University of South Bohemia
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Role of MAPKs (mitogen activated protein kinases) in regulation of nucleation and organization of microtubules

Bachelor thesis

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Annotation:

The aim of the thesis is to review the current knowledge about MAPKs and their link to cytoskeleton namely to organization and nucleation of microtubules. In the experimental part I worked out scheme for activation of MAPKs and for drug induced inhibition. Specifically activation by hypo-osmotic shock and inhibition by U0126 were optimized in *Arabidopsis thaliana* cells and seedlings and detected by western blotting using antibodies recognizing specific active phosphorylated forms of MAPKs.

Praha 22. 4. 2013   ………………………….

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Acknowledgements:

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1. Aim of the study

The aim of this thesis is to review the current knowledge about MAPKs and their link to cytoskeleton specifically to organization and nucleation of microtubules. Special attention is paid to MPK6 and its interaction with microtubules. In the experimental part I focused on testing and optimizing conditions for activation of MAPK by hypo-osmotic shock and inhibition by U0126 in *Arabidopsis thaliana*. The activation or inhibition is monitored by hyper or hypo phosphorylation of MAPKs, respectively which were detected by western blot using specific antibodies. Further I aimed to derived stable cell lines expressing C terminal GFP fusion of TubG1 (p35S::TubG1-GFP) by means of *Agrobacterium tumefaciens* mediated transformation of cells of *Arabidopsis thaliana*. Experiments were part of complex research on MAPK cell signaling to MT cytoskeleton performed in laboratory of functional cytology MBÚ AVČR guided by doc. RNDr. Pavla Binarova, CSc.

2 Introduction

2.1 Preface

Active movement is one of the fundamental attributes of life. There are multiple levels at which we can observe movement: cell, organ, organism level. One of the basic mechanisms that lies behind movement in living organisms is cytoskeleton. Cytoskeleton presents a dynamic structure that provides not only movement for cells but also structural support of the cell shape. Cytoskeleton comprises microtubules (MTs), microfilaments and intermediate filaments. MTs and microfilaments arrange movement of substances inside the cell and also movement of the whole cell. MTs are part of cytoskeleton in all eukaryotic cells. They function as a platform for cellular signaling but also guarantee locomotion of organelles and they are incorporated in cell projections, flagella, cillia and spindle bodies.

The other most fundamental attribute of life is the ability to respond to signals. Organisms created a complex communication system that governs basic cellular activities and coordinates cell actions, movement being one of them. The correct perception of signals is the basis of development, tissue repair and immunity as well as normal tissue homeostasis. Errors in processing cellular signals lead to diseases such as cancer or autoimmunity.

2.2 Cytoskeleton

Cytoskeleton is a dynamic system with the ability of flexible reaction to changes within cell and the cell environment. Constantly active three-dimensional system consists of proteins, which can polymerize into fibrous or globular structures.

Cytoskeleton is present in all eukaryotic cells. However, certain genes which code cytoskeletal proteins have proven prokaryotic origin. Amino acid sequences of these genes and tertiary structures of cytoskeletal subunits are very conservative among all organisms. This fact points at the universality and indispensability of cytoskeleton for eukaryotes and necessity of analogously functioning structure for some prokaryotes.

Cytoskeleton is important in cell division, cell growth, transport and locomotion of cells and organelles. It gives the cells or organelles solidity (e.g. interface nucleus) and flexibility. Cytoskeleton guarantees the position of the organelles (e.g. chloroplast according to light intensity) and cytoplasmic flow. The function of cytoskeleton is to mediate signal transduction and transfer of building material. It is a place where interactions among proteins occur since it serves as a frame of the cell with the ability to bind various proteins.

2.3 Types of cytoskeletal fibers

In eukaryotic cells there are multiple types of protein fibers: actin filaments, MTs and intermediary filaments. Plants have just two types of cytoskeletal fibers: MTs and microfilaments. Presence of intermediary filaments is still questionable. Both MTs and microfilaments comprise globular proteins. Microfilaments consist of actins whereas MTs of tubulins.

Microfilaments are the finest out of cytoskeletal fibers. They are 8 - 10 nm wide and are found in bundles, networks or branched on cortex or associated with MTs. Fibers of microfilaments monitor the flow of cytoplasm. Actin interacts with more than 50 different actin binding proteins (ABP). A well-known molecular motor, the myosin, also contributes to active transport inside the cell. In animal cells myosin is responsible for muscle contraction.

MTs are hollow tubes with a diameter of 24 nm. The basic building blocks are tubulin units comprising α- and β-tubulin. One MT is a circle which possess 13 tubulin units where always α-tubulins are next to each other as well as β tubulins. Dimers of tubulins form long chains called protofilaments where α-tubulin binds β-tubulin. Protofilaments have α-tubulin at one end and β-tubulin at the other end.

For eukaryotic cell, the most crucial is coordination, cooperation and regulation of microtubular and actin cytoskeleton. Actin cytoskeleton mainly mediates flow of cytoplasm however, concerning stabilization of cytoplasmic organelles, actin cytoskeleton cooperates with microtubular (Dhonukshe et al., 2005).

2.4 Microtubules

MTs are highly dynamic polymers, which take part in many physiological processes within cell such as signal transduction, vesicular transport, shape and motion of the cell, cell division, apoptosis and polarity (Valiron et al., 2001).

MTs are unstable; they are altering between period of growth and shrinkage (Vantard et al., 2000). This phenomenon is typical for MTs and it is called "the dynamic instability" (Waterman-Storer and Salmon, 1997). Dynamic instability is based on the ability of tubulin units to bind and hydrolyze guanosine-5'-triphosphate (GTP). During the process of polymerization, tubulin units are added at the fast growing (+) ends of MTs and simultaneously depleted from the slower growing (-) ends. Structure of MTs is stabilized with blocks of tubulin units with GTP bound to (+) end of MTs, so called GTP binding pocket that protects MTs from degradation. Loss of GTP binding pocket results in fast

depolymerization, which leads to shrinkage and destabilization (Mandelkov et al., 1991). Dynamic instability is connected with a term "treadmilling" (Waterman-Storer and Salmon, 1997). During this process the protofilament grows at one side and depletes at the other which make the impression of protofilament moving across cytosol. This is caused by constant addition and removal of α, β-tubulin dimers at the ends of protofilament. To demonstrate the dynamics of MTs which is one of the most fundamental properties worth emphasizing, I present the following data on the velocity of growth and depletion. The speed of MT growth was 5 μm min^{-1} in root hair and the depletion even reached 7 μm min^{-1}. Taking into account that one tubulin dimer has about 8 nm, this speed represent approximately 600 - 900 tubulin dimers.

MTs are negatively charged therefore the electrostatic forces play pivotal role. Positively charged amino acids of MT binding proteins (MBPs) can interact with negatively charged phospho moieties of MTs. Phosphorylation of MBP at their MBP-domains prevents MBP from binding serving as a negative regulatory mechanism (Drewes et al., 1998).
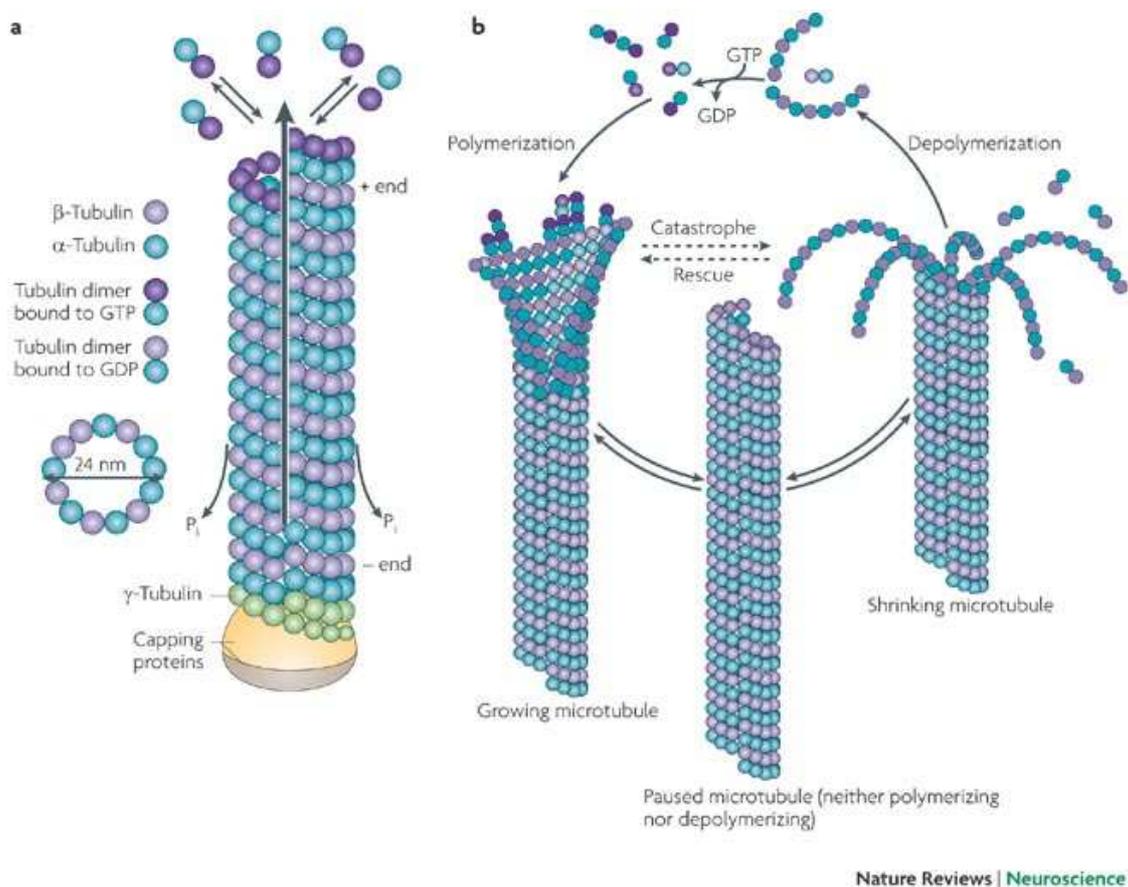
Fig. 1: MT polymerization/depolymerization; Tubulin dimers with GTP are added at the (+) end of MTs. (Conde and Caceres, 2009)

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2.5 Tubulins

MTs belong to the tubulin superfamily. The involvement of tubulins in wide range of cellular processes was already recognized in the early studies and led to formulation of multitubulin hypothesis (Fulton and Simpson, 1976). This hypothesis took into account the diversity of tubulins and addressed diverse types of tubulins different cellular functions. Up to date, 9 diverse tubulin types were identified: α, β, γ, δ, ε, ζ, η, θ, ι and κ (Libusova and Draber, 2006).

α, β and γ-tubulin are found in all eukaryotic cells and are essential for MT polymerization. Minor types of tubulins are also part of tubulin superfamily however, neither their presence among eukaryotes nor their function is universal.

δ-tubulin was first identified in alga *Chlamydomonas reinhardtii* as a product of gene UNI3 (Dutcher and Trabuco, 1998). δ-tubulin differs from other types of tubulins in few points: firstly mutants without UNI3 gene are vital and secondly there are two additional exons in UNI3 gene (Dutcher and Trabuco, 1998). It is interesting that mouse δ tubulin clone shows 43% sequential similarity to human γ-tubulin and it is associated with centrosomes independent of MTs (Chang and Stearns, 2000).

Behind the discovery of ε-tubulin lays sequential homology with already known tubulin genes. ε-tubulin together with δ-tubulin colocalizes to centrosome and its localization in the cell is dependent on the cell cycle stage (Chang and Stearns, 2000).

η-tubulin was found in alga *Parametrium tetraurelia*. It interacts with β-tubulin and it was localized at (-) end of MTs as a capping protein as well γ- and δ-tubulin (Ruiz et al., 2004).

Other minor tubulin types as well as posttranslationally modified tubulin were described mainly in alga Tetrahymena and Paramecium. In a single cell they possess great variety of microtubular structures and therefore became great model organisms for studying tubulin heterogeneity (Libusova and Draber, 2006).

FtsZ is a tubulin homologue in prokaryotes. FtsZ plays essential role in bacterial cell division; it activates other proteins needed for cell wall assembly. FtsZ stand for "filamentous temperature-sensitive Z". FtsZ mutants form filaments at certain temperature but are not capable of cell division. Alike tubulins FtsZ contains a GTP binding domain, which enables binding GTP, however, the hydrolyzing activity of GTP to GDP and phosphate is not necessary for filaments formation or division. Unlike actin-myosin, FtsZ has no motor protein associated with, and the generation of the cytokinetic force is unknown.

There is evidence that in liposomes FtsZ is able to generate force without any other proteins (Osawa, 2009).

2.6. γ-tubulin

Plant cells lack MT organizing center (MTOC) such as centrosome of animals where MTs get organized during cell division. During mitosis or meiosis in plants, there are dispersed MTOC, where especially γ tubulin plays a crucial role. Moreover even in cells containing centrosome, MTs can organize acentrosomally. Acentrosomal MT nucleation appears to be a common pathway for MT formation in all eukaryotic cells. Unfortunately this phenomenon was not described in detail so far. These facts make plants suitable model organisms for study of acentrosomal MT nucleation in eukaryotic cells.

γ-tubulin is a conserved member of tubulin superfamily that occurs in MTOC or in form of cytoplasmic protein complexes. Protein complexes of γ-tubulin are present both in cytoplasm and on membranes. Multiple studies confirmed γ-tubulin as a main player in acentrosomal MT nucleation in plants. The attachment of MT complexes to membranes provides great flexibility in acentrosomal MT nucleation (Drykova et al., 2003).

γ-tubulin was first discovered during genetic screening for interactors of β-tubulin in filamentous fungus *Aspergilius nidulans*, expressed by gene mipA (Oakley, 1989). Immuno-localization of γ-tubulin at MTOCs led to the idea that it can contribute to MT nucleation (Oakley, 1990). There are two functional genes in *Arabidopsis thaliana* TubG1 and TubG2 that code for γ-tubulins (Liu, 1994). The reason for that could be different regulation of both genes expression or selective advantage in functional redundancy (Binarova, 2008).

There are multiple isoforms of γ-tubulin, two dimensional electrophoresis indicating posttranslational modifications (Binarova et al., 2008). Studies on budding yeast reported phosphorylation of γ-tubulin where a conserved tyrosine-455 residue near the C terminus played crucial role (Vogel et al., 2001).

When comparing γ-tubulin to α- and β-tubulin, we can find similar primary and secondary structures, which turn out to be highly conserved, for example motifs taking part in GTP binding. Conversely to α- and β-tubulin possessing C terminus rich in amino acid residues, which form site for MAP interaction, γ-tubulin lacks this strongly acidic C terminal end (Burns, 1991). γ-tubulin was localized to (-) slower growing ends of MTs which suggests its responsibility for linkage of MTs and centrosome (Li and Joshi, 1995). Moreover a study using SPOT peptide technique revealed that γ-tubulin is actually a ligand of both α and β subunits which interacts laterally with γ-tubulin (Llanos et al., 1999). Structural analysis by

Aldaz et al. (2005) suggested lateral interactions in curved conformation that turned out to be a key property for MT nucleation.

γ-tubulin has a function of a key component in MTOCs however, there might be other function which are not fully understood yet. This statement is supported by several studies, for example in *A. nidulans* disruption of γ-tubulin gene resulted in loss of mitotic spindle (Oakley and Oakley, 1990) or in *Xenopus* immuno-depletion of γ-tubulin prevented the organization of MT asters of the sperm heads (Felix et al., 1994). However, surprisingly *Ceanorhabditis elegans* embryos depleted of γ-tubulin by RNAi formed microtubular asters (Strome et al., 2001).

2.6.1   Distribution of γ-tubulin in acentrosomal plants

Based on the knowledge in metazoan cells where γ-tubulin was localized in centrosomes (Stearns et al., 1991) studies in acentrosomal plants were performed. γ-tubulin was found by immuno-localization on all microtubular arrays (Liu et al., 1993). Compared with other cells, plant γ-tubulin is more abundant and occurs in different cellular compartments. Thus rearrangement of microtubular arrays such as cortical MTs, PPB, mitotic spindle and phragmoplast by continuous assembly and disassembly of MTs is rather unique for plants (Binarova et al., 2008).

2.6.2   γ-tubulin complexes and their effect on MT nucleation

γ-tubulin occurs not only on MTOCs or MT arrays but a large portion is found soluble in the cytoplasm. The first soluble cytoplasmic complexes were discovered in *Drosophila melanogaster*. There are two major cytosolic γ-tubulin complexes: γ-TuSC and γ-TuRC (Fig. 3). Human γ-tubulin small complex (γ-TuSC) has about 280 kDa and consists of two γ-tubulin molecules linked with γ-tubulin complex proteins (GCPs): GCP2 and GCP3 which are homologues of *Saccharomyces cerevisiae* Spc97p and Spc98p (Fig. 2). γ-TuSCs associate with additional GCPs GCP4, GCP5 and GCP6 into a large γ-tubulin ring complex (γ-TuRC) which is about 2200 kDa (Guillet, 2011) (Fig. 2). γ-TuRC apart from its nucleating function acts as a (-) end capping protein, γ-TuRC binds to the (-) ends and prevents their growth (Wiese and Zheng, 2000).

Top-down view   Side view

Inter-γTuSC microtubule-like contacts

Intra-γTuSC γ-tubulin gaps

Half-γTuSC overlap

Half-γTuSC overlap

γ-tubulins in a γTuSC ring

β-tubulins in a 13-protofilament microtubule

Nature Reviews | Molecular Cell Biology
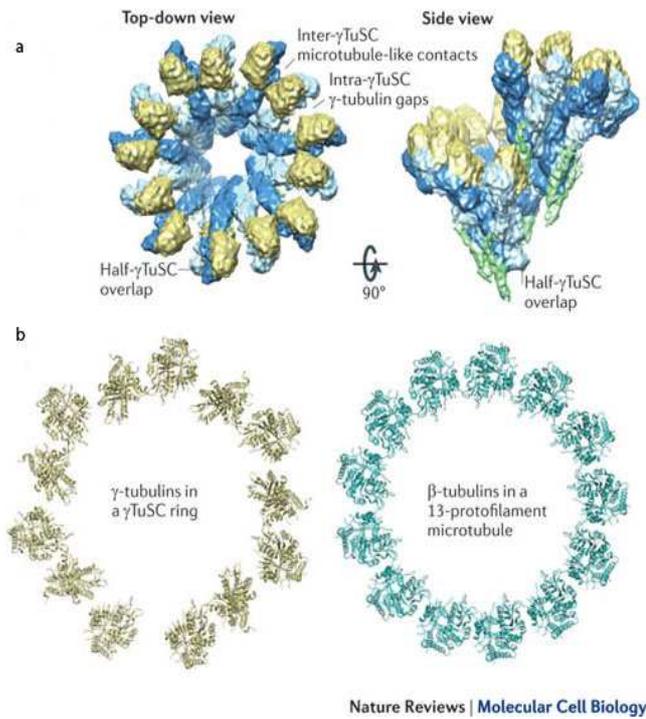
Fig. 2: a) The 8 Å cryo-electron microscopy structure of *Saccharomyces cerevisiae*. Top-down and side views of γ-TuSC. The ring has six and half γ-TuSCs per turn due to the overlap of first and seventh subunit which is demonstrated in the side view. b) Comparison of 13 γ-tubulins in γ-TuSC ring with β-tubulins in MT with 13 protofilaments. (Kollman et al., 2011)

a   b   c   d
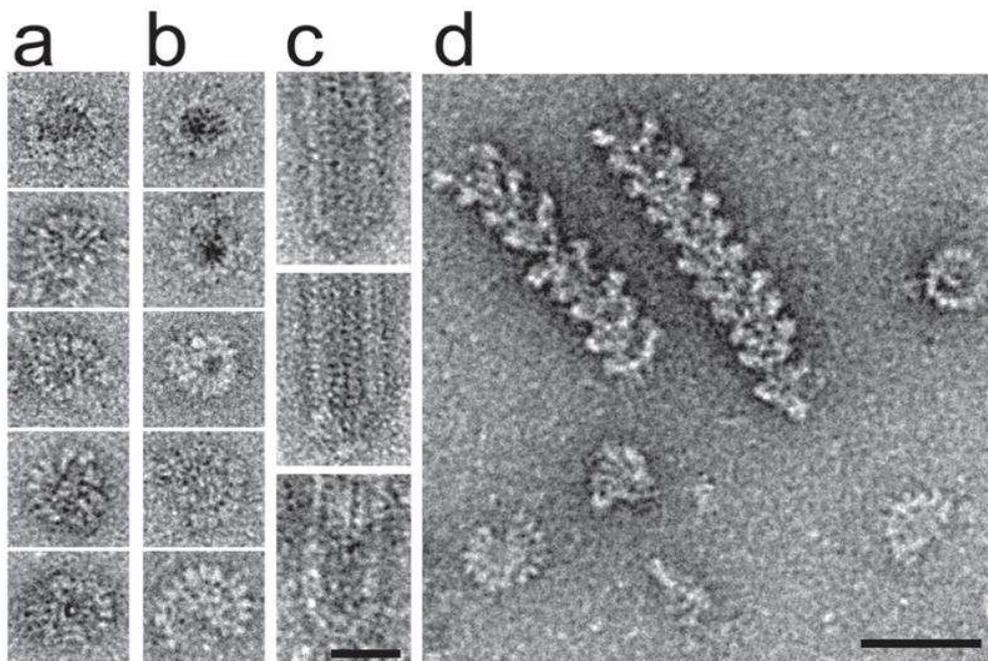
Fig. 3: Electron micrographs of γ-tubulin structures and complexes a) γ-TuSC in *Saccharomyces* b) γ-TuRC in *Drosophila* c) Capped ends formed in presence of γ-TuSC. Bar a-c 25 nm. d) Filaments of γ-TuSC grown upon co-purification with Spc110. Bar 50 nm (Kollman et al., 2010)

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MT nucleation was a subject of a great discussion. The first hypothesis suggests that γ-TuRC is absolutely essential for initiation of nucleation as it serves as a template for MT growth. In this so called template model γ-TuRC at the (-) end of the MT initiate each of the 13 protofilaments through longitudinal contacts with the γ-tubulin subunits (Zheng et al., 1995). The second hypothesis is based on a protofilament model, where in the contrast to template model γ-tubulins interact laterally (Erickson and Stoffer, 1996). After period of intensive research using various detection techniques such as biochemical analysis (Moritz, 2000), electron and fluorescent microscopy (Wiese and Zheng, 2000) there are more prons for template model however, protofilament model cannot be excluded.

A recent study by Kollman et al. (2010) revealed by usage of cryo-electron microscopy that γ-TuSC assembles in 13-fold structure similarly to MTs. There are 13 γ-tubulins per one turn of filament having (+) ends displayed for interaction with MTs which implies that the turns of filament form a MT template (Fig. 4).
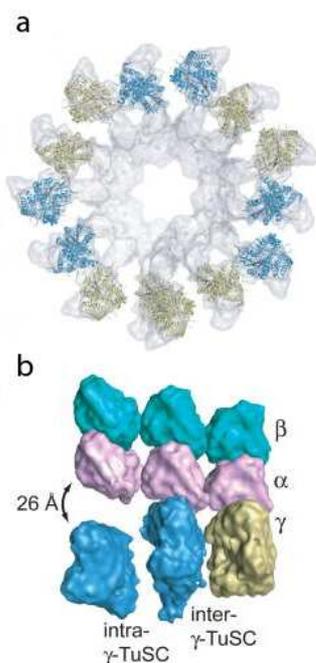
Fig. 4: a) Thirteen γ-tubulins fit into one turn of the filament resembling MT symmetry b) Longitudinal interaction of laterally connected α/β-tubulin dimers with γ-tubulin (Kollman et al., 2010)

2.6.3 Plant γ-tubulin associated with membranes

Association of MT nucleation with membrane was reported in plant as well as in animal cells (Binarova et al., 2000, McDonald et al., 1993, Vaughn et al., 1998). Specifically plant nuclear surface resemble centrosome concerning its activity in MT nucleation (Stoppin et al., 1994). Moreover, specific association of large γ-tubulin complexes with membranes was

observed by Drykova, et al., (2003). γ-tubulin also showed the nucleation ability, when bound to preexisting MTs; cytoplasmic γ-tubulin complex serves as a mediator between the cytoplasm and cortical MTs (Murata et al., 2005). In early mouse oocytes large γ-tubulin containing structures called multivesicular aggregates (MTV) are localized to the cortex. Despite the association with γ-tubulin, MTV neither prove to carry nucleation ability, nor resemble MTOCs. Nevertheless, trafficking of MTV to Golgi vesicles, degradation to smaller units and assembly of MTOCs with nucleating activity suggested that MTV are precursors of centrosomes (Calarco, 2000). Golgi complex seems to be part of MT organizing apparatus which directly stimulates nucleation in interphase cells. Subset of γ-tubulin is located at the cytoplasmic face of the organelle which suggests direct responsibility of Golgi membranes for acentrosomal MT assembly (Chabin-Brion et al., 2001). Ultrastructural studies revealed a close connection between plant membranes and acentrosomal mitotic apparatus (Helper and Wolmiak, 1984). In plants, γ-tubulin is suggested to be bound to Golgi membrane and Golgi membranes were found close to acentrosomal spindle poles positive for γ-tubulin (Drykova et al., 2003). Microtubular activities at the Golgi apparatus such as nucleation and anchoring seem to be a common pathway of MT nucleation and organization (Rios and Bornens, 2003). Addressing question on the role of membrane associated γ-tubulin in MT nucleation in acentrosomal plant cells will help to understand acentrosomal MT nucleation in eukaryotes.

In vertebrate cells a cytoplasmic TCP-1chaperonin complex enables folding of α- β- as well as γ-tubulin. Unfolded polypeptides bind the chaperonin complex and are subsequently released with the help of Mg-ATP as soluble monomeric proteins. γ-tubulin does not require any cofactors in addition to chaperonin itself in contrary to α- and β-tubulin (Melki et al., 1993). Immunoprecipitation experiments of Drykova et al. (2003) revealed co-localization of γ-tubulin along the entire plant MTs without any preference for (+) or (-) end. Tubulin dimers were not found in the majority of animal γ-TuRC however, various proportions of tubulin dimers have been reported to co-precipitate with γ-tubulin in samples from oocytes (Zheng et al., 1995) erythrocytes (Linhartova et al., 2002) As mentioned above a recent study by Kollman et al. (2010) has proven the lateral association of γ-tubulin with α- and β dimers forming 13-fold protofilament-like structure in yeast.

Ordered cortical MTs are essential for normal plant morphogenesis. Interactions between MTs take place when growing (+) ends of cortical MTs encounter already existing cortical MTs. Interestingly dependent of the angle of collision, MTs get stabilized or destabilized (Dixit and Cyr, 2004). Moreover nucleation of MTs is conditioned by pre-existing cortical

MTs which recruit cytosolic γ-tubulin to the branch points. Murata et al. (2005) suggest cytoplasmic γ-tubulin complexes shuttle between cytoplasm and side of cortical MT. Γ-tubulin plays role on both ends of MT. γ-TuRC was reported as a (-) end capping protein (Wiese and Zheng, 2000). Evidence was provided also for γ-tubulin function in regulating dynamics at the (+) end of MTs that is important for cell morphogenesis (Raynaud-Messina and Merdes, 2007).

Accumulating data support the statement that γ-tubulin has besides its nucleation and organization also role in MT stabilization. The role of γ-tubulin in organization of bipolar spindles was supported by the study of Binarova et al. (1998) who located γ-tubulin to the kinetochore region of chromosomes.

In *Saccharomyces pombe* fluent coordination of mitosis during cytokinesis is provided by dispersed MTOCs with γ-tubulin serving as an anchorage for cytokinetic actin ring (Venkatram et al., 2005). Binarova et al. (2008) suggest that similar mechanism might operate in plant cells with dispersed MTOCs where severe cytokinetic defects were observed when tubulin was downregulated by RNAi.

Above all functions of γ-tubulin mentioned, I would emphasize its role in interconnection and membrane linking with positional clues such as centrosomes during the cell cycle progression reported by Rios and Bornens (2003) in mammalian cells.

2.6.4 Conclusion γ-tubulin

Presence of γ-tubulin in various species underlines its functional indispensability in nucleation and regulation of MT dynamic. Due to the rigidity of the plant cell wall, specific microtubular arrays - cortical MTs were evolved having PPB and phragmoplast pointing at the necessity of precise control of division plane and polarity. Whereas in plants MTs associated with plasma membranes are abundant, in animal cells actin filaments are more dominant at the same locations.

γ-tubulin remains an essential protein in plant MT organization and nucleation as well as in self-organization of MT activity not only in plants but also in other eukaryotic cell types with acentrosomal MTs.

2.7 Microtubule organizing centers (MTOCs)

MTOCs are sites of MT nucleation. They are typical for eukaryotic cells and have two major functions: organization of eukaryotic flagella and cillia, and organization of mitotic/meiotic apparatus. However, the structure of MTOCs differs among distant taxons.

In animal cells there are organelles such as centrosome and basal bodies which serve as MTOCs. Centrosome consists of two perpendicularly arranged MT structures, the centrioles. The arrangement depends on the microtubular polarity, (-) ends of the MT point at the MTOC and the (+) ends point at the cell periphery. The polarity is determined by γ-tubulin a protein which is located at the (-) end of the MTs and triggers the nucleation of MTs. Coiled-coil proteins expressed by spd-5 gene provide anchoring network for MTs and key regulators. This fact also points at the structural aspects of centrosome (Hamill, 2002). Basal bodies are found at the base of eukaryotic flagella and cillia and similarly to centrosome form anchoring and nucleating site of MTs.

In yeast, some algae and fungi there is a structure called spindle pole body (SPB) which serves as a MTOC and is located at the nuclear membrane. Unlike centrosomes SPB lacks centrioles however, its function remains similar.

In plants there is no discrete MTOC which resembles centrosomes (animals) or SPB (fungi). Nucleation sites of MTs are organized rather in a dispersed fashion. There are several mechanisms underlying acentrosomal MT nucleation: launch from MTs, catalyzed nucleation at acentrosomal site or breakage of already existing MTs (Bartolini and Gundersen, 2006). Non-radial organization of MTs in contrast to centrosomes is a common phenomenon for all differentiated cells with acentrosomal sites of nucleation including muscles, epithelial and neuronal cells, as well as most fungi and vascular plant cells (Bartolini and Gundersen 2006). In addition completed genome sequencing proved that many proteins required for MT nucleation are highly conserved. This makes therefore plants suitable model system for studying the MT nucleation from dispersed sites and spindle formation in the absence of centrosomes (Binarova et al., 2008).

2.8 MAPKs origin

There are multiple synonymous names for MAP/ERK kinase reflecting their functional redundancy. MAPKs were originally called "extracellular signal regulated kinases" (ERKs) and later renamed to "MT-associated protein kinase" (MAPK) because the first protein shown to be phosphorylated by ERK was "MT associated protein" (MAP). As gradually more proteins targets phosphorylated by MAPK were identified, so the name was changed to "mitogen-activated protein kinase" (MAPK). Mitogens are proteins such as growth factors and cytokines with ability of triggering mitosis. Generally MAP/ERK signaling cascade responses to various cell stimuli through phosphorylation of cytoplasmic components and transcription factors.

Plants are sessile organisms which are not capable of moving in answer to external signaling. Plants can move just by elongation of cells or by addition of new cells at meristematic sites. To avoid the adverse effect of harsh conditions such as drought, cold, osmotic stress, ROS and pathogens, plants evolved mechanisms to sense and adapt their physiology, growth and development. The adaptations include rapid and dynamic regulation of enzymatic activities and modification of gene expression programs (Colcombet and Hirt, 2008). Phosphorylation/dephosphorylation of proteins represents the most frequent post-translational modification among all organisms. Protein phosphorylation is performed by protein kinases that are commonly organized into signaling cascades. The estimation is that 30% of proteins in eukaryotic cell are phosphorylated, so it is not surprising at all that 5% of green plant (Viridiplantae) genome code for protein kinases (International Rive Genome Sequencing Project, 2005).

MAPK cascades are conserved signaling modules found in all eukaryotic cells including plants, fungi and animals. The MAPK cascade comprises three kinases: MAPKKK, MAPKK and MAPK. MAP kinases activate each other *via* phosphorylation in a respective way. The interaction between kinases within a cascade is achieved by docking sites and/or with assistance of scaffolding proteins (Colcombet and Hirt, 2008).

MAPK are found in many different plant species which points to their conserved signaling mechanism. MAPKs are involved in many cellular processes including pathogen response, abiotic stress and morphogenic process such as embryo polarity, stomata differentiation, cell wall formation during cell division or MT organization which is of our main concern. On the one hand a few kinase namely MAPK3, MAPK4 and MAPK6 are involved in wide range of signal transduction processes, hence they are studied extensively. On the other hand there are kinases such as MKK8 and MKK10 whose functions remain unknown. The predominant involvement of few kinases might be caused by lack of monitoring tools or technical reasons that prevent from adequate detection (Colcombet and Hirt, 2008). However, apart from technical issues there are two possible explanations for this phenomenon. Firstly input signals may share a common second messenger which is the activator of such a conserved cascade. AOS (active oxygen species) (Colcombet and Hirt, 2008) or calcium (Bowler, 2000) could suit this function. Alternatively, other members of the cascade are present just in particular cell types. For example MAPK4 reacts to an osmotic shock in Arabidopsis suspension cells but not in plantlets (Droillard et al., 2004). These data support the redundancy and complexity of MAPK signal transduction networks. MAP/ERK signal cascade has an essential role in a cell.

MAPK pathway presents a cascade from external signaling to the effectors. The signal comes from the outside of cell and activates the membrane receptors that transfer the information to the nucleus, where transcription factors are targeted. Transcription factors can affect the expression of target genes. MAPK mediated transfer and amplification of extracellular stimuli is a universal model for signal transduction in eukaryotes. In plants, MAPK cascade is particularly associated with physiological, developmental and hormonal responses.

2.9 MAPK genomics

In complete *Arabidopsis* genome 20 genes encoding MAPK, 10 genes for MAPKK and over 80 for MAPKKK were identified (The *Arabidopsis* Genome Initiative, 2000; Rodriguez, 2010). The rice (*Oryza sativa*) and poplar (*Populus trichocarpa*) exhibited 15 and 21 MAPKs and 8 and 11 MAPKK in a respective order (Hamel et al., 2006). In contrast to yeast (with 6 for each MAPK) and human (with 10 MAPK and 7 MAPKK) plants have more MAPKs as well as MAPKKs which indicates the complexity of MAPK pathways in plants.

MAPKs can be divided in 4 phylogenetically distinct groups (A-D) (Fig. 1 Ichimura et al, 2002). MAPKs from group A and B have on the C terminus CD conserved domain which serves as a place for binding D domain of MAPKK, phosphatases and other protein substrates. CD domain of MAPK contains amino acid sequence where neighboring acidic residues interact with basis cluster of MAPKK (Tanoue et al., 2000). D domains are also native to other MAPKK encounters such as scaffold proteins, MAPKK substrates and tyrosine phosphatases (Bhattacharyya et al., 2006). There are two times less MAPKK than MAPK, so it is very likely that MAPKK activates multiple MAPKs (Ichimura et al., 2002). MAPKK are dual specificity kinases that activate MAPK through double phosphorylation of motif with serine/threonine and tyrosine in the activation loop. MAPKKK family has much greater variability in primary structure and has the highest number of members. 12 MEKK-like and 48 RAF-like MAPKKK was identified (Ichimura et al., 2002). MAPKKK are serine/threonine kinases which phosphorylate activation loop of MAPKK. Aside from kinase activity MAPKKK also possess a long C or N terminal region that might play role in regulation or scaffolding in order to take up MAPKKs and MAPKs. An example could be MEKK1 in *Arabidopsis* (Bogre, 2007). Both biochemical and genetic evidence concerning MAPK, MAPKK, MAPKKK multigene families point at their functional redundancy (Ichimura et al, 2002). Whereas mammalian MAPKs rather response to mitogen stimuli, in plants MAPKs evolved toward much broader range of signals (Ligterink and Hirt, 2001).

2.10 Members of MAPK pathway in tobacco and Arabidopsis

In this section I will focus on the MAPK pathway that regulates cytokinesis in tobacco, the NACK-PQR pathway and its homologues in *A. thaliana* (Fig. 5). This kinase pathway is late M-phase specific and is required for phragmoplast expansion in particular (Sasabe and Machida 2012). In tobacco it is triggered by NACK1 and NACK2 kinesin-like proteins. The signal is then transmitted by phosphorylation from NPK1 (nucleus- and phragmoplast-localized protein kinase 1) (MAPKKK) to NQK1 (MAPKK) and subsequently to NRK1 (MAPK). The final acceptor is MAP65-1a (MAP).

Instead of sensing extracellular stimuli, MAPK cascade is activated by MT motor protein NACK1. NACK1 and NPK1 have MAPK and CDK phosphorylation sites. As the cell exit metaphase NPK1 gets dephosphorylated while CDK gets inactivated (Nishihama et al., 2001). NPK1 protein from MAPKKK family has been localized to the nucleus during interface but moves to central part of phragmoplast during M phase suggesting the role in cell plate formation and cytokinesis (Nishihama et al., 2001). NPK1 promotor is predominantly active in shoot and root apices pointing at the cell proliferation regulation (Nakashima, 1998).

Arabidopsis homologues of NPK1: ANP1, ANP2, ANP3 (MAPKKK) were proved by genetic studies to contribute to cytokinesis (Nishihama et al., 1997). AtMKK6 (MAPKK) an *Arabidopsis* homologue of NQK1 show multinucleation and stub in cell walls pointing at the role in cell plate formation. However, atmapkk6 mutants are not gametophytic lethal like anp1/anp2/anp3 triple mutants which suggests the existence of another gene(s) or that the studied disruptor is not a null mutant. Only group B MAPK of *A. thaliana* such as AtMPK4, AtMPK13 exhibit structural similarity to tobacco NRK1 (MAPK) and are involved in cytokinesis. Despite the fact that MPK13 showed the highest sequence similarity, MPK4 has been identified as a downstream target of *Arabidopsis* cytokinesis pathway (Takahashi et al., 2010).
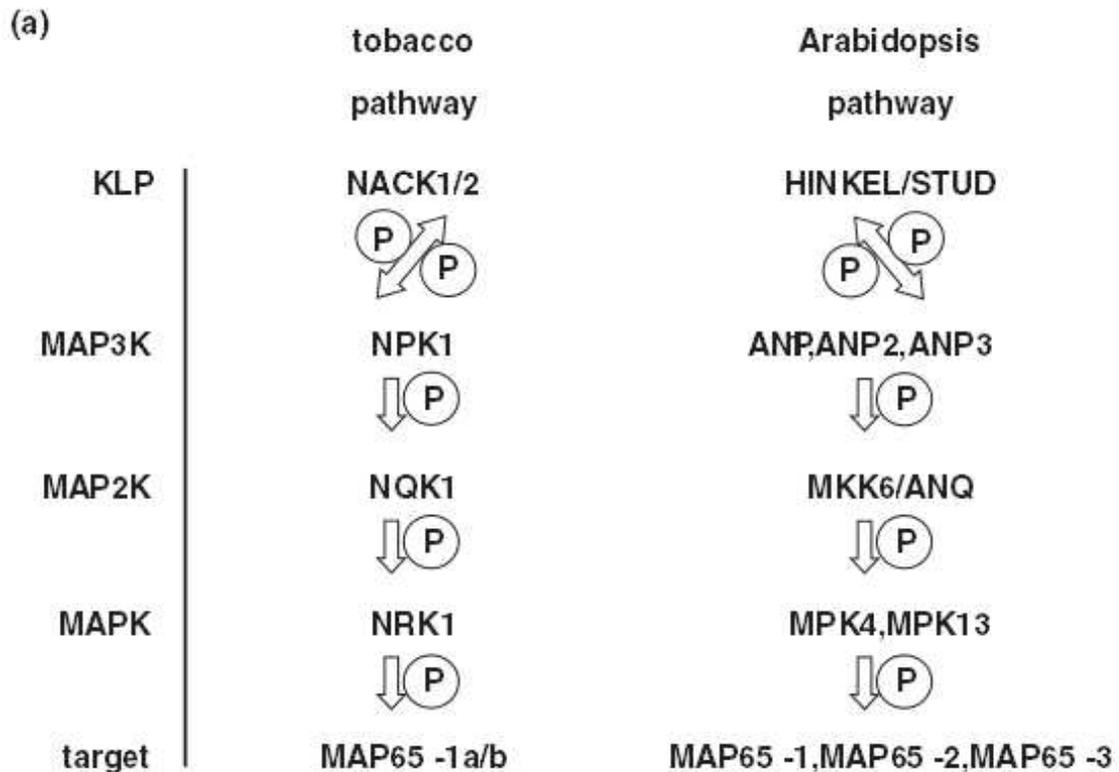
Fig. 5: Members of NACK-PQR pathway in tobacco and *Arabidopsis*. Kinesine-like proteins (KLPs) regulated cytokinesis and MAPK-dependent phosphorylation of MAP65 (Komis et al., 2011).

The upstream factors which activate NACK-PQR pathway are kinesin-like motor proteins (KLPs) NACK1 and NACK2. They have been shown to interact directly with NPK1 activate it *in vitro* as well as *in vivo* (Nishihama et al., 2002). Both are directed against the (+) ends of MTs. The truncated form of NACK1 without the motor region failed to develop the cell plate hence indicating NPK1 necessity in phragmoplast mediated cytokinesis. The *Arabidopsis* homologues of NACK1 and NACK2 have been identified by Nishihama et al. (2002) and named HINKEL and TETRASPORE/STUD respectively (Spielman et al., 1997; Strompen et al., 2002).

2.11 MAPK in cytokinesis

Already 20 years ago MAPK were found to be associated with MTOCs (Verlhac et al., 1993) and with microtubular cytoskeleton (Reszka et al., 1995) and the role in regulation of MT dynamics was suggested for MAPK in animal cells. In plant cell MAPK were located to spindle area and the area of cell plate formation implying their connection to cytokinesis (Calderini et al., 1998, Bogre et al., 1999, Calderini et al., 2001).

Plant cell division

16

Cell division is a process during which the duplicated gene material is distributed evenly between daughter cells. It comprises two stages: karyokinesis and cytokinesis. During karyokinesis nuclear membrane is dissolved, chromosomes get condensed and line up in the equatorial plane where spindle is formed. In plants the cell wall in cytokinesis is formed from the inside whereas the animal cells get constricted from the outside. Cell plate is formed in a plant specific organelle - phragmoplast which consist of MTs situated in such way that their (+) ends point towards the cell plane and (-) ends towards the daughter nuclei. Phragmoplast is a specific microtubular array which corresponds to the central spindle in animals and anaphase spindle in yeast. The expansion of phragmoplast is achieved by three major molecular processes: the MT turnover (Yasuhara et al., 1993), the synthesis of cell wall (Zuo et al., 2000) and the regeneration of cell membrane (Lukowitz et al., 1996). All these processes are crucial for phragmoplast development and inhibition of any one of them impairs cell plate formation. MT turnover which is represented by polymerazation/depolymerization of tubulin dimers at the inner and outer phragmoplast regions seems to be very important in achieving the barrel-like structure. Exponential turnover of MTs supply the vesicle machinery operating at the leading edge of the phragmoplast.

2.11.1 MAPK in MT regulation

One of the first MAPK identified to participate in cytoskeletal regulation was MMK2 and MKK3 of alfalfa (*Medicago sativa*) which become activated in MT dependent manner (Bogre et al., 1999). Despite the prediction based on homological similarities the major target of ANP pathway related to MT regulation is not MPK13 but MPK4. MPK4 is a MAPK reported to be involved in the regulation of innate immunity (Gao et al., 2008) and abiotic stress responses (Teige et al., 2004). MPK4 is activated via MEKK1-MKK1/2 pathway indicating that the specificity for MT regulation depends on ANPs not MEKK1 to co localization with MTs. Another MAPK which happen to take part in cytoskeletal regulation is MPK6. Common activation steps of MPK4 and MPK6 suggest that MPK6 is part of MEKK1-MKK1/2 pathway (Xing et al., 2009) but it can be involved in YODA-MKK4 pathway as well (Wang et al., 2007). The latest identified MAPK involved in MT regulation is MPK18 whose function is restricted to the regulation of cortical MTs (Walia et al., 2009).

There are MAPK mutants where MTs are affected. Since tubulin monomers willingly phosphorylate (Holmfeldt et al., 2009) one would suggest that MAPK can affect MTs via tubulin phosphorylation. Up to date there are no reports of tubulin phosphorylation by

MAPK leading researchers to other MAPK targets such as MAPs (Komis et al., 2011). However, based on our experience and experimental data we are convinced that γ-tubulin might serve as a phosphorylation target of MAPKs.

The involvement of MAPs (rat MAP2, yeast BIK1 and Arabidopsis MAP65) in cytokinesis varies among species. For example functions of MAPs are regulated by phosphorylation of cyclin-dependent protein kinases (CDKs) and Polo-like kinases, however, there is no report of a polo-like kinase ortholog in plants. CDKs can directly repress NACK1-regulated MAPK cascade (Sasabe and Machida, 2012). As CDK is involved in the proper course of mitosis by inhibiting aberrant MT bundles, it might be an essential feature in control of late M-phase and cytokinesis progression.

2.11.2 U0126 inhibitor as a suitable tool for MAPK studying

U0126 is a MAPK specific inhibitor directed against MEK-1 and MEK-2 (MAPKKs) whose target is Erk1/2 (MAPK) (Fig. 6). The interruption of kinase cascade by U0126 prevents transferring the signal via phosphorylation to downstream targets of MEK-1 and MEK-2 providing a great starting point for researchers interested in MAPK and MAPKK interactions. Here I give examples of two studies using U0126 as a main tool in revealing MAPK puzzle.

Rat oocytes are known to undergo spontaneous activation after leaving oviduct but remain kept at MIII arrest. Treatment with MEK inhibitor U0126 speeded up the formation of pronuclei leading us to the function of Mos/MEK/MAPK in the maintenance of MII arrest (Ito et al., 2007). The research team of Cui (2012) demonstrated that premature MAPK inactivation lead to spontaneous activation by infringing spindle integrity. Inhibition of MAPK activity by U0126 caused spindle disintegration and chromosome scatter further underlining the role of MAPK in spindle integrity.

Another study concerning BY-2 tobacco cells demonstrated that MAPK has a crucial function in cell cycle progression by affecting phragmoplast assembly. With the help of U0126 inhibitor they state that loss of MAPK phosphorylation blocks transition from telophase to early cytokinesis by impairing phragmoplast and cell plate formation hence preventing separation of daughter cells. Observing the activity of MAPK throughout the cell cycle it turned out that expression of MAPK during mitosis is lower than in interphase despite the fact that the phosphorylation level of MAPK remained unaltered. This fact points perhaps to the importance of MAPK in mitosis (Ma and Yu, 2010).
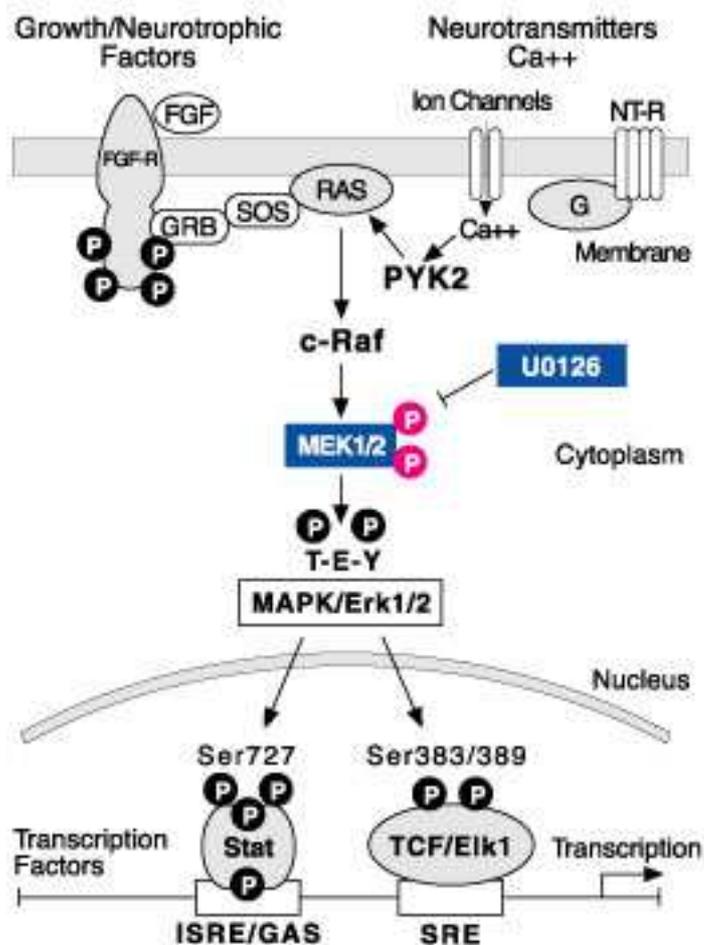
Fig. 6: Scheme of U0126 mediated inhibition of Erk1/2 in animal cell. U0126 inhibitor is directed against MEK-1 and MEK-2 (MAPKK) which are upstream of Erk1/2 (MAPK). (http://www.ebiotrade.com accessed April 2013)

2.12 MAPK targets

MAPK pathway serves as a mediator which propagates the signals to the downstream targets via phosphorylation. One has been identified as MAP65 a conserved eukaryotic protein belonging to the MT associated protein family which is associated with phragmoplast. This family is very conserved among wide range of organisms comprising Ase1 (anaphase spindle elongation factor) in yeast (Pellman et al., 1995), PRC1 (protein regulator of cytokinesis 1) in mammals (Jiang, 1998), SPD1 (spindle defective 1) in *C. elegans* (Verbrugghe and White, 2004) and Feo (Fascetto) in *Drosophila* (Verni et al., 2004). Phosphorylation of NtMAPK65 by NRK2 (MAPK) contributes to the destabilization of MTs and therefore might enhance the dynamic instability of MTs (depolymerization of MTs and polymerization of tubulins) (Bogre et al., 2008).

Tobacco NtMAPK65-1 is phosphorylated by NRK1 (MAPK) at a single site Thr-579 in the C-terminal region during late M-phase (Sasabe and Machida, 2006). In *Arabidopsis* nine members of MAP65 family were found, three of them impacting MT organization (MAP65-1, MAP65-2 and MAP65-3). AtMAPK65-1 and AtMAP65-2 show high amino acid sequence similarity to the tobacco NtMAPK65-1 and together with AtMAPK65-3 get phosphorylated by MPK4 (MAPK) (Kosetsu et al., 2010). AtMAP65-1 the most studied type that has been proved to bundle MTs but do not polymerize tubulin (Smertenko et al., 2004, 2006). MPAK65-1 has many sites for a wide range of protein kinases of course (Smertenko et al., 2006). Thanks to its structure, MT-binding regions at opposite sites, MAP65-1 can crosslink adjacent MTs with an exact spacing (Walczak and Shaw, 2010). Another member of MAP65 family in *Arabidopsis* AtMAP65-3/PLEIADE is involved in the maintaining of the phragmoplast structure (Muller et al., 2004). AtMAPK65-3 has housekeeping activity in phragmoplast through distinct C-terminal MT-binding domain (Ho, 2012). Members of MAP65 protein family share a unique feature which is the presence of ubiquitin ligase targeting sequence. It proposes regulation of their abundance by cell cycle-dependent proteolytic degradation (Hussey, 2002).

2.12.1 Regulation of MAPK by activity of MT - reciprocity

As mentioned above MAPK pathway has function in cytokinesis, specifically in phragmoplast expansion which is accomplished by regulating MT dynamics by means of MAPs phosphorylation for example MAP65. On the contrary MTs can also influence MAPK pathway at least during phragmoplast expansion or cell plate formation (Sasabe, 2012). As unbundled phragmoplast MTs depolymerize, the cascade might be quickly suppressed to avoid further MT depolymerization. There is evidence for negative feedback mechanism lying behind kinase cascade activation and MTs polymerization/depolymerization. There are two possible explanations of this mechanism. (1) The state of MTs might directly affect certain form phosphatase(s), for instance polymerized state inactive form and depolymerized state active form. (2) Depolymerization of MTs may cause structural changes to the active phosphorylated kinases resulting in increased sensitivity to phosphatases (Soyano et al., 2003).

The phosphorylation of MAP65 at the mid section of phragmoplast can lead to destabilization of the crosslink between overlapping MTs, triggering dynamic expansion of the cell plate (Sasabe & Machida, 2006). On cortical MTs the same pathway might operate to sense MT states, such as MT tension, and through the activation of the MAPK pathway

and phosphorylation of MAP65, it could allow the reorganization of MTs and dynamic shaping of cells.

Interestingly, the auxin efflux carrier PIN-FORMED (PIN) proteins also posses MAPK phosphorylation sites at their regulatory insertion loop (Benschop et al., 2007) leading us to the same MT sensing pathway which could be linked to the dynamic patterning of the direction of auxin flow (Hamant et al., 2008; Heisler et al., 2010).

2.13 MAPK6

Plant MAPK6 was shown to be involved in the control of cell plate during early root development and was localized in preprophase band (PPB), phragmoplast, trans-Golgi and plasmatic membrane (Muller et al, 2009). Today it is accepted that PPB and phragmoplast represent cytoskeletal structures involved in the control of cell division site in plants. MAPK6 was found in spot-like clusters which were tightly associated with MTs during cell division of root tips in *Arabidopsis*. Electron microscopy revealed the partial co-localization of MAPK6 with cortical MTs, patches at the plasmatic membrane as well as with the trans-Golgi vesicles. Immuno-localization experiments provided evidence that MAPK6 not only localizes to nucleus and within cytoplasm, but also associates with microsomal/vesicular endomembrane compartments of root cells. Results suggest an association of MPK6 with endomembrane belonging to complex secretory/endocytotic network that is involved in cell division and polarization of cell growth (Müller et al, 2009).

Involvement of MAPKs in the organization and/or dynamics of mitotic and cortical MTs was shown by Sasabe et al., (2006); Smertenko et al., (2006) and Walia et al., 2009). Smertenko et al. (2006) suggest that MPK4 and MPK6 affect mitosis during prophase and metaphase via reversible phosphorylation of AtMAP65-1. This model seems to be consistent with localization of MPK6 on PPB and phragmoplast (Muller et al, 2009). Sasabe et al. (2006) propose that phosphorylation of NtMAP65-1 at the phragmoplast equator reduces its MT-bundling activity *in vivo*, which enhances destabilization and turnover of MTs, perhaps facilitating phragmoplast expansion. And finally Walia et al. (2009) propose a model whereby the PHS1–MPK18 signaling module is involved in a phosphorylation/dephosphorylation switch that regulates cortical MT functions.

Recently a study by Hoang et al. (2012) revealed transcription factor AtMYB41 which requires AtMPK6 phosphorylation for its biological function. MYB family of transcription factors is known to be involved in regulation of plant development and stress responses

(Dubos et al., 2010). However, the direct downstream targets of MPK6 of MEKK1-MEKK2-MPK6 pathway are still unknown.

2.14 MAPK involvement in multiple processes

As mentioned above MAPK signaling cascade response to various cell stimuli through phosphorylation of some cytoplasmic components and transcription factors. Molecular and biochemical studies revealed MAPK activation dependent on pathogen infection, wounding, low temperature, drought, hypo- or hyper-osmolarity, high salinity, touch and ROS (Tena et al, 2001; Morris, 2001; Romeis, 2001; Zhang and Klessig, 2001).

Apart from the role in cytokinesis MAPK pathway step in: regulating cell differentiation by YODA, root hair development in terms of alfalfa SIMK and phytohormone signaling. YODA belongs to MAPKKKs which is responsible for asymmetrical cell division. In the growing root tips actin filament mesh network is involved (Sieberer et al., 2005) since actin cytoskeleton is believed to act as a rail for polar vesicular transport. SIMK belongs to MAPKs which is localized to the root tip (Samaj et al., 2002). Interestingly MAPK cascade is involved in regulation of cytoskeleton in both cytokinesis (*via* MTs) and tip growth (actin filaments).

The ability of MAPKs to take part in distinct non-interacting pathways is achieved by scaffold proteins. This phenomenon is very well described in Ste5 of budding yeast. Ste5 ensures scaffolding of Ste11-Ste7-Fus3 (MAPKKK-MAPKK-MAPK) in response to pheromone signal. However, without pheromone signal the scaffolding does not take place and another Ste11-Ste7 target is activated (Zalatan et al., 2012).

Multiple roles of MAPK pathway in response to various stimuli is demonstrated by example of SIMK. SIMK is involved in root tip growth, ethylene response and in osmotic stress (Munnik, 1999). Ethylene a stimulator of hair root elongation seems to be obvious activator of SIMK when taking into account the involvement of SIMK in root growth. It remains an open question why two different stimuli (ethylene response and osmotic stress) use a common pathway.

Similarly NPK1 also appears in various cellular processes such as regulation of phragmoplast expansion, pathogen response. ANP1 which is an *Arabidopsis* orthologue of tobacco NPK1 is suggested to take a part in the response to oxidative stress and pathogenic infections.

3 Materials and methods

3.1 Material

3.1.1 Plant material

Arabidopsis thaliana ecotype Columbia and Landsberg erecta were cultured in MS medium (Murashige and Skoog, 1962) enriched with 3% sucrose and growth regulators: 2.5 μM 1-naphtaleneacetic acid (NAA 0.5 mg ml^{-1}) and 0.25 μM kinetin (KIN 0.05 mg ml^{-1}) with pH of 5.7. Cell cultures have been kept in dark under constant shaking.

Arabidopsis thaliana ecotype Columbia seeds were sterilized, sowed and kept for two days in cold (8°C) and dark. Seedlings were grown on solid MS medium (0.22% w/v MS, 0.05% w/v MES (Sigma), 0.05% w/v sucrose (Lach-ner) and 1% w/v phytoagar (Duchefa)) in controlled environment room with 16h day at 24 ^{0}C.

3.1.2 Bacterial strains

Escherischia coli strain DH5α was used to amplify plasmid DNAs for testing for mutations with MAXI kit.

Agrobacterium tumefaciens strain GV3101 was used for transformation of *Arabidopsis thaliana* plants. Bacteria were cultured at 28°C with the addition of certain antibiotics: 50 μg ml^{-1} rifampicin (Fluka),50 μg ml^{-1}gentamicin (Sigma),2 μg ml^{-1}tetracycline (Sigma), kanamycin 50 μg ml^{-1} (Duchefa) and spectinomycin 50 μg ml^{-1} (Sigma).

3.2 Methods

3.2.1 *Agrobacterium*-mediated transformation

Arabidopsis cells were transformed according to Mathur et al. (1998). The *Arabidopsis* cell cultures were grown for one day so most of the cells were just entering the mitotic stage. At that time *A. tumefaciens* carrying designed vector was applied together with a phenolic compound. Phenolic compounds like acetosyringin are produced by wounded plants and cause chemotaxis of bacteria. The cell cultures were checked every second day for new transformants under fluorescence microscope. If the transformation was successful, antibiotics were applied to select specific cells and to prevent bacterial overgrowth.

Bacteria with incorporated plasmids were stored in glycerol stock at -80 ^{0}C. They were grown at 28 ^{0}C in liquid YEP medium with specific antibiotics for one day and plated to solid YEP medium with specific antibiotics for another day. A tip of bacteria was placed in

the cell culture using inoculating loop. 100mM phenolic compound acetosyringon (Sigma) was applied to the cell culture.

liquid YEP medium: 1% yeast extract (w/v) (Oxoid), 1% peptone (w/v) (Oxoid), 0.5% NaCl (w/v)

solid YEP medium: 1% yeast extract (w/v) (Oxoid), 1% peptone (w/v) (Oxoid), 0.5% NaCl (w/v), 1.5% agar (w/v) (Oxoid)

3.2.2 Preparation of samples from cell fractions of *Arabidopsis thaliana*

Cell cultures were homogenized in liquid nitrogen and placed for 20 min in extraction buffer with inhibitors of proteases and phosphatases in the ratio 1 g of cell to 2 ml of buffer.

extraction buffer: 50mM HEPES (Serva) pH 7.5; 75mM NaCl (Lach-ner); 1mM EGTA (Serva); 1mM MgCl$_2$ (Sigma); 1mM NaF; 10% glycerol and protease and phosphatase inhibitors (Tab. 1)

Tab. 1: Protease and phosphatase inhibitors

protease inhibiotrs	dithiothreitol (DTT)	10 µl ml^{-1}
	aprotinin (AP)	5 µl ml^{-1}
	leupeptin (LEU)	5 µl ml^{-1}
	pepstatin (PEP)	5 µl ml^{-1}
	antipain (ANT)	5 µl ml^{-1}
	soybean trypsin inhibitor (STI)	5 µl ml^{-1}
	2 mM pefablock (PFB)	20 µl ml^{-1}
phosphatase inhibitors	60 mM β-glycerolphosphate	60 µl ml^{-1}
	0,5 mM Na$_3$VO$_4$	*10 µl ml^{-1}
	*Activation: 10 µl 0,5 M Na$_3$VO$_4$ + 85 µl H$_2$O + 5 µl H$_2$O$_2$	

The extract was centrifugated using eppendorf 5810R centrifuge at 10 000 rpm and the supernatant was obtained.

3.2.3 Bradford protein assay

The amount of protein present in the samples was determined by Bradford protein assay using Coomassie Blue Brillant R250 and measuring absorbance at 595 nm (Bradford, 1976).

Bradford reagent: 1% Comassie Brilliant Blue G-250 (w/v) (LKB Bromma); 10% phosphoric acid (v/v), 5% ethanol (v/v)

3.2.4 SDS-PAGE

The protein SDS polyacrylamide gel electrophoresis was performed according to Sambrook (1989). SDS-PAGE is a method used for protein separation according to their molecular weight. The factor of charge is eliminated by addition of SDS (sodium dodecyl sulfate) a strong detergent which binds to polypeptides in constant weight ratio thus the intrinsic charges of polypeptides become negligible compared to the negative charges contributed by SDS.

Every gel comprised separating and stacking solution. The composition of these gel solutions are summarized in the Tab. 2.

Tab. 2: Polyacrylamide gels

	8% separating gel 7.5 ml	4% stacking gel 5 ml
30%AA, 0.8% BisAA	2 ml	0.67 ml
1.5M Tris pH 8.8 0.4%SDS	1.9 ml	-
0.5M Tris pH 6.8 0.4%SDS	-	1.25 ml
H_2O	3.6 ml	3 ml
TEMED (Sigma)	3.8 µl	5 µl
10%APS (Sigma)	37.5 µl	50 µl

TEMED (N, N, N', N'-tetramethylethylenediamine) and APS (ammonium persulfate) are added at the end hence they start the polymerization reaction. The separation solution is topped with butanol to form flat surface and washed out before pouring the stacking solution. The separation gel is left polymerazing for 2 hours whereas the stacking gel is left polymerizing just half an hour. The polymerized gels are placed into electrophoretic apparatus containing electrophoretic buffer.

The samples are dissolved in sample buffer and heated to 95 0C which causes denaturation of native proteins forming unfolded polypeptides. 8 µg of protein is loaded per one well. The electric field of 20mA and 300V per gel is applied and causes negatively charged proteins to migrate across the gel to the positive electrode. The electrophoresis takes about 1,5 hour in 8 0C.

electrophoretic buffer: 25mM Tris (Lach-ner), 19mM glycine (Lach-ner) and 0.08% SDS (w/v)

sample buffer: 62mM Tris (Lach-ner) pH 6.8; 10% SDS (Sigma); 0.01% bromophenol blue; 500mM DTT

3.2.5   Western blot

Western blot is used to detect specific proteins from cell extract. First the proteins are transferred on a membrane from an electrophoretic gel. Secondly the membrane is blocked and primary and secondary antibodies are applied in a respective order. Finally proteins were visualized using enhanced chemiluminiscence.

A sandwich from acrylamide gel, nitrocellulose membrane (Whatman), wet filter papers and wet fiber pads is put together and placed into blotting apparatus filled with blotting buffer. The electric field of 100 V and 400 mA is applied for 80 minutes at room temperature with cooling unit attached. As a result negatively charged proteins move to positively charged electrode where nitrocellulose membrane is placed.

The proteins transferred to membrane were stained in Ponceau dye to visualize whether the protein transfer was successful and to visualize molecular size markers. Subsequently the membrane is washed in TTBS buffer 3 times 10 min and blocked for 1 hour with 5%skimmed milk (5%BSA when detecting phospho proteins). Primary antibodies (Tab. 3) are diluted in 5% skimmed milk (5%BSA) and incubated with the membrane overnight. The specifications of primary antibodies used are summed up in Tab. 1. Secondary anti rabbit IgG antibody conjugated with horseradish peroxidase (Promega) was diluted 1:10 000 with 5% skimmed milk (5%BSA) and applied for 1 hour. Secondary antibody was washed out 3 times 10 minutes.   At the end secondary antibody was visualized using enhanced chemiluminiscence detection kit ECL (Thermo Scientific) and Kodak films. The duration of exposition was dependent on the signal strength and varied between 10 seconds and 10 minutes.

blotting buffer: 39mM glycine (Lach-ner), 48mM Tris (Lach-ner), 0.037 SDS (w/v) (Sigma), 10% methanol (v/v)

TTBS buffer: 20mM Tris (Lach-ner), 150mM NaCl (Lach-ner), 0.1% Tween 20 (Sigma) pH 7.4

Tab. 3: List of used primary antibodies and their specifications

primary antibody	supplier	specificity	primary antibody dilution	blocking agent used	animal type
antiGFP	Abcam	entire GFP molecule	1:2000	5% skimmed milk	rabbit mAb
anti-γ-tubulin (AthTU)	(Drykova, 2003)	C terminus of γ-tubulin	1: 2000	5% skimmed milk	rabbit mAb
anti-MPK6	Sigma	C terminus of AtMPK6	1: 3000	5% skimmed milk	rabbit mAb
anti-pERK	Cell Signaling	phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	1: 2000	5% BSA	rabbit mAb
anti-phosphoserine (pSER)	Invitrogen	proteins containing phosphorylated serine residues	1: 250	5% BSA	rabbit mAb

3.2.6 Fluorescence microscopy

All fluorescence images were collected with Olympus oil immersion objectives 60x/1.35 with U-MGFPHQ filter using the Olympus IX-81 inverted microscope with Olympus Disc Scanning Confocal Unit (DSU). The microscope was equipped with Hammamatsu Orca/ER digital camera and the Cell R^{TM} detection and analyzing system.

Následující pasáž o rozsahu 13 stran obsahuje utajované skutečnosti a je obsažena pouze v archivovaném originále bakalářské práce na přírodovědecké fakultě JU.

6 Conclusion

Dissection of dynamic cytoskeletal structures and the emerging field of MAPKs deserve attention of various scientific communities such as biologist, chemists and physicists to solve the riddle of cell signaling regulation.

I have summarized the recent data on cytoskeletal proteins specifically γ-tubulin and mitogen activated protein kinases with the focus on MAPKs in plants. *Arabidopsis* MPK6 is discussed in details. Within the experimental part methods for activation and inhibition of *Arabidopsis* MAPK were optimized. Specific protocols for handling, treatment and detection of MAPKs in *Arabidopsis* cell cultures and seedlings were developed. The data obtained by my experiments present basic protocols for recent and further studies of MAPK and MTs/γ-tubulin interactions.

The knowledge of cell signaling and the response of the cytoskeleton can elucidate downstream targets of MAPK cascade and their role in MT nucleation and organization.

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8 Abbreviations:

AthTU anti γ-tubulin antibody

ABP actin binding protein

γ-TuSC γ-tubulin small complex

γ-TuRC γ-tubulin large ring complex

KLPs kinesine-like motor proteins

MAP microtubule associated protein

MAPK mitogen activated protein kinase

MAPKK mitogen activated protein kinase kinase

MAPKKK mitogen activated protein kinase kinase kinase

MTs microtubules

MTOC microtubule organizing centers

PPB preprophase band

ROS reactive oxygen species