cultured cells lacking the multicellular component of plant tissues (Nishihama et al., 2001). Therefor when the Arabidopsis homologues of NPK1 (ANPs) were studied in whole seedlings of appropriate mutants, growth deficiencies were revealed which could not be explained merely by assuming restricted defects in cytokinesis. In brief, vegetative organs such as the hypocotyl and the root exhibit considerable radial swelling which is similar to defects of such organs following prolonged treatments with microtubule drugs, or of cytoskeletal mutants devoid of or defective in the function of microtubule associated proteins. Strikingly similar phenotypes were recorded for another Arabidopsis mutant, namely *mpk4* which is deficient in the MPK4 MAPK (**Fig. 14**).

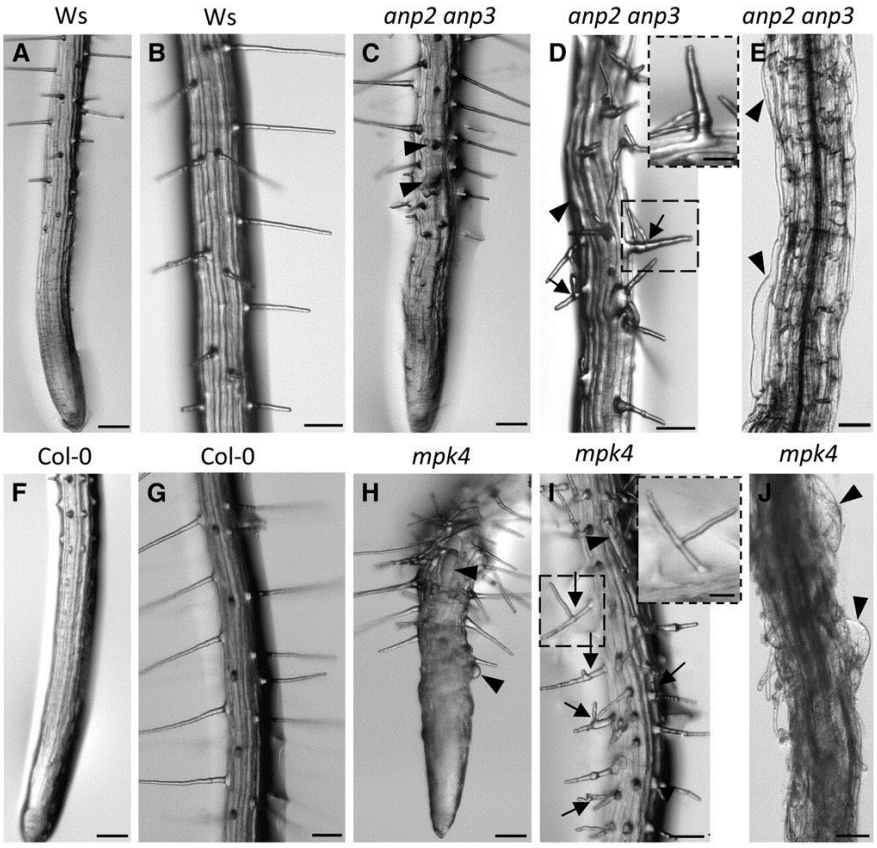

Fig. 14. Documentation of root growth phenotypes of *anp2anp3* and *mpk4 Arabidopsis thaliana* mutants by comparison to the respective Wassilevskaya (Ws) and Columbia (Col-0) phenotypes. (A, B) Overview of the appearance of Ws roots. (C-E) Ectopic and/or branched root hair formation (C,D) and cell bulging in the *anp2anp3* mutant. In both cases *anp2anp3* and *mpk4* mutants exhibit root swelling and ectopic and branched root hair formation. (F,G) Overview of *Arabidopsis thaliana* Col-0 root. (H-J) Root swelling, ectopic and branched root hair formation and cell bulging in the primary root of the *mpk4* mutant (From Beck et al., 2010 **Included Publication 8**).

Perturbation of cortical microtubule organization by any pharmacological or genetic means, is regularly resulting in radial swelling of vegetative organs since in the absence of cortical microtubules or under their inability to reorient, cell growth is prevalently isotropic (Baskin et al., 1994). For this reasons, we characterized microtubule organization in selected cell types of *anp2anp3* and *mpk4* Arabidopsis mutants.

In this case, we found that epidermal cells of the hypocotyl and of the root exhibit randomly organized and highly bundled cortical microtubules. Cortical microtubule defects in both mutants were originally observed in epidermal cells of roots, hypocotyls and cotyledons, appropriately expressing a GFP-MBD microtubule marker (**Fig. 15**) which allowed us to observe microtubule organization in living material.

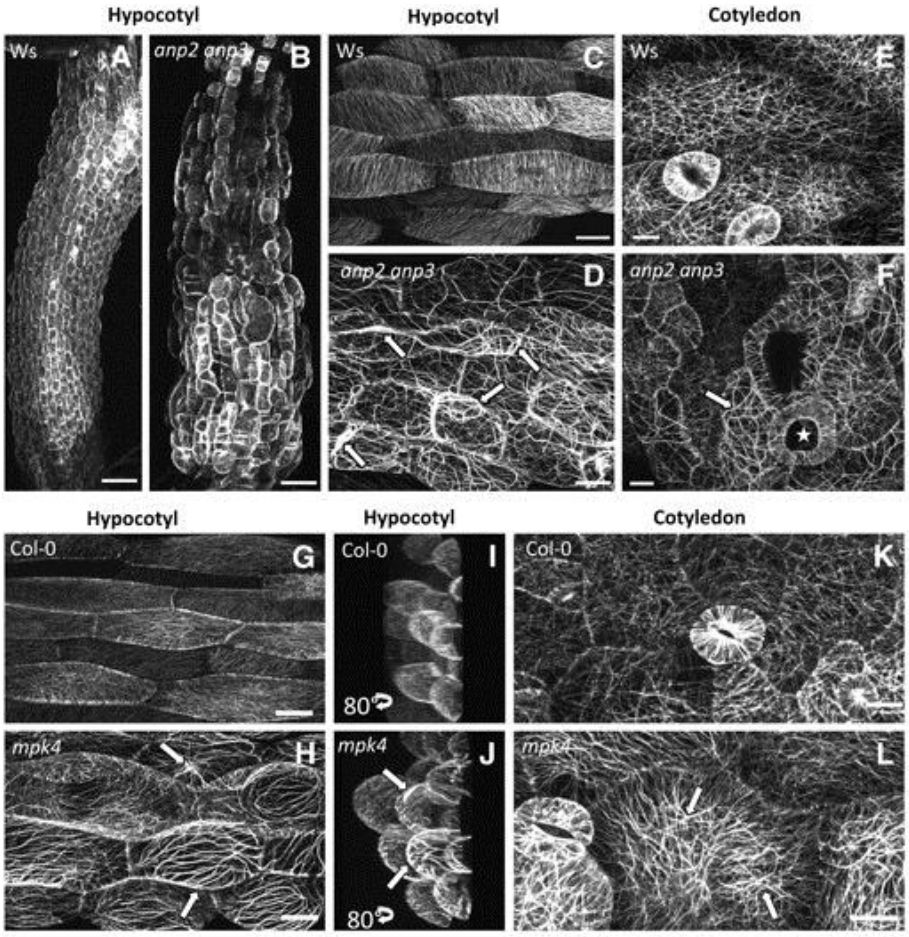

Fig. 15. *In vivo* localization of microtubules in epidermal cells of hypocotyls, cotyledons, and roots using the GFP-MBD microtubule marker in *anp2anp3* and *mpk4* mutants. (A-F) Visualization of cortical microtubule organization in the hypocotyl and cotyledon of wild type and mutant plants. Note the extensive bundling and the irregular configuration of the bundles (D,F, arrows). (G-L) Cortical microtubule organization in the hypocotyl and cotyledon of Col-0 and *mpk4* plants showing cortical microtubule bundling in *mpk4* plants. (M-Q) Cortical microtubule organization organization in root hairs of wild type and mutant plants. Mutant root hairs exhibit abnormal morphology, branched (N,Q, arrowhead), or swollen root hair bases (P) concomitant to microtubule bundling (arrows) or disturbed microtubule orientation compared with wild-type root hairs (M,O). (R-U) Cortical microtubule organization in root epidermal cells of wild type and mutant plants (From Beck et al., 2010 **Included Publication 8**).

Microtubules within bundles showed extensive crosslinking by electron dense crossbridges when visualized by TEM. Since it is known that the major crosslinker of cortical microtubules is the microtubule associated protein MAP65-1 (and its redundant homologue MAP65-2; Lucas et al., 2011, 2012) we examined its abundance by means of Western blot and we found that it is drastically upregulated in both *anp2anp3* and *mpk4* mutants. By comparison, the cell division specific homologue MAP65-3 is downregulated, possibly explaining the cytokinetic defects observed in both *anp2anp3* and *mpk4* mutants (Krysan et al., 2002; Kosetsu et al., 2010). Upregulation of MAP65-1 suffices to explain cortical microtubule bundling (Mao et al., 2006). However, MAP65-1 is also target of *MPK4*-mediated phosphorylation at its microtubule binding carboxyl terminal domain

(Smertenko et al., 2006). Phosphorylation of MAP65 proteins at their microtubule binding domain is a general mechanism of negative regulation of their microtubule binding and for this reason we addressed MAP65-1 phosphorylation levels in both *anp2anp3* and *mpk4* mutants by means of Phos-Tag pendant SDS-PAGE (Beck et al., 2010 **Included Publication 8**; Bekešová et al., 2015 **Included Publication 9**) and subsequent western blot analysis. In this case, abolishment of ANP2, ANP3 and *MPK4* activities in the respective mutants resulted in diminished phosphorylation of MAP65-1.

Drug treatments with the microtubule depolymerizing agent oryzalin showed that bundled microtubules of *anp2anp3* and *mpk4* mutants were considerably resilient, suggesting that apart from affecting cortical microtubule dynamics, deficits in the MAPK module may also affect microtubule dynamics. The technical problem that arose during the early attempts of following the dynamics of microtubules in such mutants related to the inability of diffraction limited microscopy methods such as CLSM or spinning disc microscopy to track individual microtubules within bundles. For this reason we employed structured illumination microscopy (SIM) a superresolution method that allows imaging at ca. 100 nm resolution and superior contrast compared to the aforementioned methods (Gustafsson, 2000).

By means of SIM, it was possible to track end dynamics of individual or bundled microtubules of the *mpk4* mutant using appropriate kymographic analysis (Komis et al., 2014 **Included Publication 10; Fig. 16**). In this case, dynamics and especially plus end growth and shrinkage of both individual and bundled microtubules were found to be significantly reduced in the *mpk4* mutant compared to the Col-0 wild type.

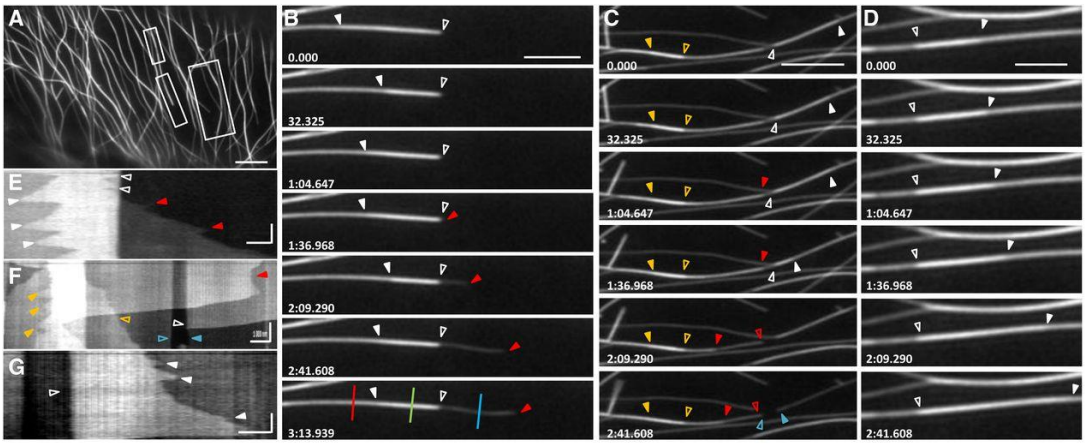

Fig. 16. Documentation of intrabundle microtubule dynamics in hypocotyl epidermal cells of the *mpk4* mutant of *Arabidopsis thaliana* by means of SIM. A. Overview. (B-D) Selected stills from different microtubule bundles of (A). (E-G) The respective kymographs from (B-D; From Komis et al., 2014 **Included Publication 10**).

Apart from allowing the dissection of microtubule dynamics in the *mpk4* mutant this study (Komis et al., 2014 **Included Publication 10**) was the first time lapsed imaging of cortical microtubule dynamics using SIM. Since then we established its validity for superresolution studies not only of microtubules but also of other subcellular structures and organelles of diverse cells in living *Arabidopsis thaliana* seedlings expressing appropriate fluorescent protein markers (e.g., **Fig. 17**;

Komis et al., 2015 **Included Publication 11**, Komis et al., 2018b **Included Publication 12**). Within limits we showed that SIM can be used to deliver superresolution images of high quality, but also to track the dynamics of diverse structures including cortical actin and ER (Komis et al., 2018b **Included Publication 12**).

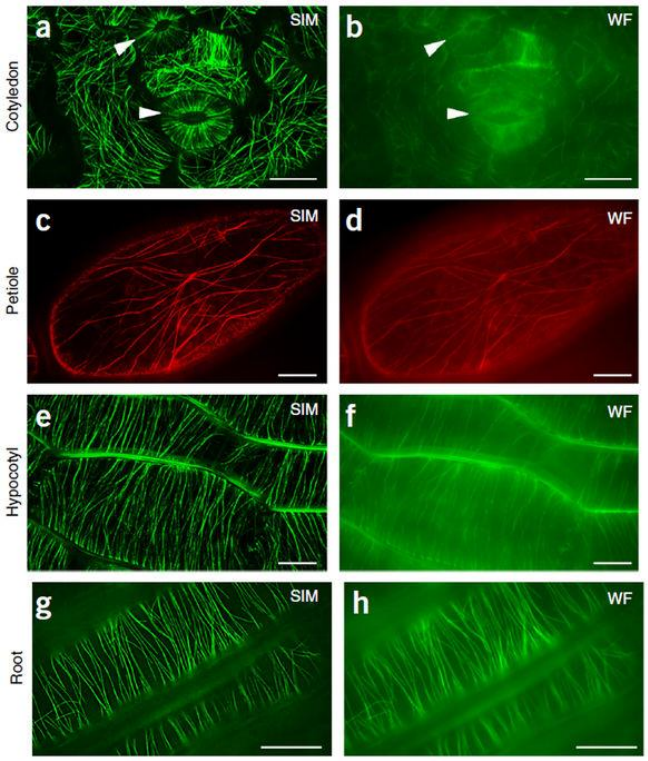

Fig. 17. Visualization of cortical microtubules in epidermal cells of cotyledons, petioles, hypocotyls and roots by means of SIM and widefield epifluorescence microscopy using different microtubule markers such as GFP-MBD, RFP-TUA5 and GFP-TUA6 (From Komis et al., 2015 **Included Publication 11**).

4.2.1. Interplay between MAPKs and MAPs in the regulation of the cortical microtubule array

Our studies in the *Arabidopsis thaliana anp2anp3* and the *mpk4* mutants revealed a tight connection between MAPK signaling and the cortical microtubule cytoskeleton which is so far restricted to microtubule bundling regulation. MAP65 proteins emerged as examples of microtubule binding affinity regulation through reversible phosphorylation. The carboxyl terminal domain of MAP65 proteins contains target motifs for MAPK phosphorylation which comprise of proline directed serine and/or threonine residues (S/TP motifs; Komis et al., 2018a) and are commonly targeted by protein kinases related to cell cycle regulation. In this context MAP65-1 was found to be phosphorylated at least in vitro by the MPK4 and MPK6 MAPKs and by cyclin dependent kinases (CDKs; Smertenko et al., 2006) while recently it was found to be also targeted and phosphorylated by Aurora kinases (Boruc et al., 2017).

4.3. Relevance of MAPK activity to mitosis

As described before, the tobacco homologue of ANPs (NPK1) defines a specific MAPK cascade related to the progress of cytokinesis. The tobacco module includes NPK1 MAPKKK, NQK1 MAPKK and NRK1 MAPK. Arabidopsis homologues are the ANP MAPKKKs, the MKK6 MAPKK and the MPK4 MAPK (Takahashi et al., 2010). The module remains inactive prior to cytokinesis via an autoinhibitory fold of NPK1 which brings its carboxyl terminus in contact with the catalytic domain. This autoinhibitory interaction is raised late in mitosis through the interaction of NPK1 (and ANPs) with the kinesin motor proteins NACK1 and NACK2 (HINKEL). The module is then rendered active and becomes transported to the phragmoplast midplane via the motor activity of NACK1/2 (Ishikawa et al., 2002; Nishihama et al., 2002). Thereon, the activated module phosphorylates and regulates three members of the MAP65 family (namely MAP65-1, MAP65-2 and MAP65-3; Sasabe et al., 2011) and the lipid binding protein PATELLIN2 (Suzuki et al., 2016).

In our studies we observed the occurrence of enlarged multinucleated cells in both *anp2anp3* and *mpk4* mutants and for this reason we documented the course of mitosis and cytokinesis in living mutant seedlings expressing a GFP-MBD microtubule marker. Our results showed that although both the mitotic spindle and the phragmoplast form normally, the processes of mitosis and cytokinesis proceed with considerably reduced rates, which in the latter case may lead to abortive cell plate formation (**Fig. 18**; Beck et al., 2011 **Included Publication 13**). It is notable that although cytokinetic regression was shown before (Kosetsu et al., 2010), the mitotic delay was noted in our study suggesting that the ANP – MKK6 – MPK4 pathway may assume roles before phragmoplast formation and be somehow implicated in mitotic spindle formation and the dynamics of sister chromatid segregation.

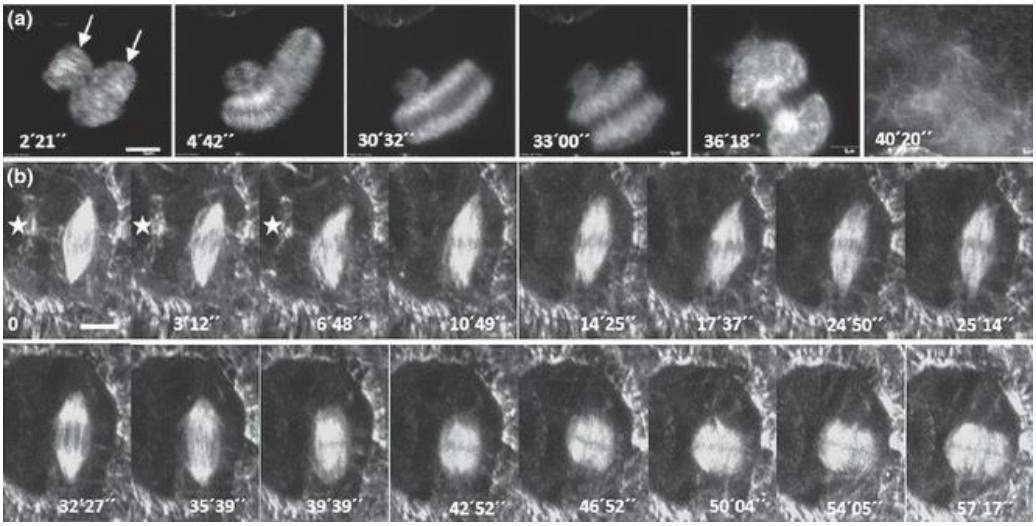

Fig. 18. Mitotic and cytokinetic deregulation in the *anp2anp3* mutant documented by time lapsed imaging. (a) Shows two synchronous spindles with the lower one corresponding to polyploid state. (b) Shows the dramatically delayed mitotic and cytokinetic course (From Beck et al., 2011 **Included Publication 13**).

Abortive cytokinesis in both *anp2anp3* and *mpk4* mutants will lead to the formation of multinucleated cells as evidenced by the routine observation of large coenocytes bearing numerous

cell wall stubs and nuclei of variable size (Beck et al., 2011 **Included Publication 13**). When such multinucleated cells entered mitosis, we frequently observed the formation of multiple mitotic spindles which occasionally could fuse to a single one pending on their proximity (**Fig. 19**).

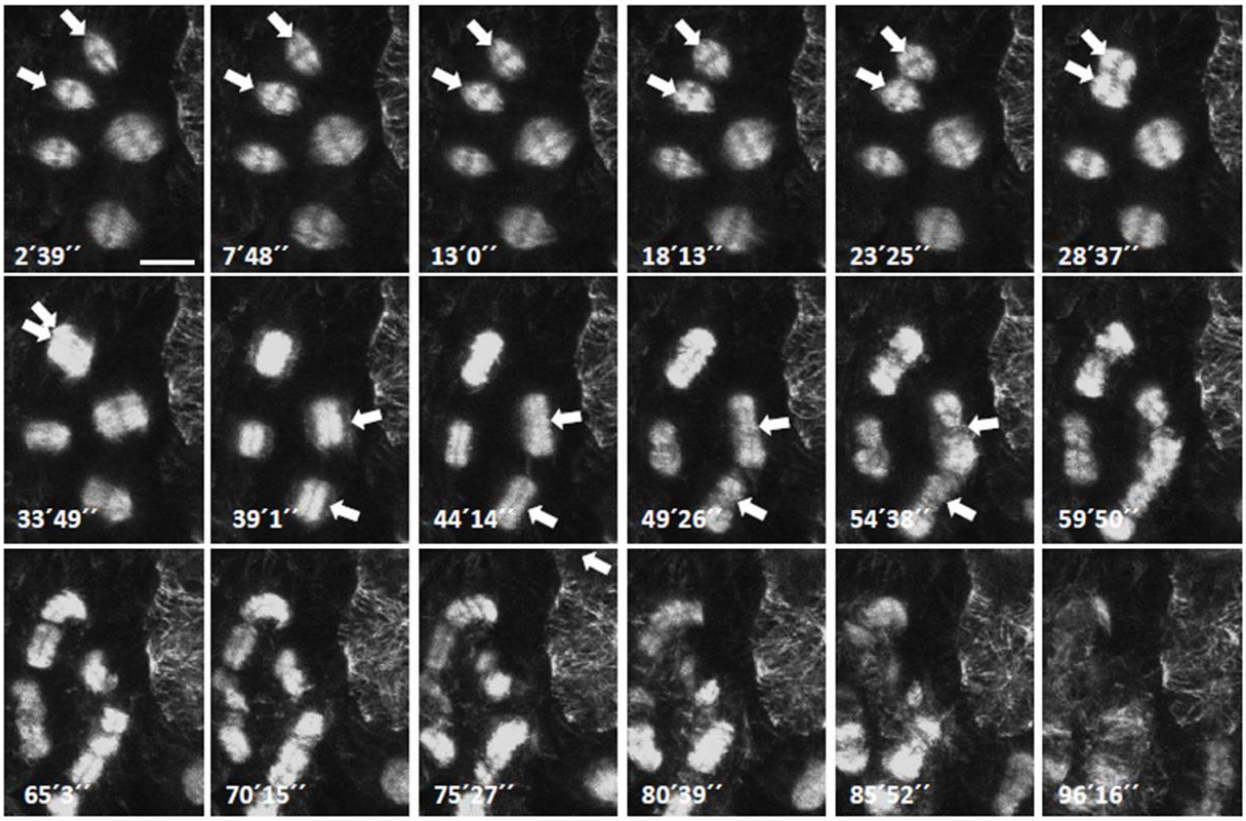

Fig. 19. Mitotic and cytokinetic progress in a multinucleated, cotyledon pavement cell of *Arabidopsis thaliana anp2anp3* mutant, showing the formation of 5 synchronous mitotic spindles. Progressively the two top left of them fuse to a single one, while later on the two bottom right phragmoplast also join during the course of cytokinesis (From Beck et al., 2011 **Included Publication 13**).

4.4. Relevance of MAPK activity to cell division plane orientation

However the cortical microtubule organization is of key importance to larger scale developmental events such as tissue patterning and organ differentiation and shaping, since they underlie cell division plane (CDP) orientation and polarity of cell division. The principal role of cortical microtubules in such cases, involves the formation of a premitotic cortical microtubule annulus which is called the preprophase microtubule band (PPB; Lipka et al., 2015). The role of the PPB in the definition of CDP has fascinated plant cell biologists ever since its discovery by Jeremy Pickett-Heaps (Pickett-Heaps and Northcote, 1966) and although many aspects of its function have been elucidated over the years, its precise role remains unresolved. What is known is that the PPB coincides with the plane of centrifugal expansion of the cytokinetic phragmoplast during cell plate deposition. This is somehow related to the deposition of molecular imprints at the cortical division zone, long after the decomposition of the PPB which takes place shortly after the onset of mitosis.

These cortical division zone markers, are diverse proteins with or without microtubule binding capacity. Such proteins include TANGLED (Walker et al., 2007), FASS/TONNEAU (Camilleri et al., 2002), PHRAGMOPLAST ORIENTING KINESINS 1/2 (POK1/2; Müller et al., 2006), and the respective mutants show phenotypes consistent with cell division plane orientation defects.

4.4.1. MPK6 downstream of YODA regulates tissue patterning in the root

Cell division plane (CDP) orientation in plants is a major determinant of cell specification, tissue patterning and organ shape and development in the absence of cell motility. Examples of how decisive CDP positioning can affect cell fate can be found in embryo development, the ontogenesis of the stomatal lineage and the tissue architecture of the root among others.

In this respect, stomatal ontogenesis emerged as a model of how MAPK cascades may be implicated in cell fate decisions. The stomatal lineage during leaf epidermis tissue patterning is specified by the differential regulation of three paralogous basic helix-loop-helix transcription factors which are SPEECHLESS, FAMA and MUTE (reviewed in Komis et al., 2018a). These transcription factors are transcriptionally or post-translationally regulated by a MAPK cascade that encompasses YODA (MAPKKK), MKK4,5,7 and 9 (MAPKKs) and MPK3,6 (MAPKs; Bergmann et al., 2004; Wang et al., 2007b). Components of the same pathway and particularly YODA and MPK6 were further shown to regulate cell division patterns in the developing embryo (Lukowitz et al., 2004). In both stomatal and embryo development, the YODA pathway is related to the positional control of cell division. Since CDP determination and establishment are also crucial in vegetative tissue patterning, we examined the role of components of the YODA pathway in the development of the root.

Our first study was conducted in three allelic *mpk6* mutants of Arabidopsis which showed aberrant cell file organization in the primary root and the occurrence of oblique cell walls suggesting defects in CDP orientation. Consistent with its putative role in CDP orientation, MPK6 was found to localize both in the PPB and the phragmoplast (Müller et al., 2010 **Included Publication 14**).

To further build on these results we expanded our studies to loss-of-function and gain-of-function mutants of YODA (designated *yda* and *ΔNyda* respectively) which were previously shown to exhibit abnormal stomatal and embryo development (Bergmann et al., 2004; Lukowitz et al., 2004). Such mutants showed compromised development and aberrant tissue patterning of the primary root. These defects were concomitant to the observation of oblique cell plates in all root cell files and consistent with the respective formation of oblique PPBs and phragmoplasts (Smékalová et al., 2014 **Included Publication 15**).

Subsequent experiments showed that the relation of MPK6 to CDP orientation is likely occurring at two levels. Directly, with the association of MPK6 with PPBs and phragmoplasts as shown before, and through interactions with cytoskeletal proteins such as MAP65-1 (Smékalová et al., 2014 **Included Publication 15**) and EB1c (Kohoutova et al., 2015), or indirectly through the transcriptional regulation of CDP marker proteins such as TANGLED and POK1 kinesin (Smékalová et al., 2014 **Included Publication 15**).

MPK6 localization to PPBs and phragmoplasts was seemingly dependent on MPK6 activation since cytoskeletal structures are devoid of MPK6 in *yda* while MPK6 is significantly enriched in *ΔNyda* (**Fig. 20**) consistent with the localization of activated MAPK species with mitotic and cytokinetic microtubule systems reported before (Beck et al., 2011 **Included Publication 13;** Winnicki et al., 2015).

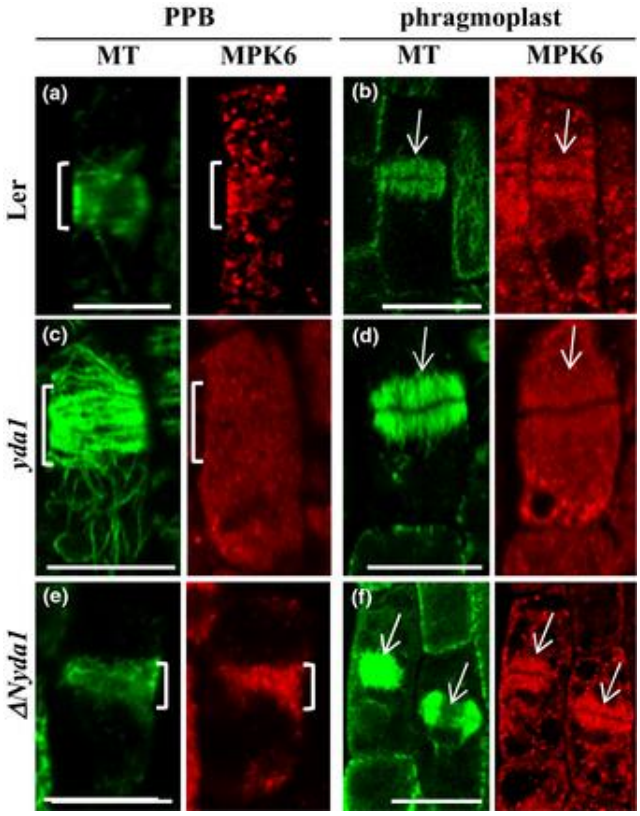

Fig. 20. Differential localization of MPK6 in PPBs and phragmoplasts of *Arabidopsis thaliana* ecotype Landsberg erecta (wild type), yda and *ΔNyda*. The general trend shows depletion of MPK6 from PPBs and phragmoplasts in the loss of function *yda* mutant and high enrichment in the constitutively active *ΔNyda* suggesting an activity based mechanism of MPK6 recruitment to these structures (From Smékalová et al., 2014 **Included Publication 15**).

5. Conclusions and future directions

Conditional and developmental rearrangements of microtubules and actin microfilaments are always inducible by extracellular stimuli. Major mechanisms like microtubule bundling by members of the MAP65 family, control of plus end dynamics by EB1c or severing by the microtubule severing protein KATANIN1 are considered targets of several signaling pathways leading to microtubule reorientations (e.g., Beck et al., 2010 **Included Publication 8**; Beck et al., 2011 **Included Publication 13**; Lindeboom et al., 2013; Smékalová et al., 2014 **Included Publication 15**; Kohoutova et al., 2015). Additionally the actin cytoskeleton can be likewise modified through the targeting of proteins like profilin and capping protein (Limmongkon et al., 2004; Li et al., 2012; Pleskot et al., 2012, 2013). In this way signaling pathways are integrated with microtubules and actin

microfilaments during conditional cellular responses or during developmental processes such as cell growth and differentiation and cell division.

To further understand how this integration is materialized focus must be given to a broader identification of cytoskeletal proteins that are potential targets of reversible phosphorylation or interaction with other signaling components like bioactive lipids. Software based prediction strategies (e.g., see Šamajová et al., 2013) or searches in existing databases may provide a clue as to which proteins are amenable to signaling based regulation.

Such an example is KATANIN1 of *Arabidopsis thaliana* which was predicted to be phosphorylated by MAPKs (Šamajová et al., 2013). Given the established importance of KATANIN1 in cell growth directionality related cortical microtubule reorientation (Lindeboom et al., 2013) and its newly identified role during PPB formation and maturation as well as in mitotic and cytokinetic progression (Komis et al., 2017), it is important to understand whether KATANIN1 may be targeted by reversible phosphorylation in the regulation of the above processes. From mammalian systems it is known that phosphorylation of the p60 catalytic subunit of katanin, is implicated in mitotic spindle sizing and it is mediated by Aurora kinases (Loughlin et al., 2011). Similar functions are expected to be deciphered in plants.

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7. List of abbreviations

AAA-ATPase	ATPases Associated with diverse cellular Activities
ANP	Arabidopsis homologue of Nucleus and Phragmoplast localized Protein kinase
BDM	Butanedione monoxime
CDP	Cell Division Plant
CDZ	Cortical Division Zone
CESA	Cellulose Synthase
DAG	Diacylglycerol
DAGK	Diacylglycerol kinase
DAPI	4',6-diamidino-2-phenylindole
EB1	End Binding protein 1
ER	Endoplasmic Reticulum
ERK1/2	Extracellular signal Related Kinase1/2
fABD2	Fimbrin Actin Binding Domain 2
GFP	Green Fluorescent Protein
GTPase	Guanosine Triphosphatase
IP3 (or InsP3)	Inositol Triphosphate
JNK	cJun N-terminal Kinase
MAP4	Microtubule Associated Protein 4
MAP65	Microtubule Associated Protein 65
MAPK(KK)	Mitogen Activated Protein Kinase (Kinase Kinase)
MBD	Microtubule Binding Domain
MKK6	Mitogen Activated Protein Kinase Kinase 6
MKP1	MAPK Phosphatase 1
MPK4	Mitogen Activated Protein Kinase 4
MPK6	Mitogen Activated Protein Kinase 6
NACK1	NPK1-ACTIVATING KINESIN 1
NAE	N-acylethanolamine
NPK1	Nucleus and Phragmoplast localized Kinase
NQK1	Next kinase after NPK1. Q is next to P in alphabet
NRK1	Next kinase after NQK1. R is next to Q in alphabet
p38	Mammalian stress activated protein kinase
PA	Phosphatidic Acid
PC	Phosphatidylcholine
PD 98059	MAPKK inhibitor
PIP2	4,5 biphosphate phosphatidyl inositol

POK1/2	Phragmoplast Orienting Kinesin ½
PPB	Preprophase microtubule Band
R59022	DAGK inhibitor
RFP	Red Fluorescent Protein
RVD	Regulatory Volume Decrease
RVI	Regulatory Volume Increase
SB 203580	p38 Inhibitor
Ser	Serine
Thr	Threonine
TUA6	Alpha Tubulin 6
Tyr	Tyrosine
U0 126	MAPKK inhibitor

8. List of publications included in habilitation thesis

Included Publication 1. **Komis G**, Apostolakos P, Galatis B. 2001. Altered patterns of tubulin polymerization in dividing leaf cells of Chlorophyton comosum after a hyperosmotic treatment. New Phytologist 149: 193-207

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Included Publication 5. **Komis G**, Apostolakos P, Gaitanaki C, Galatis B. 2004. Hyperosmotically induced accumulation of a phosphorylated p38-like MAPK involved in protoplast volume regulation of plasmolyzed wheat root cells. FEBS Letters 573: 168-174

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