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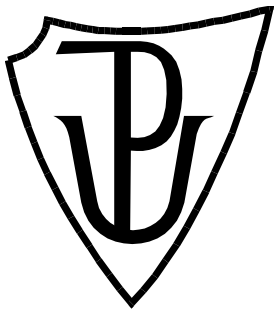

Role inhibitorů GSK na vývoji mitózy rostlin

BAKALÁŘSKÁ PRÁCE

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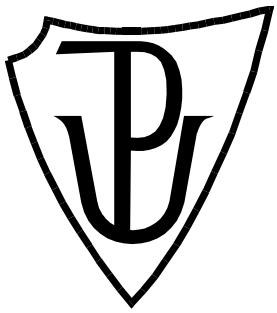

Role of GSK inhibitors on mitotic progression of plants

Bachelor thesis

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Form of study:	Full
Supervisor:	**Georgios Komis, Ph.D.**
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Poděkovani

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Bibliografická identifikace

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Abstrakt

Předložená bakalařská práce byla zaměřena na studium inhibice GSK a její vliv na buněčné dělení. Rostlinné buňky mají definovaný tvar buněčnými stěnami tvořenými mikrotubuly. Ty v rostlinách během mitózy formují jedinečné útvary – preprofázní svazky, které nadále definují orientaci dělení, a fragmoplast umožňující tvorbu nové buněčné stěny. Mitóza je typ buněčného dělení, jehož cílem je zachovat genetický materiál ve dvou dceřiných buňkách. Mitóza sestává z pěti základních fází, u nichž je pozorovatelná úloha mikrotubulů při rozdělování sesterských chromatid do dvou stejných skupin a následném formování jader a nové buněčné stěny. Ví se, že glykogen syntasa kinasa 3, také známá jako SHAGGY-like kinasa, je serin/treonin kinasa podílející se na různých signálních dráhách včetně regulace cytoskeletonu. Mezi jeden z inhibitorů aktivity těchto kinas patří i chlorid litný. V této práci byla studována regulace cytoskeletonu GSK3 kinasami v průběhu mitotického dělení v kořenových spičkách *Arabidopsis thaliana*. Vliv GSK3 byl sledován na organizaci mikrotubulů v průběhu mitózy po ošetření vzorků chloridem litným. Výsledky ukázaly afekty na vývoj mitózy a cytokinezi. Byly sledovány změny v orientaci preprofázního svazku, v některých případech nebyl kompletní, dále byly sledovány defekty tvorby fragmoplastů, kdy se nespojily s buněčnými stěnami a znemožnily tak tvorbu nové buněčné stěny mezi nově vznikajícími rostlinnými buňkami.

Kličova slova	Arabidopsis, GSK, buněčné dělení, mikrotubuly, kořeny, chlorod litný
Počet stran	57
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Abstract

The present thesis was focused on the study of GSK inhibition and its role in cell division. Plant cells shape is defined by cell walls which are made of microtubules. These formulate unique arrays in plants – the preprophase bands that define the plane of cell division, and the phragmoplast that enable the formation of the new cell wall. Mitosis is the type of cell division aiming to the preservation of genetic material in two daughter cells. Mitosis consists of five essential stages during which is shown the role of microtubules in the separation of sister chromatids in two equivalent groups and then in formulation of new nuclei and cell wall. It is known that glycogen synthase kinase 3, also known as a SHAGGY-like kinase, is serine/threonine kinase participating in signaling pathways including cytoskeleton regulations. One of the GSK3 inhibitors is lithium chloride. In this work was studied the regulation of cytoskeleton by GSK3 during mitotic cell division in root tips of *Arabidopsis thaliana*. The role of GSK3 was observed into microtubule organization during mitosis after lithium chloride treatment. Results shown affects in the progression of mitosis and cytokinesis. Changes in preprophase band orientation were observed while in some cases when these were not complete, then were observed defects in the development of phragmoplasts and caused delays in their expansion between cell walls resulting in impossible development if new cell walls between two new plant cells.

Keywords	Arabidopsis, GSK3, cell division, microtubules, roots, lithium chloride
Number of pages	57
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Language	English (Czech)

Obsah

1 Introduction

The plant cytoskeleton bounds plant cells and guides the formation of rigid cell walls. This is important for plant development by defining the orientation of cell growth, by controlling the symmetry of cell division, and by assisting with separating genetic material into two daughter cells.

The plant cytoskeleton is promptly responsive to conditional and developmental cues and as such it is able to be very rapidly remodelled. To this extent, the cytoskeleton is targeted by cell signaling mechanisms which include reversible protein phosphorylation of specific cytoskeletal substrates interaction with secondary messengers such as cyclic nucleotides (Means et al., 1982), bioactive phospholipids (Pleskot et al., 2013), and intracellular Ca^{2+} (Means et al., 1982).

Since the cytoskeleton is implicated in cell division, its regulation by protein phosphorylation is of key significance to drive cytoskeletal rearrangements during the process. It is known, that many microtubular proteins are regulated by cyclin dependent kinases (Sato and Toda, 2010) as well as by MAPKs during the progress of mitosis and cytokinesis. Since the above protein kinases belong to a group that encompasses classes sharing common targeting mechanisms, and since this group of kinases also includes GSK3s with renowned roles in cytoskeletal regulation, we examined in the present thesis the effects of lithium, a broad specificity GSK3 inhibitor to the organization of microtubule arrays in immufluorescently labeled dividing root cells of *Arabidopsis thaliana*.

2 Current State of the Topic

2.1 An Overview of the Plant Microtubule Cytoskeleton

Higher plant cells are bound by a rigid cell wall which is prohibiting cell motility in the classical sense. Therefore, aspects of cell specification which in turn define tissue patterning and organ identity and form, require decisions at the cellular level which will occur *in situ* (Robert et al., 2015) and not through cell migration as it happens during development of animals (Naylor and Davidson, 2017; Nagy and Goldstein, 2017). In this context the microtubule cytoskeleton plays a pivotal role since it underlies three key features of plant development: it defines directionality of cell growth by directing cellulose microfibril deposition (Chen et al., 2016; Griffiths et al., 2015; Gutierrez et al., 2009; Paredez et al., 2006); it controls symmetry of cell division by defining the cell division plane at any stage of plant development (Kimata et al., 2016; Shao and Dong, 2016; Martinez et al., 2017) and finally it drives the partitioning of genetic material to two physically separated daughter cells via the cytokinetic deposition of the nascent cell plate (Miart et al., 2014; Murata et al., 2013).

2.2 The Microtubule Cytoskeleton during Cell Growth

Plant cells, plant organs, and entire plants grow by means of hydraulic cell enlargement. At the phase of vigorous cell growth, internode cells of the freshwater plant Nitella may develop turgor pressure higher than 100 atm and the forces generated have to be both tamed to preserve cellular integrity and to be directed to promote cell elongation (Green, 1968). This is achieved by appropriate spatial deposition of cellulose microfibrils in the overlying cell wall and in turn, this deposition is blueprinted by the cortical microtubule cytoskeleton and its organization (Komis et al., 2015; Li et al., 2015 and references therein). As shown in the composite scheme (Fig. 1) the spatial organization of cortical microtubules coincides with cellulose microfibril patterns and cortical microtubules provide tracks for regulating the trajectories of transmembrane cellulose synthase complexes as they catalyze the polymerization of nascent cellulose microfibrils (Paredez et al., 2006). Moreover, microtubules promote the targeted exocytosis of vesicular delivery of cellulose synthases (CESA) to the plasma membrane (Gutierrez et al., 2009).

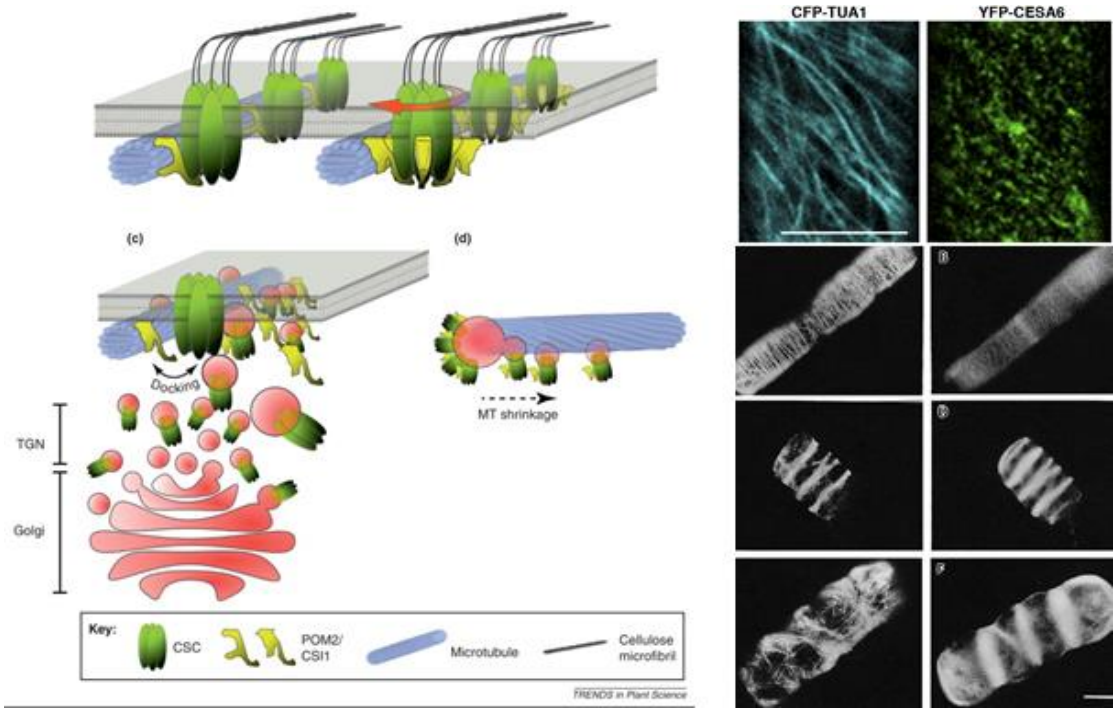

Figure 1. The role of cortical microtubules on cell growth and cytomorphogenesis. A. Schematic demonstration of the role of microtubules underlying CESA motility and membrane targeting (adapted from Bringmann et al., 2012). B**.** Experimental demonstration on the dependence of CESA trajectories (YFP-CESA6) on microtubule orientation (CFP-TUA1; taken from Li et al., 2012) C. Coincidence of microtubule (left column) and cellulose microfibril localized thickenings (right column) during mesophyll cell morphogenesis of wheat (taken from Wernicke and Jung, 1992).

Conclusively, cortical microtubules play a pivotal role in a major event of plant development by regulating the directionality of cell growth and by molding cell shape. As will be shown later, changes in cell growth directionality are many times conditioned by the external environment and this requires a rapid reorganization of the cortical microtubule network, making it responsive to signal transduction pathways that regularly implicate reversible protein phosphorylation events catalyzed by different protein kinases.

2.3 The Microtubule Cycle during Cell Division

Commitment to cell division requires drastic changes at the molecular and subcellular level and it is hallmarked by the global reorganization of the cytoskeleton. As mentioned before, during interphase, the plant microtubule cytoskeleton is dedicated to blueprint cellulose deposition in the surrounding cell wall to support cell growth and cytomorphogenesis, but once a cell becomes committed to cell division, the microtubule cytoskeleton changes priorities and becomes devoted to the equipartition of the duplicated genetic material to two daughter cells that will be in the end of

mitosis physically separated by the microtubule-driven deposition of the daughter cell wall, the cell plate. All the events that pertain to cell division are intimately associated with the microtubule cytoskeleton.

Before the onset of mitosis and during the G to S2 transition of the cell cycle when the cell is typically still in the interphase cortical microtubules predetermine the cell division plane by forming a cortical microtubule annulus, the preprophase band (PPB; Müller et al., 2009). The PPB starts as a broad ring of parallel microtubules and it narrows in a timely manner before the onset of mitosis (Dhonukshe and Gadella, 2003). At that point, the PPB coexists with a polar, perinuclear microtubule array which is the precursor of the mitotic spindle. At this point, the PPB disintegrates and mitosis is initiated (Fig. 2).

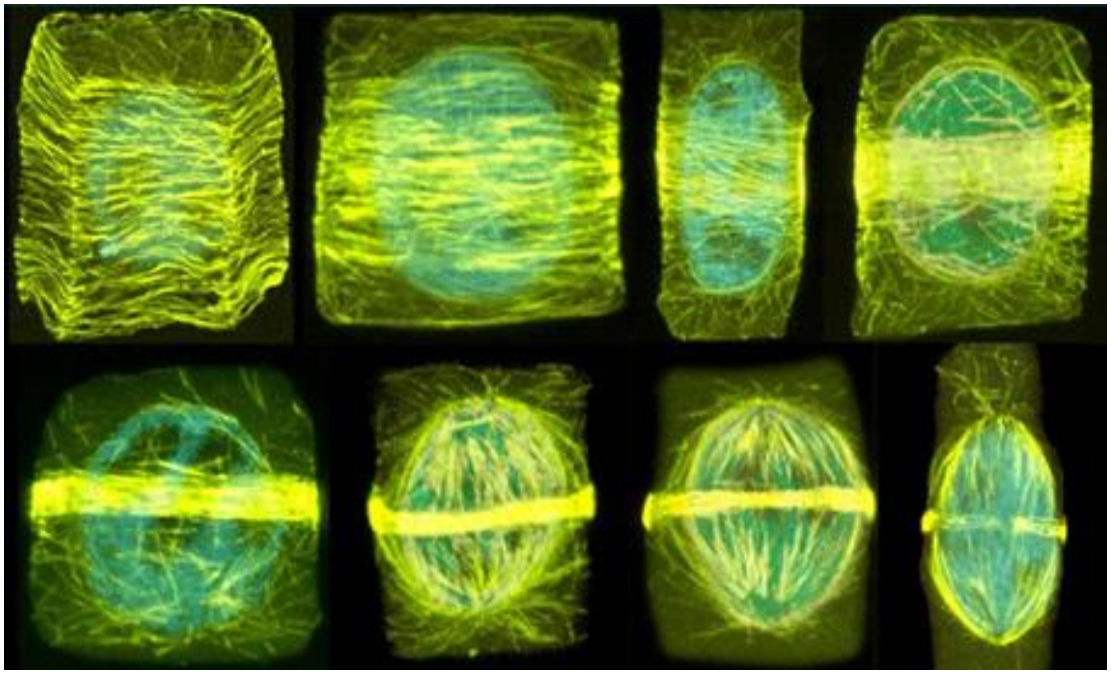

Figure 2. Formation and progressive narrowing of the PPB as visualized in maximum intensity projections of confocal laser scanning microscopy images of tubulin immunolabeled (yellow) onion root cells counterstained with the DNA-specific dye DAPI (blue) to show chromatin configuration. Image is taken from Plant Cell Biology on DVD, B. Gunning, 2009.

By some yet unknown mechanism the PPB marks unexceptionally the cortical sites where the cell plate will fuse with the parent cell walls at the end of cytokinesis and in this sense, the PPB is considered a dominant marker of cell division plane (Rasmussen et al., 2011). And although the microtubule PPB ceases to exist upon the entry into mitosis, there are persistent molecular markers which are somehow recruited at the PPB site and remain until the completion of mitosis (Fig. 3). Such markers include the protein TANGLED (Walker et al., 2007), the protein phosphatase type 2A subunit FASS/TONNEAU (Spinner et al., 2013; Kirik et al., 2012), the microtubule-associated protein AIR9 (Buschmann et al., 2006, 2015), the functionally redundant kinesins POK1/2 (Müller et al., 2006), the Ran GTPase effector RanGAP1 and other RanGAPs (Xu et al., 2008; Stöckle et al., 2016) and the calmodulin-regulated kinesin KCBP (Buschmann et al., 2015), although the mechanism of their recruitment and their precise function in the PPB site are not yet known.

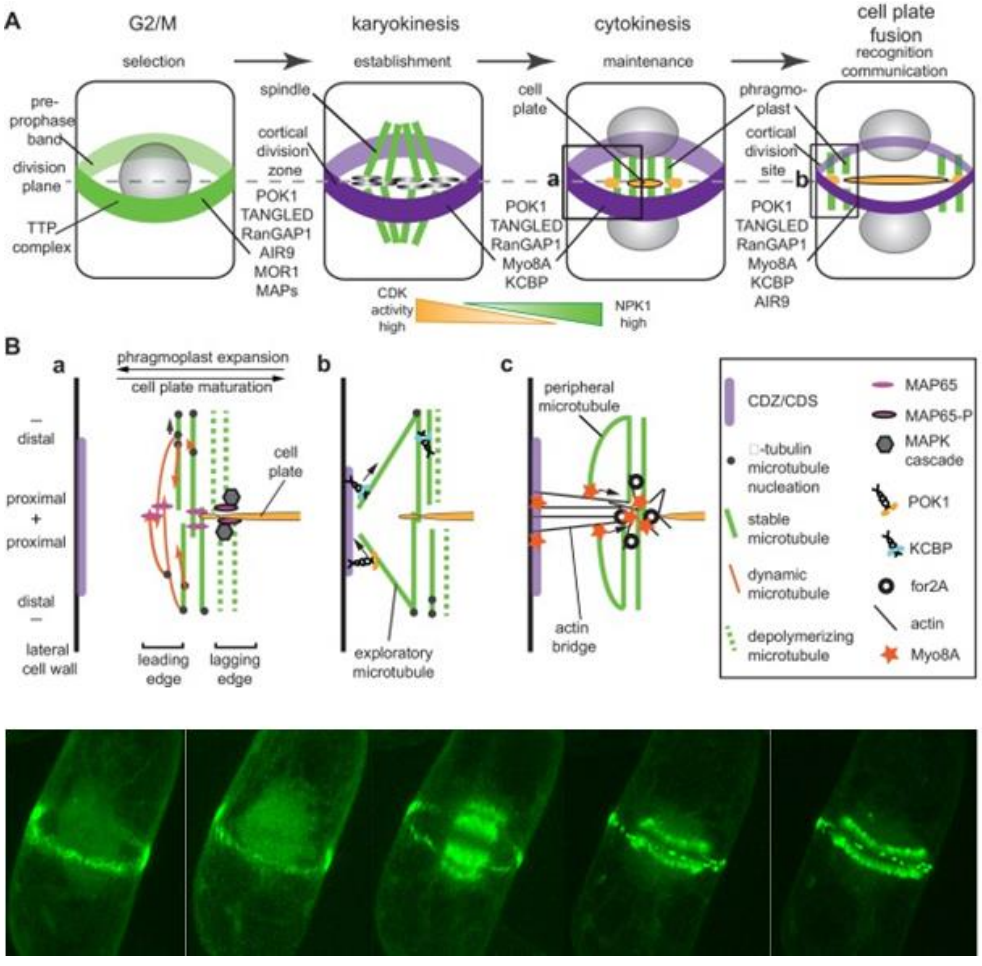

Figure 3. The recruitment of cell division plane molecular markers at the site of the PPB. A. Scheme denoting the markers and their specific stage of recruitment (taken from Müller and Jurgens, 2016). B. Timelapsed imaging of the localization of GFP-KCBP showing its persistence at the cortical PPB site during the entire progress of mitosis and cytokinesis (taken from Buschmann et al., 2015).

After the dissolution of the PPB, the nuclear envelope breaks down and exposes the condensed chromatin to the cytoplasmic environment. At this stage, the chromosomes interact via their kinetochores with the microtubule bundles and the mitotic spindle is formed (prophase to prometaphase). The purpose of the mitotic spindle at this stage is to orient chromosomes so that kinetochores will be aligned to the equatorial plane which is an arbitrary geometrical position coinciding with the previously defined cell division plane set by the PPB (Fig. 4). During the mitotic progression, microtubules undergo dramatic spatiotemporal rearrangements and finally regulate the accurate partitioning and positioning of the sister chromatids to the two opposite poles of the mitotic spindle (Yamada and Goshima, 2017).

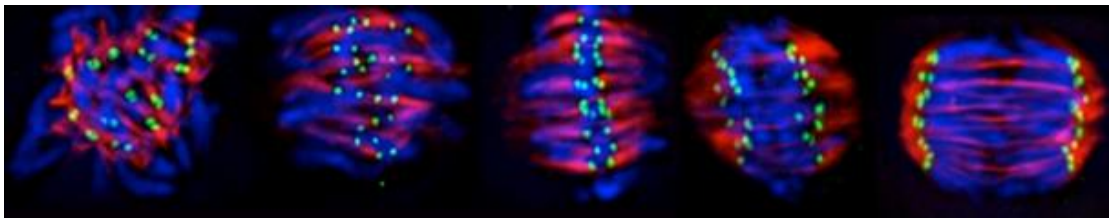

Figure 4. Mitotic spindle transitions during the course of kinetochore coalescence to the equatorial plane and subsequent separation of sister chromatids. First two panels show the gradual arrangement of kinetochores from prophase to prometaphase, the third panel shows the alignment of kinetochores to the equatorial plane (metaphase) forming the chromosome or metaphase plate and the last two panels show two distinct stages of anaphase leading to the segregation of the sister chromatids to the two opposite poles of the mitotic spindle (adapted from Zhang and Dawe, 2011). Chromosomes/sister chromatids are stained with DAPI (blue) while mitotic spindle microtubules and kinetochores are immunolabeled with appropriate antibodies and appear red (microtubules) and green (kinetochores).

2.4 Regulation of Microtubule Transitions by Reversible Phosphorylation Events

Conditional and developmental responses of cell growth and division require rapid adaptation of the cellular machinery to such abrupt changes. Blue light, for example, is known to promote cell elongation (Lindeboom et al., 2013) which requires symmetry breaking in the organization of the cortical microtubule array from an isotropic arrangement to a biased system that mostly comprises of parallel microtubules perpendicular to the cell growth axis (Panteris et al., 2013). Similarly, the mitotic transitions of microtubule arrays from the PPB to the mitotic spindle and finally to the phragmoplast need to be promptly accommodated to the available time frame of cell division since delays in the progression of some stages may lead to abortion of the mitotic/cytokinetic process (e. g., Beck et al., 2011).

In both cases, microtubular rearrangements are coordinated with the aid of a consortium of microtubule-associated proteins, the affinity of which towards the microtubule surface is mostly regulated by reversible phosphorylation events (e. g., Kohoutova et al., 2015; Smekalova et al., 2014; Beck et al., 2010) or with interactions with bioactive lipids such as phosphatidic acid (Zhang et al., 2012). Since the microtubule is a polyanion with a net negative charge under the physiological cytoplasmic pH conditions, binding of microtubule-associated proteins is mostly electrostatic and it relies on the existence of positively charged microtubule-binding domains (Yu et al., 2015).

A prototypical microtubule-associated protein with such properties is MAP65-1, the founding member of the Feo/PRC1/MAP65 family. MAP65-1 contains a bipartite microtubule-binding domain in its carboxyl-terminal end (Smertenko et al., 2004) which is predicted to be targeted and phosphorylated by members of the CGMC group of protein kinases (which includes, cyclin-dependent protein kinases or CDKs; mitogen-activated protein kinases or MAPKs and glycogen synthase kinases or GSKs). Phosphorylation-dependent regulation of MAP65-1 affinity for the microtubule surface has been experimentally verified (Smertenko et al., 2006; Beck et al., 2010)

2.5 Important Protein Kinases in the Regulation of Cytoskeleton

Plant reactions at the molecular level primarily include quick modification of the enzymatic activity of gene expression. The most common mechanisms of the regulation are posttranslational modifications. Such modifications include phosphorylation,

glycosylation, hydroxylation, methylation, acetylation, and ubiquitination, and affects almost all processes at the cell level (Prabakaran et al., 2012). Proteins are phosphorylated by kinase enzymes that catalyze the transfer of the γ-phosphate group of ATP to an acceptor site (typically a hydroxyl group of Ser, Thr or Tyr) in the substrate molecule.

Since the microtubule cytoskeleton is promptly responsive to environmental challenges and to temporally-regulated developmental processes, it is expected that its conditional reorganization would be a target of such regulatory mechanisms, and this is indeed correct expectation since tubulin itself (Ban et al., 2013; Bekesova et al., 2015), as well as a variety of microtubule-associated proteins (e. g., EB1c, Kohoutova et al., 2015; MAP65-1, 2 and 3, Beck et al., 2010; Sasabe et al., 2011) are targeted by such activated protein kinases and become controlled by reversible phosphorylation events.

2.5.1 Cyclin Dependent Kinases

The cell cycle control machinery is regulated by cyclically activating and inactivating key proteins and protein complexes. The most common way for switching the protein on and off is phosphorylation by a CDK-activating kinase (CAK) and dephosphorylation of specific substrates on serine/threonine residues. The phosphorylation reactions are carried out by protein kinases and dephosphorylation by phosphatases. Protein kinases are present in proliferation cells throughout the cell cycle. They are activated only at appropriate times during the cell cycle. This switching on and off of these kinases are under control of cyclins. Cyclins bind to the cell-cycle kinases before they become enzymatically active. These kinases are known as cyclin-dependent protein kinases (CDKs). Hereby, changes of cyclin levels are important for regulation of CDKs during the cell cycle. For the maximal activity of CDKs is necessary phosphorylation at one site by a specific protein kinase and dephosphorylated at two other sites by a specific protein phosphatase. There are different types of CDKs according to different cell cycle phases and cyclins for help drive the cell through the phases. (Alberts et al., Essential Cell Biology 3rd ed.)

CDKs allow the organism to regulate growth and adapt to changes of environment, differentiate and are decisive for plants. In plants are expressed six types of CDKs, CDKA-CDKF (Joubes et al., 2000). The CDKA plays role in both G1 to S and G2 to M-phase progression. The CDKB is limited from late S to M-phases. The CDKC and CDKE are related to some vertebrate CDKs (Umeda et al., 2005).

What is of interest to the present thesis is that CDKs belong to the same phylogenetic branch as mitogen-activated protein kinases (MAPKs or MPKs; see next section) and glycogen synthase kinases (GSKs). This particular group is called CGMC (from **C**DK, **G**SK, **M**APK, and Cdc2-like kinase; **C**LK) and among other features, it shares the common mechanism of targeting substrates. Briefly, all the above kinases phosphorylate Serine or Threonine residues in their target proteins which are arranged in proline-directed motifs (i. e., SP or TP motifs). Many cell cycle proteins contain such motifs and thus may be redundantly regulated by members of the CMGC family of protein kinases. One such example protein is MAP65-1 a microtubule crosslinking protein which is active during interphase and promotes cortical microtubule bundle formation but is excluded from mitotic microtubule systems such as the mitotic spindle until late anaphase when its microtubule-based localization is reinstated and MAP65-1 decorates the spindle as well as the succeeding phragmoplast (Mao et al., 2005).

Previous study showed that MAP65-1 can be targeted by either CDKs or MAPKs in SP-motifs, particularly in its carboxyl-terminal microtubule binding domain (Smertenko et al., 2006), rendering the protein unable to bind to the microtubule surface. In plants CDK and MAPK activities are temporally discriminated, hence targeting of MAP65-1 from both classes provides a tool for the temporal regulation of MAP65-1 affinity for the microtubule surface during the course of mitosis.

2.5.2 Mitogen Activated Kinases

An important role in cell signaling in plants is assigned to mitogen-activated protein kinases (MAPK; Rodriguez et al., 2010), which are typically arranged in three-tiered and processive signaling cascades (Mishra et al., 2006). MAP kinases are involved in key processes such as cell proliferation, differentiation and cell death in all eukaryotic organisms from yeast to humans (Qi and Elion, 2005; Raman et al., 2007; Colcombet and Hirt, 2008; Keshet and Seger, 2010). In plants the MAP kinases are typically involved in responses to abiotic stress (Moustafa et al., 2014), hormone signaling (Smekalova et al., 2014), including abscisic acid (de Zelicourt et al., 2016), auxins (DeLong et al., 2002) and jasmonates (Ahmad et al., 2016). MAPKs are also implicated in plant responses to biotic interactions and the induction of innate immunity triggered by pathogens (bacteria, fungi, nematodes, Meng and Zhang, 2013; Hamel et al., 2012), and to chemoprotective reactions against herbivores (Hettenhausen et al., 2015).

Signaling cascades of MAP kinases comprise of highly conserved modules in all eukaryotic cells (Zhang et al., 2006). Each cascade harbors at least three protein kinases which are activated and function sequentially; thus each member of the cascade (MAPKKK, MAPKK, and MAPK) is phosphorylated by the preceding kinase and thereby activate each other. Therefore, all members of a typical MAPK cascade, serine/threonine, and tyrosine residues, which are modified by the addition of phosphate group (Ichimura et al., 2006).

MAPKs form a complex system of interrelated pathways where one or more components of a signaling pathway might affect others. This cross-communication is called crosstalk and is typical for complex signaling pathways, among which MAPKs are included (e. g. Jonak et al., 2002). Some kinases of the same type may be involved in diverse physiological processes (Colcombet and Hirt, 2008). MAPK signaling pathway begins with the phosphorylation of serine/threonine kinases MAPK triple kinase (MAP3K), proceeds through the activation of a dual specificity MAPKK (MAP2K) and concludes with the dual phosphorylation of the MAPK (dual phosphorylation is the simultaneous phosphorylation of the MAPK on a TXY motif, i. e., a motif that contains a Thr and a Tyr residue that become phosphorylated by only one kinase, the MAPKK; Colcombert and Hirt, 2008). In the end of the cascade, the activated MAPK is able to phosphorylate many substrates including transcription factors regulating gene expression (Andreasson and Ellis, 2010).

As discussed before, the role of MAPKs is not restricted to conditional signaling, since different MAPKs have essential developmental roles, including control of embryo development (Lukowitz et al., 2004; Bush and Krysan, 2007; Lopez-Bucio et al., 2014), stomatal lineage specification and ontogenesis (Bergmann et al., 2004) and post-embryonic vegetative development (Müller et al., 2010; Smekalova et al., 2014) to name some few. At the cellular level, MAPKs have been shown to regulate the process of cytokinesis by controlling the rate of centrifugal phragmoplast expansion (Beck et al., 2011) as well as the control of cortical microtubule organization and rearrangements during cell expansion and differentiation (Beck et al., 2010). To this extent, MAPKs, have been shown to associate with compartments of membrane trafficking including the plasma membrane and the trans-Golgi network and with microtubule arrays (Müller et al., 2010; Beck et al., 2010). The association of MAPKs with microtubules is of particular interest since it implicates their function in the process of cytokinesis and the control of cell division plane orientation.

The dominant MAPK cascade which is controlling cytokinetic progress comprises of the ANP family of MAPKKKs, the MKK6 MAPKK and the MPK4 MAPK (Beck et al., 2010; Kosetsu et al., 2010). Among the targets of MPK4 which are involved in the process, are MAP65-1, MAP65-2 and MAP65-3 (Sasabe et al., 2011) and the cell plate specific protein patellin (Suzuki et al., 2016).

In the control of cell division plane orientation, the dominant MAPK is MPK6 acting downstream of YODA MAPKKK and MKK4/5/7/9 MAPKKs. In this case the role of MPK6 still remains obscure since it may involve direct phosphorylation of cytoskeletal substrates such as MAP65-1 (Smekalova et al., 2014) or EB1c (Kohoutova et al., 2015), but it may also implicate the transcriptional transactivation function of MAPKs, regulating the expression levels of proteins related to CDP such as TANGLED, POK1/2, and GCP4 to name some few (Smekalova et al., 2014).

2.5.3 Glycogen Synthase Kinases

2.5.3.1 General Properties of GSKs

Glycogen synthase kinases 3 (GSK3), also known as a SHAGGY-like kinases, represent a highly conserved family of serine/threonine protein kinases with roles in diverse biological processes in eukaryotes. This enzyme was originally characterized in the animal insulin signaling pathway and is responsible for the final step in the synthesis of glycogen - the phosphorylation of glycogen synthase (Lawrence et al., 1986). However, the activity of GSK3 is also involved in cell fate specification, meiotic chromatin segregation in oocytes, developmental signaling, regulation of cytoskeleton, programmed cell death while its deregulation is involved in human pathogenesis including cancer development or an emergence of Alzheimer's and other neurodegenerative diseases. Mammalian GSK3s consist of two isoforms namely GSK3α and GSK3β which have divergent C- and N-terminal domains that are important for regulation of their function but harbor conserved kinase domains (Saidi et al., 2012; Jonak and Hirt, 2002; Qi et al., 2013; Acevedo et al., 2007).

Mammalian GSK3s have been critically involved in the development and the aggression of neurodegenerative diseases which are accompanied by amyloid aggregations (also called neurofibrillary tangles) of the microtubule-associated protein tau (Takahashi et al., 2017). Tau is a multitasking protein that primarily crosslinks microtubules in neuronal cells (Bakota et al., 2017). The key significance to its function is that its primary sequence contains multiple Ser/Thr residues that are targeted for

phosphorylation by a pleiade of Ser/Thr protein kinases (Rudrabhatla 2014) including CDKs (Castro-Alvarez et al., 2014), CLK1 (Jain et al., 2014), the apoptosis signal-regulating kinase 1 (Song et al., 2014), but most importantly GSK3s (Maqbool et al., 2016).

Our knowledge on the association of GSK3 signaling with the regulation of the cytoskeleton does not derive solely from their connection to neurodegenerative diseases, but also through the involvement of GSK3s in the regulation of the actin and the microtubule cytoskeleton during cell migration (Sun et al., 2009), the regulation of centrosome duplication prior to mitotic spindle assembly (Yoshino and Ishioka, 2015) but also the dynamics of mitotic spindle and cytokinesis progression (Kalive et al., 2012; Harwood et al., 2013; Portegijs et al., 2016).

2.5.3.2 The GSK family in Plants (Arabidopsis)

The GSK family of plants as exemplified in the case of the genetically tractable model plant *Arabidopsis thaliana* comprises of ten different genes which can be classified into 4 distinct classes based on sequence homology. Class I includes ASKα, γ, ε, class II includes ASKι,dζ, and BIN2, Class III contains ASKβ and θ and Class IV contains ASKκ and ASKδ (Fig. 5).

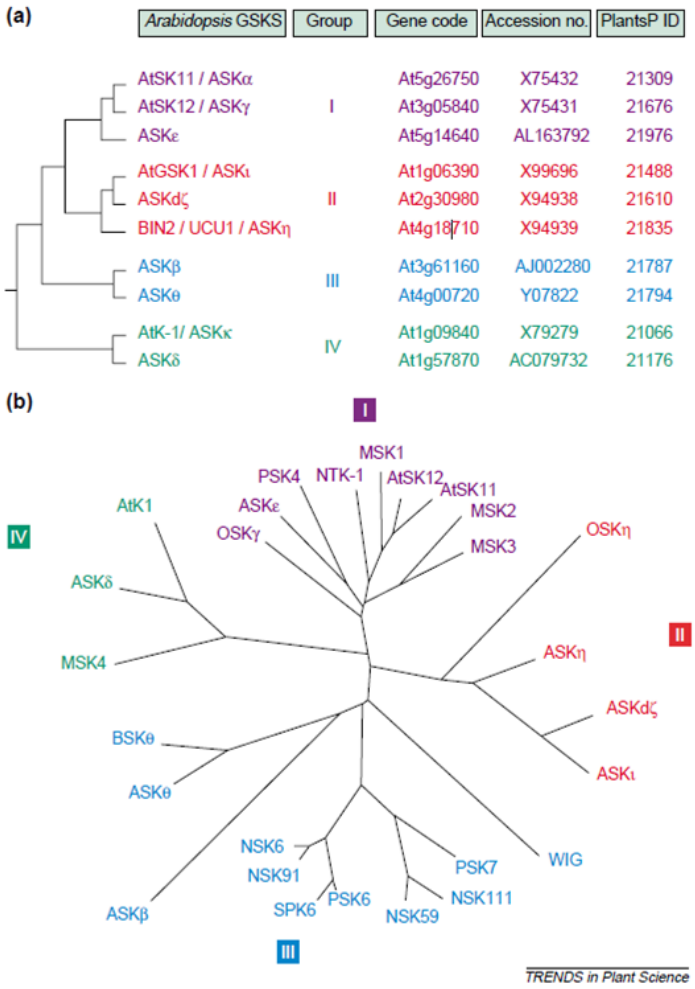

Figure 5. The phylogenetic classification of *Arabidopsis thaliana* GSK3s taken from Jonak and Hirt, 2002.

Plant GSKs have been very much understudied and the very few studies have provided expression patterns of some GSKs (Pay et al., 1993; Jonak et al., 1995), plastid localization in one case (Kempa et al., 2007) but most importantly a tight connection between brassinosteroid signaling, BIN2 activation and regulation of YODA pathway in stomatal ontogenesis by the YODA pathway. As a result of this involvement, BIN2 constitutive activation in *ultracurvata1-1* semidominant mutants (*ucu1-1*) phenocopies the loss-of-function *yda* mutation and so far represent the only *GSK3* mutant with a discernible phenotype (Berna et al., 1999; Perez-Perez et al., 2002). The *ucu1-1* (and its allelic *ucu1-2*) mutant shows severe dwarf phenotype with a disturbed growth of the hypocotyl and the leaves but reasonably such developmental phenotypes can be also extended to the root development (Perez-Perez et al., 2002; Fig. 6).

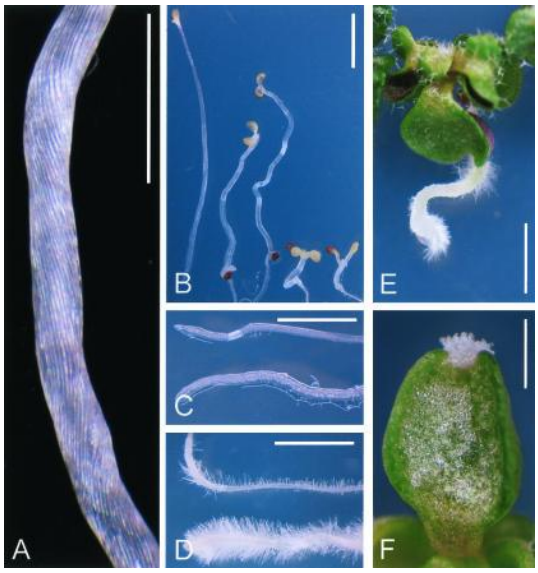

Figure 6. The vegetative phenotype of ucu1-1 mutant a semidominant BIN2 constitutive activator. The image was taken from Perez-Perez et al., 2002.

From the very few studies, GSK3 kinases have been implicated in innate immunity induction through pathogen-associated molecular patterns (Stampfl et al., 2016) and in oxidative stress responses (Dal Santo et al., 2012) however, their major involvement is the integration of brassinosteroid signaling. As mentioned before, BIN2 is an upstream negative regulator of the YODA pathway which is in turn activated by the brassinosteroid receptor BRI1 and through this pathway, BIN2 is involved in stomatal lineage specification. Moreover, a chemical biology, the inhibitor-based study revealed that general inhibition of Arabidopsis GSK3s can trigger brassinosteroid signaling (De Rybel et al., 2009).

2.5.3.3 Pharmacology of GSKs (Lithium Chloride and Bikinin)

There have been identified more than 30 GSK3 inhibitors. Most are ATP-competitors binding to the ATP-binding pocket in the kinase domain (An et al., 2012 and references therein). GSK3 inhibitors can be used as the treatment for several diseases like neurodegenerative diseases, e. g. Alzheimer's disease, bipolar affective disorder, diabetes and diseases caused by unicellular parasites which express GSK3 homologs.

Lithium (Li) has been identified as a broad range inhibitor of GSK3 kinases. Thereon, the lithium effects on GSK3 activity have stimulated the search for more inhibitors of GSK3 especially for the treatment or amelioration of neurodegenerative diseases such as Alzheimer's disease. Most pharmacological inhibitors of GSK3 have common properties (An et al., 2012). Interactions of lithium with GSK3 are similar to those with inhibitors of CDKs which pertains to the structural similarities of the GSK3

and CDK families. The options for modulating physiological functions of the GSK3 are attractive because of its multi-protein complexes where are targeted sites between GSK3 and other proteins (Meijer et al., 2004). Specificity of lithium for GSK3 is questioned because it is able to inhibit alternative targets such as diacylglycerol kinases (Sakane et al., 2016). Use of lithium is limited in *in vivo* studies in plants because it induces ion toxicity. Depending on its concentration in culture media, it can influence plant growth by both stimulatory and inhibitory ways. Lithium cations are transported by similar mechanisms used by potassium or calcium ions. Lithium can cause necrotic spots on leaves, inhibition of growth by reduction of biomass production, compete with water and act like dehydrating agent or act like cofactor for the activation of some enzymes while it causes oxidative stress and inhibit DNA synthesis in higher concentrations than 20 $mg \cdot l^{-1}$ in many plant species. In lower concentrations however, lithium stimulates plant growth (Shahzad et al., 2016)

Lithium chloride (LiCl) induces the inhibition of GSK3 activity leading to the dephosphorylation of GSK3 substrates via protein phosphatases. Mice embryos cultured in LiCl-supplemented nutrient medium, exhibited abortive cleavage after the first mitotic division and resulted in abnormalities in chromatin and cytoskeleton. This finding suggests that LiCl perturbs preferentially cytokinesis (Acevedo et al., 2007).

In the case of Alzheimer's disease, treatment with lithium resulted in considerably lower levels of phosphorylation of tau and subsequently to reduced levels of tau neurofibrillary tangles. Moreover, LiCl treatment also enhanced microtubule binding with tau, and promoted microtubule assembly. Thus, lithium reduces levels of pathogenic insoluble tau by GSK3 inhibition and can be effective therapeutically (Noble et al., 2005).

Bikinin (Fig. 7) is a specific inhibitor of plant GSK3s. It is a small nonsteroidal molecule that acts like a competitor of ATP and binds directly with BIN2. Seven of Arabidopsis GSK3s are potential targets of bikinin. The strongest inhibition activity was found in Arabidopsis I and II group of GSK3 subfamilies (De Rybel et al., 2009). It was identified in a chemical genetics screen as an inhibitor of GSK3s. Bikinin can also mimic brassinolide and induce brassinosteroid response which participates in plant development, salt and pathogen stress, and in response to wounding. For its activity is important the bromine in position 5 of the pyridine ring and the carboxylic acid group. It can be used for brassinosteroids studies. It competes with the binding site for ATP in ASKs and therefore, inhibits ASKs activity. It was shown that synthesized bikinin

derivatives are in many cases more active than bikinin itself, for example, iodobikinin or methyliodobikinin. The methyliodobikinin was more active *in vivo* than bikinin because it showed increased plant tissue permeability (Rozhon et al., 2014). Bikinin inhibits BIN2 and activates brassinosteroid signaling (Bjornson et al., 2016). It can increase susceptibility to infection by *Tobacco Mosaic Virus* so the activation of brassinosteroid signaling leads to inhibition of viral defense response in *Nicotiana benthamiana* (Deng et al., 2016). High concentrations of bikinin (around 30 μM) caused abnormal structures with many turgescent cells in wheat embryos. The treatment of wheat embryos with bikinin indicated that seedling growth is regulated by brassinosteroid signaling (Bittner et al., 2015).

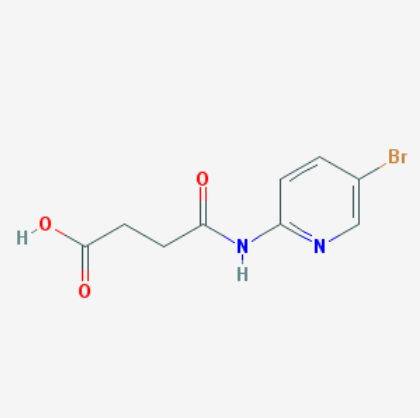

Figure 7. The molecular structure of bikinin, an ATP-competitive inhibitor of plant GSK3s, the monoamide of succinic acid with 2-amino-5-bromopyridine (Adapted from De Rybel et al., 2009).

2.6 Scope of the Thesis

2.6.1 The Architecture of the Root Meristem of Dicots

The structure of the primary root emerges through a highly regulated cell division consortium in the root apical meristem (RAM) and the subsequent expansion and differentiation of these cells. All cell types in the root arise from the RAM, which is initiated during the early stages of embryogenesis (Altamura et al. 2007). The RAM is characterized by the quiescent center cells with a low mitotic activity (QC; Doerner, 1998) flanked by stem cell active initials (Stahl and Simon, 2005). The set of these two cell types is called the stem cell niche (Spradling et al., 2001). Depending on their position, stem cells divide and differentiate into various cell types of the root with different functions (Stahl and Simon, 2005) whereas cells from quiescent zone centers are slowly-dividing cells (approximately 500 to 600 in the number of cells in mature maize; Clowes, 1959). The quiescent center acts as a reservoir of cells that can survive the stress load and regenerate the meristem when necessary. Apart from these two types of cells, RAM harbors predetermined cells that are vigorously dividing either periclinally or anticlinally. The purpose of the periclinal (or formative) divisions is to form the distinct root cell files (lateral root cap, epidermis, cortex, endodermis, pericycle, protoxylem and protophloem) while the anticlinal division promotes the growth of cells of each file to maintain the uninterrupted basipetal root growth (Wachsman et al., 2015).

Root cell division can take place at three planes: a plane parallel to the root surface (anticlinal), a plane perpendicular to the root surface (periclinal), or a radial plane (radial) that can be either periclinal or anticlinal (Fig. 8).

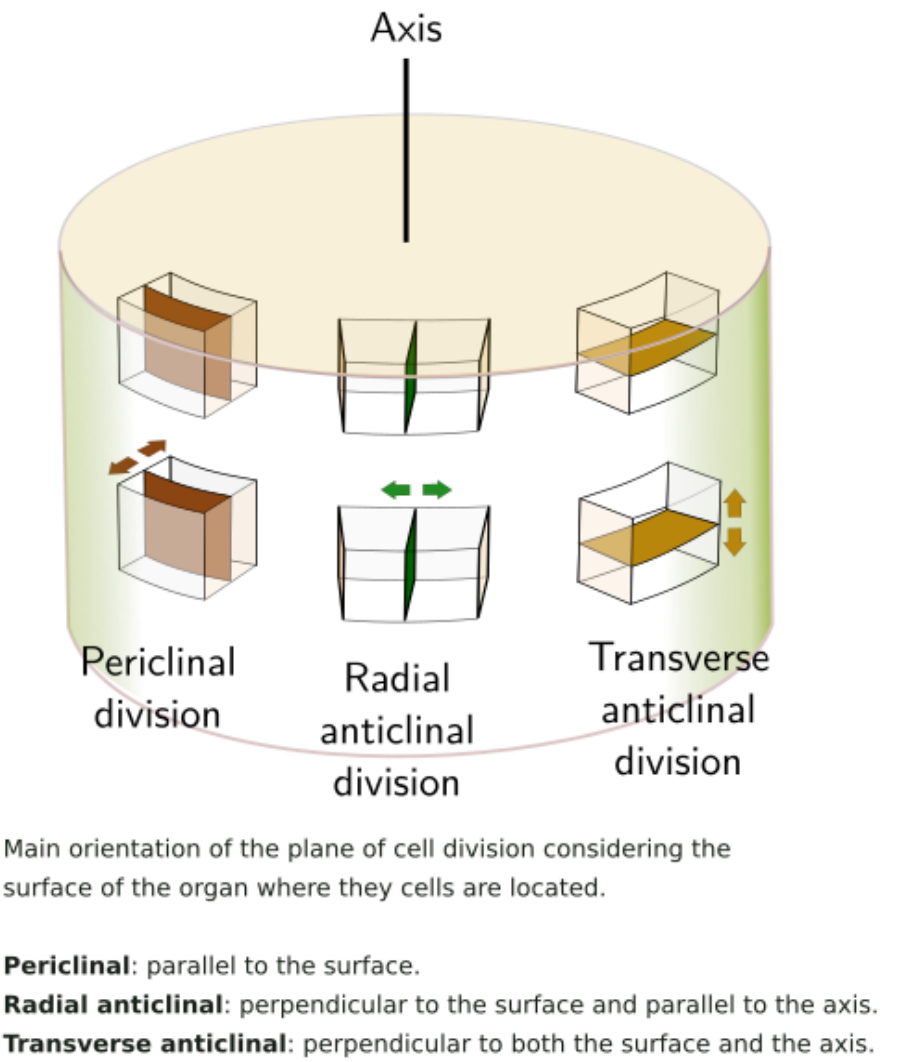

Figure 8. A visual guide of cell division planes in the root of *Arabidopsis thaliana* (taken from Atlas of Plant and Animal Histology: https://mmegias.webs.uvigo.es/02-english/1-vegetal/guiada_v_meristemos.php)

These modes of cell division lead to the increase of the length and the width of the root. Individual periclinal cell divisions at the root tip create the root cap (or calyptra), which extends forwardly and protects the root apex. The central cells of the root cap are often oriented in vertical rows and are quickly worn out during penetration of the root into the soil. These cells also secrete rich glycoproteins mucigel, thereby reducing the friction between the root and the soil matrix during root elongation (Oades, 1978). Stem cells give rise to a population of rapidly dividing progenitor cells that respond to developmental cues. Progenitor cells divide asymmetrically and produce clonally related cells of the root meristem. After a series of divisions, these cells differentiate in the tissues constituting the epidermis, the bark (cortex), endodermis and vascular tissue in the root elongation zone and the root differentiation zone (Perilli et al., 2012). In

a centripetal manner, the root is radially organized into specific cell layers. These include the lateral root cap which protects the underlying cells, the epidermis, the cortex, the endodermis, the pericycle and the central cylinder, which comprises of the protoxylem and protophloem, founders of the root vasculature. Stem cells located on the center plane of the root meristem activity keeps producing the abovementioned root tissue cells. Figure 9 shows the arrangement of cells in the stem cell niche of the primary roots of *Arabidopsis thaliana*.

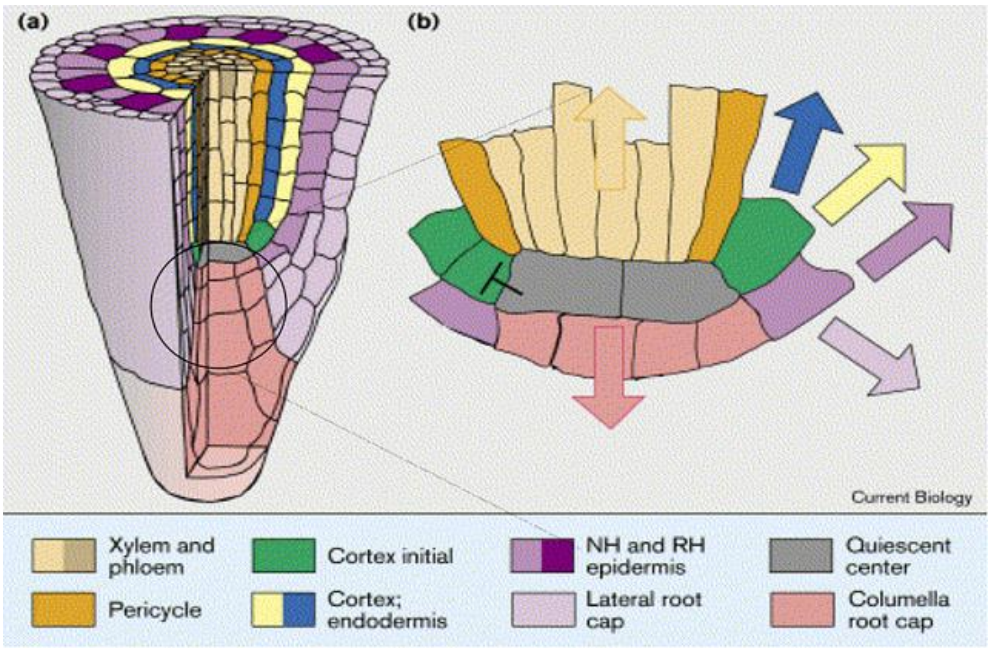

Figure 9. The schematic arrangement of cells in the root tip of *Arabidopsis thaliana*. (b) Enlarged schema of cell organization in the stem cell niche (taken from Benfey et al., 2000)

2.6.2 Immunolocalization of Proteins in Arabidopsis Root Wholemount

Detailed microscopic visualization of cell structure and localization of proteins requires specific and highly sensitive method. Such a method is immunofluorescence labeling of proteins which is now a widely used laboratory technique based on the principle of antigen-antibody binding. The antigen is typically a protein that we want to localize and which specifically binds to an antibody (immunoglobulin) labeled with a fluorescent label - a fluorophore. The fluorophore absorbs light of a certain wavelength (λexc) which depends on its chemical structure and becomes activated in order to emit light of a different wavelength (λem). The emitted light (fluorescence signal) is then detected by the microscope detector unit and is used to reconstruct the image of the fluorescently labeled structure.

In immunolabeling techniques, two types of antibody sources are used. Monoclonal antibodies which are copies of a single molecule that bind to a specific area of the antigen, whereas polyclonal antibodies are a heterogeneous mixture of immunoglobulins (Ig) and have the ability to bind to different sites of the same antigen. Moreover, immunofluorescence detection can be classified into two different methods, the direct and the indirect. In the direct the antigen is detected by the respective antibody which is coupled to the fluorophore. In the case of using monoclonal antibodies only one labeled antibody can bind to the antigen enabling the accurate detection of the protein under investigation. However, the disadvantage of this technique is a weak fluorescent signal that is provided by a single molecule of fluorophore bound to the antibody. This can be overcome by using the indirect approach, in which the antigen is detected by an antibody (it is called primary antibody) which in turn is recognized by anti-idiotypic, fluorophore labeled IgGs (secondary antibody). Typically, the secondary antibody comes from a polyclonal mixture therefore it can bind to various sites of the primary antibody. In the end, a single epitope can be labeled by more than one fluorophores providing the basis of signal amplification in the case of indirect immunofluorescence.

Most previously developed immunolocalization methods for visualizing plant tissues require preparation of sections, but this preparation is laborious, time-consuming and unsuitable for large numbers of samples. An effective alternative is the immunolocalization by whole mount method for *in situ* localization of proteins in intact

tissues. This method is relatively simple and provides high resolution without the need for preparing tissue sections. It also allows the preservation of tissue integrity which is very important for development studies. The whole mount assay is particularly suitable for the localization of proteins within cells root tips, lateral roots, hypocotyls, cotyledons and embryos of *Arabidopsis thaliana.* The method has been successfully used for tobacco (*Nicotiana tabacum*) and tomato roots (*Lycopersicon esculentum*) (Sauer et al., 2006). The main limitation of this protocol refers to the low permeability of tissues and the depth to which antibodies can diffuse within the tissue.

The basis of the whole mount method can be summarized in five steps: tissue fixation, cell wall digestion and membrane permeabilization, blocking of non-specific antibody binding sites and sequential incubation with primary and secondary antibodies (Sauer et al., 2006). Each of these steps involves an extended process that must be followed. In this study the protocol by Sauer et al. (2006) was used, after modifications (described in Materials and Methods), but the principles remain the same individual steps. The first step in sample preparation is fixation of the plant material. Fixation is required to immobilize antigens and preserve cell morphology which is very important for successful immunolocalization. Improper fixation can lead to a reduction of the fluorescence signal or to changes in morphology. The best results were achieved by fixation with formaldehyde with the addition of small amounts of glutaraldehyde. To ensure the entry of antibody inside the cells the barriers of the cell wall and the plasma membrane need to be removed. This is achieved by enzymatic digestion of cellulose and pectins and by a detergent-based permeabilization step to extract lipids from the membranes. The time and temperature of enzyme digestion needs to be carefully controlled in order to avoid under- or overdigestion which can compromise labeling efficiency and structure integrity respectively. Residual aldehydes from the fixation step can hamper localization specificity by covalently attracting antibody molecules to non-specific sites. Such aldehyde groups are reduced to hydroxyl groups by incubation with a strong reducing agent - a sodium borohydride ($NaBH_4$). Subsequently membranes are permeabilized to allow entry of antibodies inside the cell and to facilitate the access of antibodies to antigens. The permeabilization solution is a buffered mixture of an organic solvent (dimethyl sulfoxide; DMSO) and some detergent (in the present thesis NP-40 was used but it can be substituted for Triton X-100). Prior to antibody it is necessary to prevent the non-specific interaction with other antigens in the sample by using

a solution of an inert protein (typically bovine serum albumin; BSA) dissolved in a buffer.

The first five steps of whole mount method can be considered preparatory since they precede the actual application of antibodies. As already mentioned, the binding of antibodies to the antigen is the very essence of immunolocalization methods in which whole mount method belongs. In the standard indirect immunofluorescence scheme followed herein, the antibodies are applied sequentially with very thorough washes in between. Primary antibody is directed against the protein of interest, while the secondary antibody is an anti-IgG mixture raised against the animal species from which immunoglobulins of the primary antibody were isolated. The fluorophore conjugate of the secondary antibody, mainly depends on the excitation and emission spectra that are allowed by the laser lines and the filter cubes of the microscope used in our laboratory.

2.6.3 Microscopy methods

Plant cells and their structures are very small objects that are not visible to the naked eye. Therefore, their study requires various microscopic techniques to observe the subcellular structures at various magnifications and in conjunction with immunofluorescence detection it provides a reliable tool for visualization of very small objects. Cellular structures and proteins can be studied by using these microscopic techniques that were also used in this study:

2.6.3.1 Confocal laser scanning microscope Spinning disc

The principle of the confocal laser scanning microscopy (CLSM) is the formation of optical sections taken with the aid of a focused excitation laser beam that passes through the sample and scans the object point by point. Fluorophores excited by the beam emit photons which are then detected by the microscope's detection system (a photomultiplier tube in the case of CLSM). The energy of the laser beam excites the targeted area, whereas the emitted light passes through a single pinhole blocking scattered light. Limitation of the confocal microscope is image acquisition rate. A single laser beam is limited by an extremely precise control through galvanometric mirrors which are used for the raster of the scanning beam passing through the sample. It is also limited in the number of photons emitted by the sample during a delay in sensing pixels. Confocal microscopes scan a sample in a rate of 1 microsecond per pixel, depending on the dimensions of the data, the rate varies from one-half to more than two seconds per frame. Therefore, most of the confocal laser microscopes are useless for studying

dynamic processes in living cells that take place within milliseconds (Davidovits and Egger 1969, 1971).

Some limitations of confocal scanning microscopy can be overcome by imaging the sample with multiple excitation beams operating in parallel. This can be achieved by passing light through the Nipkow disc which contains thousands of apertures arranged in a rotating spiral and enables the overall illumination of the sample at the same time (Petraň et al., 1968).

Figure 10 shows the microscope used in this study.

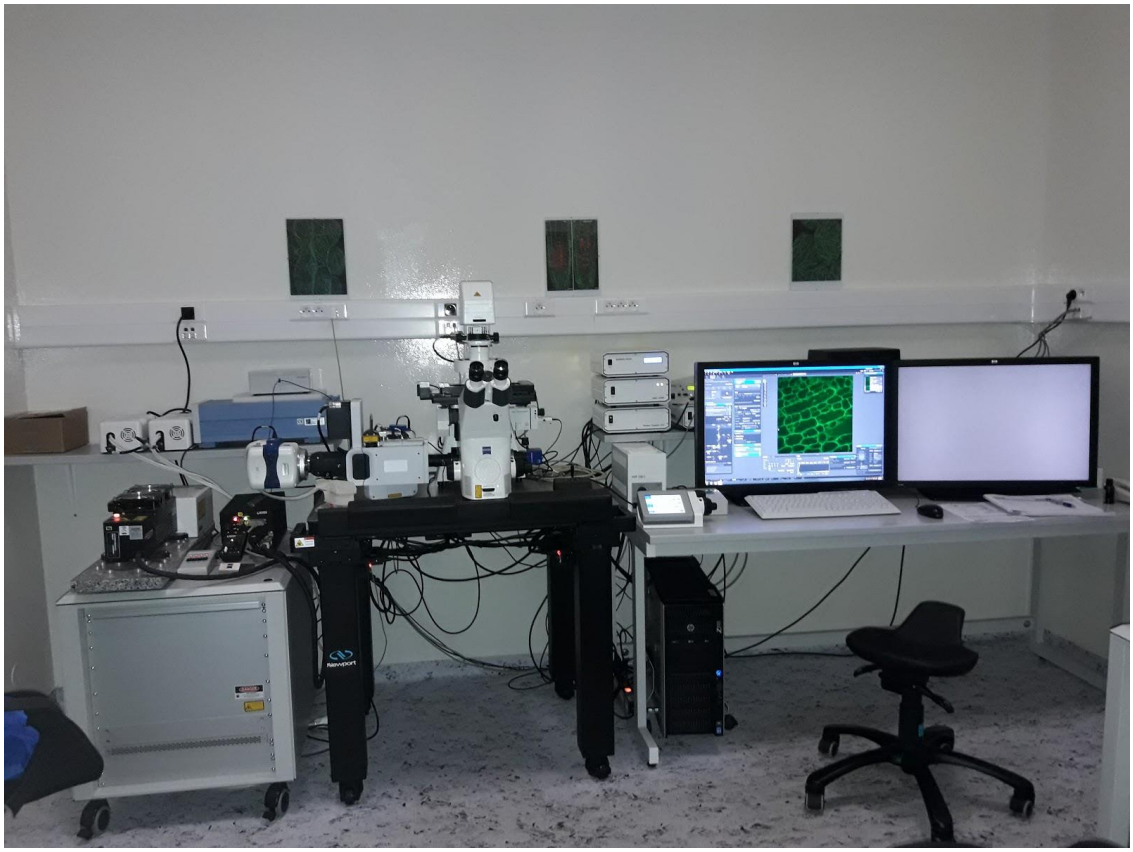

Figure 10. The Zeiss Cell Observer SD spinning disc platform hosted by the Department of Cell Biology (CR Hana) that was used in the present study.

3 Material and Methods

3.1 Plant Material

Arabidopsis thaliana (L.) Heynh, ecotype Columbia (Col-0)

3.2 Observation of Seedlings Phenotype

Plant cultivation was carried out under sterile conditions *in vitro* in a growth chamber. Seeds were plated on the square Petri dishes with a solid MS medium (Murashige and Skoog) at half concentration of substances ($^1/_2$ MS) and without vitamins.

3.3 Preparation of Seeds

The surface sterilization of seeds was made before sowing seeds on the culture medium. The sterilizing solution was prepared by diluting a stock of 10% v/v solution of sodium hypochlorite (NaClO) at a 1% v/v solution and then one drop of the Tween 20 detergent was added. Incubation took around 10 minutes with occasional vortexing.

After this, every manipulation with seeds was carried out in a sterile laminar flowbox and with the use of only sterile instruments and solution. For solution exchange, after sterilization, an automatic micropipette was used. The sterilization solution was nearly completely removed and replaced with 1 ml of 70% v/v aqueous ethanol. The tube was inverted few times for a total of not more than 30 seconds and then the ethanol solution was removed. Seeds were then washed 3-5 times with 1 ml of sterile distilled water before being transferred to the solid ½ MS medium. It was necessary to shake microtubes with water because of residues of ethanol solution and sterilizing solution which would prevent germination. After the last wash, a small volume of water was allowed in the tube to be used for transferring the seeds to medium.

3.4 Preparation of Culture Medium and Sowing

Arabidopsis seeds were cultivated under sterile conditions *in vitro* on ½ MS medium. Sterile medium was poured into square Petri dishes (120 mm × 120 mm) in a sterile laminar flowbox. Thereafter, approximately 100 seeds were seeded on each sterile plate by using micropipette (100 µl). For easier sowing of seeds on sterile plates the tip of the pipette was slightly cut at the end. All instruments used for sowing seeds were sterile or were sterilized by Spitaderm disinfectant solution or 70% v/v aqueous ethanol. The closed dishes were sealed with parafilm and placed in a horizontal position in the refrigerator (4 °C, dark) for 1-4 days in order to stratify seeds and synchronize their germination and seedling growth. The Petri dishes with seeds were then transferred to

a culture chamber (phytotron) where they were placed vertically and cultured for another 3-4 days to allow germination and seedling growth (23 °C, 16 h light / 8 h dark).

3.5 Lithium Chloride Treatment

One half of the grown seedlings was treated with lithium chloride (LiCl). For this purpose the seedlings were immersed into a 10 mM LiCl solution in liquid ½ MS medium and incubated for 4 hours at room temperature accodring to standard inhibitor treatment regimes (De Rybel et al., 2009). For control the rest of the seedlings were immersed in plain liquid ½ MS and were incubated in parallel with the LiCl treated ones for the same time.

3.6 The Whole Mount Method for Immunofluorescence Labeling

This method is a variant of immunofluorescence techniques, which aims to the preservation of the entire organ integrity (in this particular case the root) while preserving immunoreactivity of the proteins of interest (in this case, tubulin as a constituent of microtubules). For this is required a special procedure for sample preparation. For this study, a published protocol was followed (Sauer et al., 2006) after certain modifications which are mentioned in the following sections.

1) Fixation

Fixation is the process of chemical immobilization of intracellular molecules aiming to the preservation of structure and antigenicity. For this purpose the ideal fixative comprises a mixture of a monovalent (formaldehyde) and a bivalent (glutaraldehyde) aldehyde, buffered at a pH and mixed in a solution with additives necessary (such as EGTA and $MgSO_4$) for the preservation of microtubules. Formaldehyde is derived from the alkaline hydrolysis of a 8% w/v paraformaldehyde solution. For the preparation of this solution, 16 g of powdered paraformaldehyde was dissolved in 150 ml of ultrapure Milli-Q® water under continuous stirring with a magnetic stirrer and gentle heating (at appx. 70 °C for about 30 min). The hot solution had a milky appearance. To clear the solution and promote hydrolysis of PFA, one tablet of solid pure KOH was dipped into the solution with the aid of tweezers, until the latter was colorless and transparent. After the solution cooled down, 4-10 ml of glycerol was added and the solution was complemented to its final 200 ml volume by Milli-Q® water. The resulting solution contains monomeric formaldehyde and can be stored indefinitely at room temperature.

The solution is toxic, so it is always necessary to work with it under a hood and with protective clothing.

The fixative used here (see List of Solutions, Tab. 1) was dispensed as required into the wells of twelve-well plastic plates for cell cultures (ca. 3 ml of fixative per well). Control and lithium chloride-treated seedlings were transferred to hollow plastic baskets (see Fig. 11) with their bottom lined with a 50 μm plastic net which were then placed in fixative-filled wells of the culture plate. Fixation was carried out under the hood for a minimum of 1 hour at room temperature and was extended in some occasions for overnight at 4 °C. Prior to fixation plastic baskets were very thoroughly washed with detergent, tap water and then Milli-Q® water. The fixative was removed from the plates by using a Pasteur pipette and the samples were washed at least 4 times by 10 minutes with buffer (½ MTSB). Solutions were exchanged from outside of the baskets to prevent damaging of the delicate root tips.

2) Enzymatic Digestion of the Cell Wall

Antibody molecules are impermeable to the plant cell wall which has a cut-off pore size of ca. 20 kDa (Fleischer et al., 1999). To ensure diffusion of antibody molecules within root cells and at considerable depth of the root it is necessary to degrade cell wall components post-fixation and this is done by using an appropriate cell wall digesting enzyme cocktail. After all post-fixation washings were completed, baskets with fixed seedlings were removed from the ½ MTSB solution, carefully blotted on filter paper and placed into other wells containing cell wall digesting enzyme cocktail (see List of Solutions, Tab. 1.) and the seedlings were incubated for 25-30 min at room temperature. After enzyme digestion, baskets with seedlings were placed to clean wells containing ½ MTSB. The enzymatic solution was stored at -20 °C and could be reused 5-6 more times. Seedlings were washed twice in MTSB for 10 minutes each and then two more times with phosphate buffered saline (PBS) for 10 minutes each. For working with a larger number of samples, plastic baskets were transferred using tweezers into ½ MTSB-, or PBS- prefilled wells. Same approach was also used after the reduction and antibody incubation (see below).

3) Reduction

Even after extensive washing it is expected that residual unreacted aldehyde groups are remaining in the sample and such groups can covalently bind antibody molecules to

non-specific sites causing background fluorescence in the sample. A potent way to deal with this problem is to reduce aldehyde to non-reactive alcohol groups and this is done by treating the fixed samples with $NaBH_4$ as a reducing agent. Therefore, after the last wash with PBS, samples were treated with 1 $mg \cdot ml^{-1}$ of $NaBH_4$ in PBS for 15 min at room temperature. After reduction, the baskets were transferred into wells with PBS buffer (without $NaBH_4$) and then washed extensively with PBS until the solution stopped to effervesce.

4) Permeabilization

Although the barrier of the cell wall is breached by cell wall digesting enzymes, the diffusion of antibody molecules in the root cells is still hampered by the intact plasma membrane. This is circumvented by extracting the membrane lipids using a buffered detergent solution (see List of Solutions, Tab. 1). Samples were incubated in extraction solution for 30 min 1 h and subsequently the solution was removed with a Pasteur pipette and the samples were washed with PBS 4 times for 10 minutes each.

5) Blocking

Non-specific antibody binding can also occur non-covalently through hydrophobic or electrostatic interactions of immunoglobulin molecules with different subcellular structures. This type of non-specificity is prevented by incubating the sample with a solution of an inert protein such as bovine serum albumin (BSA; see List of Solutions, Tab. 1). The blocking solution was applied for at least 1 hr at room temperature but it was occasionally was prolonged to overnight incubation at 4 °C.

6) Incubation with Primary Antibody

The primary antibody is used to recognize the epitope of interest in the sample. For indirect immunofluorescence, this antibody is unconjugated. The primary antibody solution was prepared by diluting the antibody in 3% w/v BSA in PBS at a ratio of 1:500. As the primary antibody was chosen rat monoclonal anti-α-tubulin (see Tab. 1). Plastic baskets with seedlings were transferred directly from the blocking solution to the antibody solution which was placed into the wells of 24-well culture plates containing 700 µl of antibody solution in each well (Fig. 12). The culture plate was covered with a lid and the samples were incubated overnight at room temperature. Following antibody incubation, samples were then transferred back into larger wells with PBS

buffer and washed at least 6 times for 10 minutes each - the first 4 times with PBS and then 2 times with 3% w/v BSA in PBS. Frequently, the washing after primary antibody incubation was extended overnight. The diluted primary antibody was stored at -20 °C and could be reused for 4-6 more times with no evident decline in labeling efficiency.

7) Incubation with Secondary Antibody

In the indirect variant of immunofluorescent detection the primary antibody is recognized by fluorophore-conjugated species specific immunoglobulins. Each secondary immunoglobulin molecule can be derivatized with 3-9 fluorophore molecules and each primary immunoglobulin can bind up to 3 secondary immunoglobulin molecules. Therefore and by comparison to direct immunofluorescence where the primary antibody is directly conjugated, the indirect regime provides the means for a tremendous enhancement of the signal and markedly increased signal-to-noise ratios in the final image. Prior to application of the secondary antibody the samples were blocked with 3% w/v BSA in PBS as in step 6 for at least 1 hour. After this the plastic baskets were lightly blotted on filter paper and transferred into the wells of a 24-well plate filled with 700 µl of the solution of the secondary antibody. The solution was made by diluting the secondary antibody in 3% w/v BSA in PBS at a ratio of 1:1000 and the incubation of the seedlings was carried out for 3 hours at 37 °C and was continued overnight at 4 °C. As the secondary antibody was chosen Alexa Fluor 488 anti-rat. After incubation, the secondary antibody solution was discarded and the samples were washed at least 6 times for 10 minutes each in PBS buffer.

8) DAPI Staining

Since the purpose of the present thesis is to delineate different mitotic/cytokinetic stages, samples were counterstained with the DNA dye 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI). DAPI binds to the minor groove of AT-rich stretches of DNA and exhibits a characteristic blue fluorescence with a λem_{max} at 488 nm and after excitation at a λexc_{max} at 340 nm. Conveniently, DAPI emits fluorescence only when bound to DNA. The DAPI working solution was prepared by dilution of stock solution (10 mM in DMSO) at a ratio of 1:2000 with PBS buffer and then the seedlings were incubated for 10-15 min at room temperature. The samples were then washed 3 times for 10 minutes each with PBS.

9) Mounting of Samples

During imaging with the laser light sources used to excite the fluorophores of the sample, free radicals are produced and these can irreversibly destroy the fluorophores (photobleaching). To protect the fluorophores and preserve fluorescence emission, samples must be mounted in the presence of a strong antioxidant which in this case is paraphenylene diamine. The solution is prepared as shown in Tab. 1 and containing a strong buffering agent (100 mM Tris-Cl pH 8.8) and high concentrations of glycerol (up to 90% v/v). The alkaline pH is chosen because it allows maximum quantum yield of the fluorophores, while the glycerol content is adjusted to such high values as it offers a refractive index close to that of the coverslip, the immersion oil and the objective thus minimizing potential spherical aberrations that may confound the final image. For the mounting three to four seedlings were carefully transferred onto glass slides into a drop (ca. 50 µl) of mounting medium (see List of solutions, Tab. 1), covered with 22×34 mm rectangular coverslips and sealed with clear nail polish. Thus prepared samples were stored in appropriate microscopy sample cassettes (e. g. Heathrow Scientific) at -20 °C and then observed using a confocal spinning disc microscope.

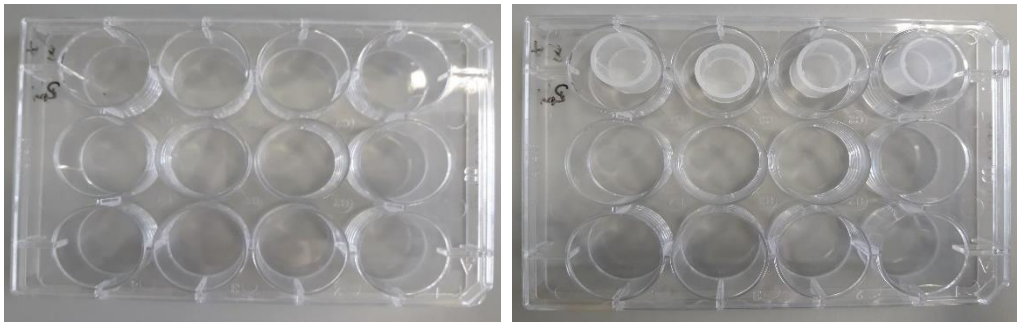

Figure 11. Plastic wells of a 12-well plate

Figure 12. Plastic wells of a 24-well plate for antibody incubation

3.7 Programs for Outcome Analysis

Processing of microscopic images - export and editing images by using programs:

- ZEN Blue 2012 (Zeiss)
- Zen Black 2012 (Zeiss)
- Microsoft Powerpoint 2016

3.8 Instrumentation

- Analytical balance (XA 110/2X, RADWAG)
- Automatic pipettes (10 µl – 10 ml, Eppendorf)
- Laboratory fume hood (M 1200, MERCI)
- Incubator (Biotrade)
- The Culture chamber (phytotron, Weiss Gallenkamp)
- Laboratory fridge (ERB 34633W, Electrolux)
- Laboratory balance (S1502, BEL Engineering)
- Laminar flowbox biohazard (Faster, FERRARA)
- Magnetic stirrer (MSH-420, BOECO)
- Confocal laser scanning microscope with operating programme ZEN Blue 2012 (Spinning disc, Axio Observer Z1, ZEISS)
- Microwave (MGE21, HITACHI)
- Freezer (Liebherr)
- Desktop pH meter (PC 2700, Eutech Instruments)
- Combi-Spin (FVL-2400N, bioSan)

3.9 List of Solutions

Most of the chemicals used in the experiment come from Sigma-Aldrich unless otherwise stated.

Table 1. List of used solutions

The sterilizing solution	
1% v/v sodium hypochlorite, 0,05% v/v Tween 20	
10 ml	10% v/v sodium hypochlorite
90 ml	Distilled water (dH_2O)
Supplemented with dH_2O to 100 ml with addition of drop of Tween 20	

Table 1. List of used solutions *(continuation)*

Solid ½ MS medium without vitamins	
2,2 $g \cdot l^{-1}$	Murashige and Skoog medium (Duchefa)
10 $g \cdot l^{-1}$	Sucrose
8 $g \cdot l^{-1}$	Phytagel
Supplemented to 1 liter with dH_2O, pH adjustment to 5,8 (KOH), sterilized by autoclaving	
Liquid ½ MS medium without vitamins	
2,15 $g \cdot l^{-1}$	Murashige-Skoog medium (Duchefa)
10 $g \cdot l^{-1}$	Sucrose
Supplemented to 1 liter with dH_2O, pH 5,8 (KOH), sterilized by autoclaving	
Lithium chloride	
Lithium chloride was prepared as a 100 mM stock solution in Milli-Q® water and diluted appropriately with MS medium to a final working concentration of 10 mM	
Solutions for whole mount	
PFA solution for fixation	
16 g	paraformaldehyde
150 ml	Ultrapure Milli-Q® H_2O
Fixative solution	
2,5 ml	8% w/v paraformaldehyde
7,5 ml	1× MTSB
4,4 ml	Ultrapure Milli-Q® H_2O
300 µl	glutaraldehyde
25 µl	Triton-X
A total of 15 ml of fixative solution for five wells of 12 well culture plates (3 ml for each well)	
1× MTSB	
3,775 g	PIPES
0,3075 g	$MgSO_4 \times 7\ H_2O$
0,475 g	EGTA
Supplemented to 250 ml with Milli-Q® H_2O, pH 6,8	

Table 1. List of Used Solutions *(continuation)*

0,025 g / 250 ml	sodium azide
Enzymatic solution	
0,2 g	2% w/v Meicelase
0,2 g	2% w/v Cellulase
0,1 g	1% w/v Macerozyme
10 mg	0,05% w/v Pectolyase Y23
10 ml	MTSB
Incubation: 20-30 min, room temperature	
½ MTSB buffer for dissolving the enzymes	
100 ml	1×MTSB
100 ml	Ultrapure Milli-Q® H_2O
PBS (Phosphate-buffered saline)	
Stock solution (10× PBS)	
80 g	NaCl (0,8 M)
2 g	KCl (2,7 mM)
11,5 g	$Na_2HPO_4 \times 2\ H_2O$ (6,5 mM)
2 g	KH_2PO_4 (1,5 mM)
Supplemented to 1 l with Milli-Q® H_2O, pH 7,3 (0,1 M KOH)	
Working solution (1× PBS)	
100 ml	PBS (10×)
900 ml	Ultrapure Milli-Q® H_2O
Reduction solution	
15 ml	PBS (1×)
Add of aligned micro weighing spatula with $NaBH_4$ for each sample, total ca. 15 mg of $NaBH_4$ for 5 samples.	
Permeabilization solution	
10 ml	PBS (10×)
10 ml	DMSO (10% v/v)

Table 1. List of used solutions *(continuation)*

2 ml	Nonidet P-40 (2% v/v)
78 ml	Ultrapure Milli-Q® H_2O
Blocking solution	
3% w/v BSA in PBS	
1,5 g	BSA
50 ml	PBS (1×)
Before use, store for a few minutes in a freezer (-20 °C) for better dissolution of BSA	
Primary antibody solution	
15 ml	3% w/v Blocking buffer (BSA+PBS)
30 µl	Monoclonal rat anti-α-tubulin (clone YOL1/34; 1:500)
700 µl of antibody solution for one sample (well). Antibody solution was kept at -20 °C	
Secondary antibody solution	
10 ml	3% w/v Blocking buffer (BSA+PBS)
10 µl	Secondary antibody: Alexa Fluor 488 anti-rat IgG (1:1000)
DAPI	
Stock solution	
10 $mmol \cdot l^{-1}$ in DMSO	
Working solution	
Dilution 1:2000, 0,5 µl of 10 mM DAPI/1 ml PBS	
30 ml	PBS
15 µl	DAPI
Mounting medium	
100 mg	paraphenylenediamine
30 µl	DMSO
1 ml	Tris (1,5 M, pH 8,8)
9 ml	100% v/v Glycerol
Solution was mixed with a glass rod, pH ca 8,8, stored in dark at -20 °C	

4 Results and Discussion

In the present thesis, the wholemount immunofluorescence method was used to address microtubule organization in distinct cell division stages of the *Arabidopsis thaliana* root tip meristem in control or in LiCl-treated seedlings. In this way, stage-specific effects of LiCl treatment could be identified and lead to hypotheses regarding GSK3 function during the mitotic cycle of plants. Representative results are going to be presented in a comparative fashion, showing together wild type and LiCl treated cells in the following sections. For staining nuclei was used DAPI pseudo in red color to improve contrast in figures.

4.1 Preprophase

Preprophase in plants is a distinct premitotic stage which is hallmarked by the definition of cell division plane, i. e., the plane upon which the daughter cell wall will be deposited during the process of cytokinesis. The distinction of preprophase is purely plant specific and is strictly dependent on the global reorganization of cortical microtubules to eventually form the PPB. As explained before, during preprophase and once the PPB has formed, many markers of the cortical cell division zone accumulate and collectively are thought to permanently mark the future site of cell plate fusion at the parent cell walls.

In control Col-0 seedlings, early premitotic cells form a cortical annular microtubule band, the PPB. PPB comprises of parallel microtubules (Fig. 13 A) that show uniform width throughout the entire cell circumference (Fig. 13 B). Such cells show already condensed chromatin (Fig. 13) while the nucleus is surrounded by a conspicuous perinuclear microtubule system without particular polarity (Fig. 13) showing microtubule connections with cortical sites.

After LiCl treatment, PPB formation seems to be compromised. The PPB at surface optical planes appears to be malorganized and incomplete (Fig. 13) while at middle planes, microtubules do not seem to be restricted around the nuclear surface (Fig. 13).

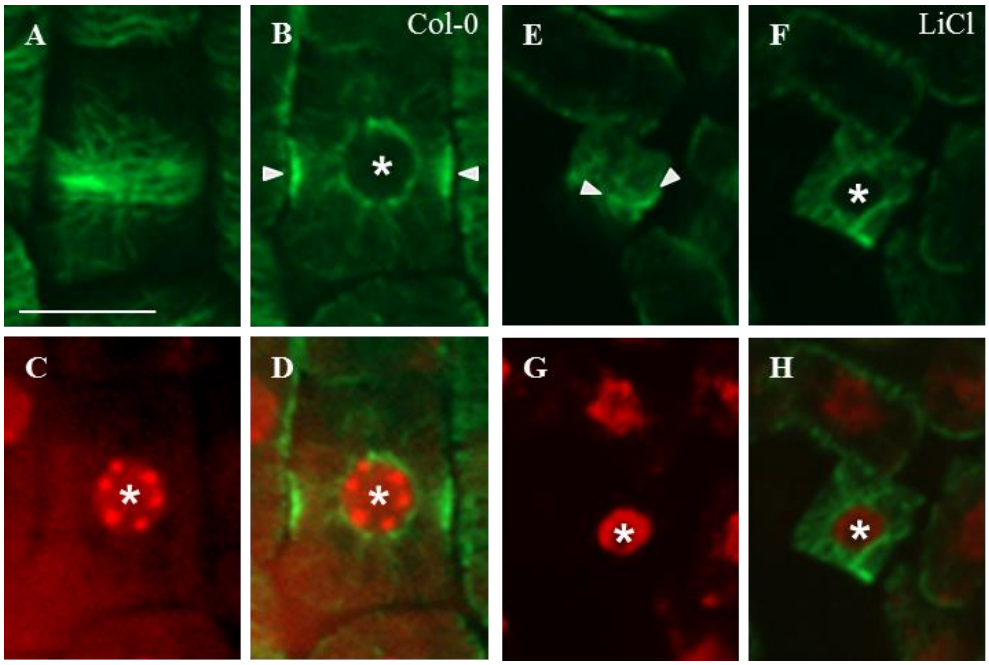

Figure 13. Comparison of PPB organization between Col-0 (A-D) and LiCl treatment (E-H). In Col-0, the PPB appears as a well-organized microtubule band in the cell cortex (A). In the midplane (B), the PPB (arrowheads) coexists with a perinuclear microtubule array (asteris denotes nuclear position). C, D. The nucleus after chromatin staining with DAPI pseudo (C) at midplane and (D) is the merge of both channels. E, F. PPB organization after LiCl treatment at the surface (E) and middle (F) optical planes. In this case, PPB appears incomplete and malorganized (arrowheads in E; asteris denotes nuclear position). G, H. Chromatin (G) and merge of both channels (H). Scale bar: 10 μm

These observations suggest that LiCl treatment perturbs PPB formation and imply that GSK3s targeted by LiCl may be involved in the process. PPB formation is an event that requires control of mechanisms of microtubule lateral association (i. e., bundling). Prospective proteins involved in this process are members of the MAP65 family of microtubule-associated proteins. Members of this family with a proven role in PPB formation include MAP65-1/2 and MAP65-5 (Van Damme et al., 2004). At least MAP65-1/2 were shown to be targeted by CMGC kinases and become negatively regulated by phosphorylation on their carboxyl terminus harboring their microtubule binding domain (Smertenko et al., 2006). Overexpression of MAP65-1 or inhibition of its phosphorylation in non-phosphorylatable mutants generated by site-directed mutagenesis (e. g., Chang et al., 2005; Smertenko et al., 2006; Boruc et al., 2017) results in overbundling and ectopic localization which may explain the aberrations observed herein. MAP65-1 is known to be targeted by CDKs and MAPKs (including MPK4 and MPK6; Smertenko et al., 2006; Beck et al., 2010; Sasabe et al., 2011; Smekalova et al., 2014) but also by Aurora kinases (Boruc et al., 2017) which rather target the dimerization domain of MAP65-1 in its aminoterminus.

Due to the effects observed in the presence thesis, it can be postulated that LiCl treatment and GSK3 inhibition interferes with the microtubule binding of proteins responsible for microtubule bundling during PPB formation.

4.2 Prophase/Prometaphase

The onset of mitosis in plants coincides with two different events. The dissolution of the PPB and the breakdown of the nuclear envelope, exposing the condensed chromosomes to the cytoplasmic environment and to the mitotic prospindle which is progressively forming during the process of PPB disassembly and assumes bipolarity (Fig. 14). Although these two events are temporally coupled, they occur independently of each other as in some case, PPB disassembly may be delayed but spindle formation may occur nonetheless as it happens in mutants of the microtubule severing protein katanin (e. g., Panteris et al., 2011) or after microtubule stabilization following treatment with the microtubule stabilizing agent taxol (Panteris et al., 1995). During prophase/prometaphase, the chromosomes appear as an entangled chromatin mass which encounters the microtubule mitotic spindle via kinetochores. The purpose of the process is to form the mitotic spindle complex which at this stage, aims to bring chromosome kinetochores aligned at the equatorial plane to ensure faithful segregation. The initial formation of the mitotic spindle and its first encounters with the released chromosomes does not seem to be affected by LiCl treatment, suggesting that nuclear envelope breakdown, mitotic spindle formation, and kinetochore – spindle associations are independent of GSK3 activity.

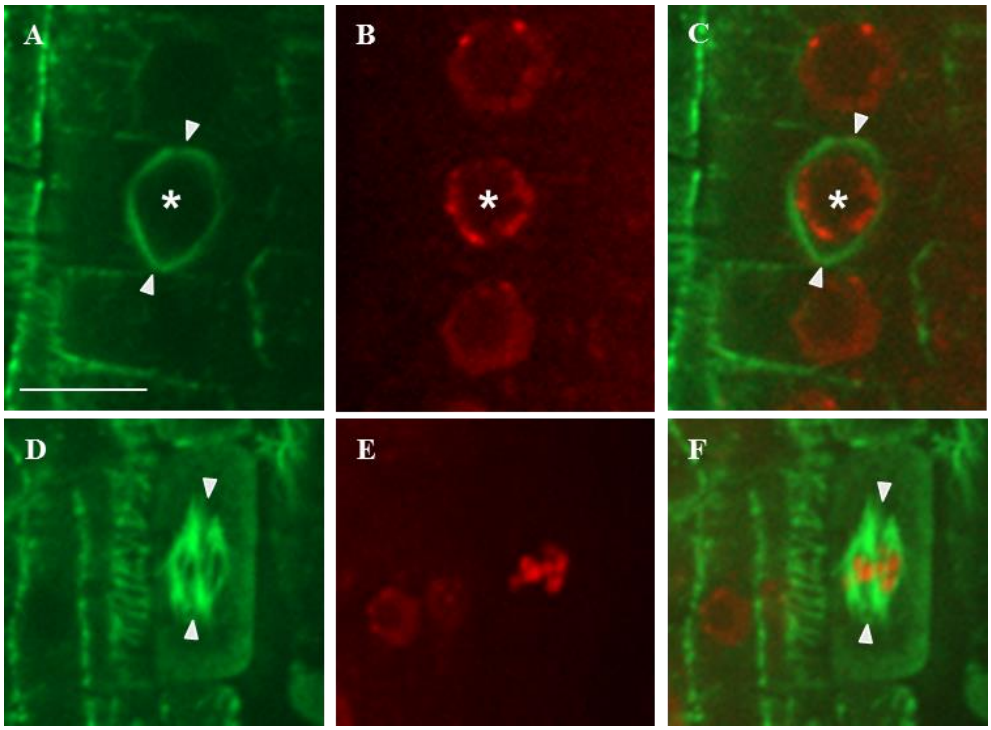

Figure 14. Prophase/prometaphase spindle formation/organization is not affected by LiCl treatment. (A-C) Typical prophase cell of Col-0, showing perinuclear spindle assembly (A, C, arrowheads). Arrowheads denote spindle poles and asterisks denote nuclear position. (D-F) Prometaphase cell after LiCl treatment. Again spindle is bipolar (D, F, arrowheads) while chromosomes (E) stained with DAPI are expectedly dispersed. Scale bar: 10 μm

Apart from PPB formation and premitotic dissolution, nuclear envelope breakdown (NEB) is a major hallmark of mitosis onset in both animals and plants. As studied exhaustively in mammalian cells, NEB is dependent on the disassembly of nuclear envelope complexes which is ultimately driven by disassembly of the peripheral nuclear cytoskeleton and nuclear pore complexes by phosphorylation of proteins such as lamins and nucleoporins respectively (Fernandez-Alvarez and Cooper, 2017). At least in the latter case, GSK3s were shown to have a dominant role (Hinchcliffe et al., 1999). Regarding PPB disassembly, it was not impaired by lithium treatment since we have never observed residual PPBs in lithium treated roots suggesting that this process is independent of GSK3s.

Finally and regarding the process of spindle assembly itself, it is concluded that unlike the effects of other kinase inhibitors, LiCl did not prevent its formation neither did it affect its shape and characteristics. This finding is in sharp contrast to the effects of CDK inhibitors, which were previously shown to affect mitotic spindle formation in mitotic root cells of *Vicia faba* (Binarova et al., 1998), or to the effects induced by genetic disturbance of the MAPK MPK4 of *Arabidopsis thaliana* (Beck et al., 2011).

However, the effects of CDK and MAPK inhibition at the early stages of spindle formation are subtle.

4.3 Metaphase

Metaphase represents an instant in the mitotic cycle of eukaryotes which is marked by the alignment of kinetochores along the equatorial plane. During this stage, the mitotic spindle comprises of well discernible microtubule bundles called kinetochore or spindle fibers which interact with kinetochores at their plus ends, while their minus ends converge to the respective spindle poles. In plants, where the mitotic spindle is formed in the absence of dominant microtubule nucleating centers and polar organizers such as the mammalian centrosomes, or the yeast spindle pole bodies, the polar spindle area may appear broad, or narrow with focusing mechanisms being uncovered up to date. Organization of metaphase spindle is critical for the equipartition of sister chromatids into the future daughter cells, as perturbations in its assembly may eventually lead to aneu- or polyploidy.

In *Arabidopsis thaliana* control root wholemounts, two slightly different types of metaphase spindle were discernible. The majority of metaphase cells exhibited broad mitotic spindles with the little convergence of kinetochore fibers at the spindle pole regions (Fig. 15 A, C) and good alignment of chromosomes at the spindle equator (Fig. 15 B). In the second category, mitotic spindles of metaphase cells showed similar characteristics but with more focused polar convergence of spindle fibers (Fig. 15 D, F). Again, chromosomes were uniformly arranged via their kinetochores at the spindle equatorial plane (Fig. 15 E).

In lithium treated metaphase cells, the congregation of chromosomes at the spindle equatorial plane is not visibly affected however, we observed defects in spindle kinetochore fibers at the polar regions (Figure G-I). In this case, spindle shape was rather asymmetric with one broad and one narrow pole.

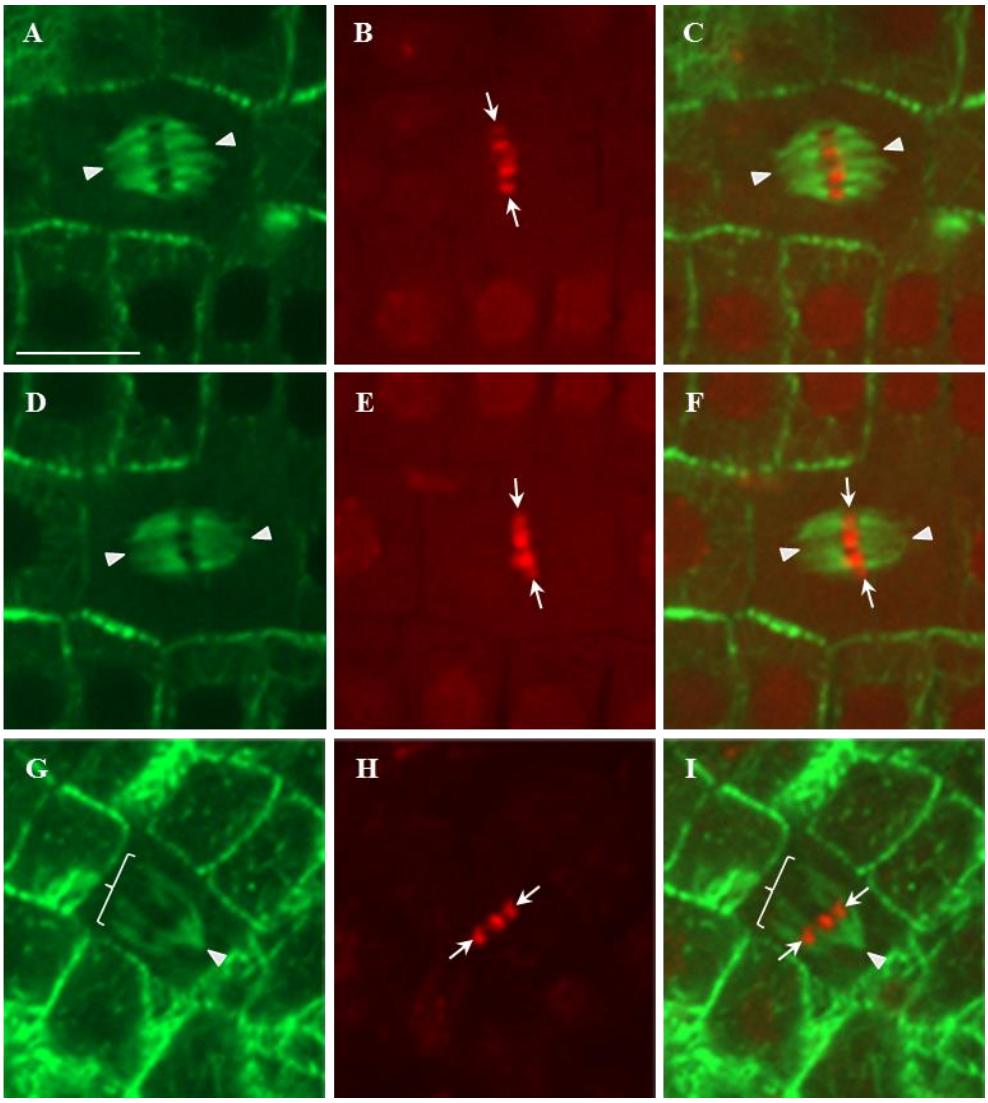

Figure 15. Metaphase spindle organization in *Arabidopsis thaliana* Col-0 control (A-F) and LiCl-treated seedlings (G-I). A-F. The mitotic spindle is strictly bipolar with well-defined broad (A, C, arrowheads) or focused (D, F, arrowheads) poles. Chromosomes (B, E) define the equatorial plane (B, C, E, F, arrows). G-I. After LiCl treatment some metaphase spindles assume the asymmetric shape with one broad and one narrowly focused poles (G, I) although congression of chromosomes to the equatorial plane (H, I) is not impaired. Scale bars: 10 μm

Organization of the mitotic spindle can be a subject of targeting by many kinases, the inhibition of which may impair spindle form, polarity and chromosome positioning with detrimental effects on the subsequent mitotic progress.

Inhibition of CDKs with the chemical inhibitor bohemin, induces the formation of astral, monopolar spindles and the circular organization of chromosomes (Binarova et al., 1998). Similarly, metaphase spindle organization is affected in MPK4 knock out mutants but also after treatment of mitotic cells with the MAPK inhibitor PD98059 (Beck et al., 2011). In all cases, the chromosome – kinetochore fiber attachments as well as the formation of kinetochore fibers are not disturbed, but rather the overall spindle

form is affected, suggesting that all categories of CMGC kinases are somehow implicated in the focusing of the mitotic spindle to the spindle poles and to the establishment of spindle bipolarity.

4.4 Anaphase

Anaphase is the mitotic stage during which, equivalent sister chromatid groups segregate faithfully to the two poles of the spindle. From thorough studies in animal cells, anaphase can be discriminated into two distinct stages: anaphase A and anaphase B. During anaphase A, sister chromatids move towards the poles via microtubule disassembly at the microtubule plus ends at the vicinity of the kinetochores (Asbury, 2017). This microtubule disassembly is mostly driven by depolymerization inducing kinesin-8 motor proteins which are also acting during the metaphase congression of chromosomes at the spindle equatorial plane (Stumpff and Wordeman, 2007; Sharp and Rogers, 2004). Anaphase B occurs by pulling apart half-spindles, resulting in spindle elongation without changes in the spindle form. Anaphase B is more complicated than anaphase A and requires mechanisms promoting antiparallel microtubule gliding at the spindle midzone as well as regulated microtubule disassembly of the minus ends (which reside at the pole regions; Scholey et al., 2016).

In plants, it is possible to discriminate different stages of anaphase based on the distance between sister chromatid groups, but it is not clear whether the discrimination between anaphase A and B is valid. This can be depicted in tubulin immunolabeled root wholemounts of control Col-0 plants, where it was possible to discriminate two stages of anaphase. In the first stage, the mitotic spindle exhibited robust spindle fibers (Fig. 16 A-F) and sister chromatid groups at variable distances from the spindle poles (Fig. 16 B, C; E, F). At a later stage which is precursor of telophase/early cytokinesis, the spindle comprises of a robust interzonal – midzone – microtubule system, situated between the two well-separated sister chromatid groups, and two polar microtubule caps (Fig. 16 G, I), covering the sister chromatid groups which at this stage occupy their final position at the spindle poles (Fig. 16 H).

The most important observation in some but not all anaphase cells after LiCl treatment is that sister chromatid groups fail to separate and appear entangled at the equatorial plane at the early stages of anaphase. This is accompanied by minor spindle malformations whereby the spindle asymmetry observed in metaphase is maintained during anaphase as well resulting in unequal polar areas (Fig. 16 J-L).

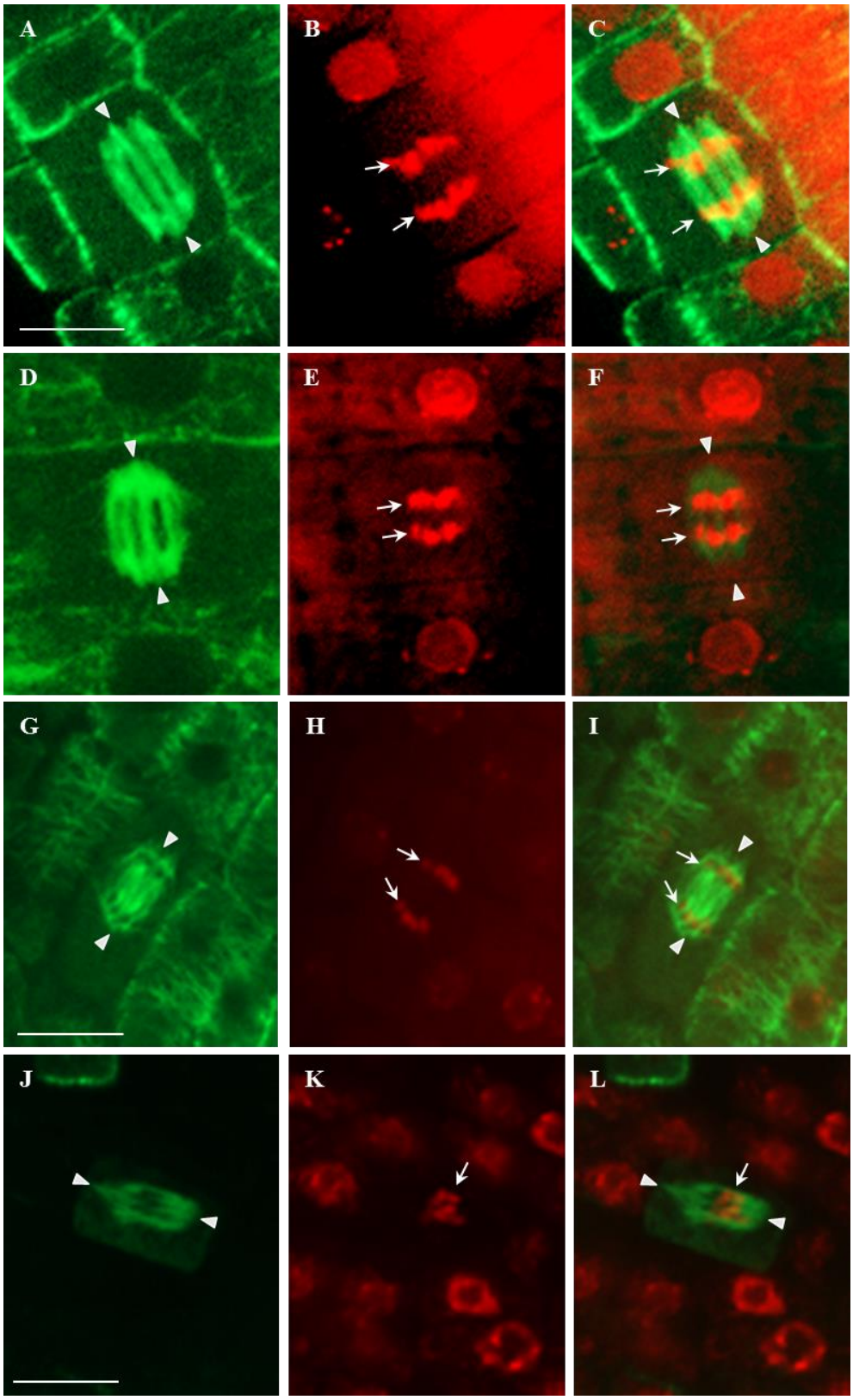

Figure 16. Anaphase progression, spindle assembly and sister chromatid segregation in control *Arabidopsis thaliana* Col-0 root epidermal cells, or following LiCl treatment. A-F. Early stages of anaphase in control Col-0. Arrowheads show spindle poles and arrows show sister chromatid groups. G-I. Later anaphase stage showing well separated sister chromatid groups (arrows). Arrowheads denote spindle poles. J-L. Abnormal anaphase spindle with asymmetric poles (arrowheads) and failure to separate sister chromatid groups (K, arrow). Scale bars: 10 μm

4.5 Telophase/Cytokinesis

Telophase is considered the onset of cytokinesis and it is marked by two events. The first is the formation of the microtubular cytokinetic apparatus, the phragmoplast. The second event is the gradual reinstatement of the nuclear envelope and the progressive decondensation of sister chromatids, in order to form the daughter nuclei.

The phragmoplast is a microtubule system that comprises of two antiparallel microtubule subsets. In orthogonal view, the phragmoplast appears as a disc the plane of which coincides with the plane earlier defined by the PPB. At the start of cytokinesis, microtubules occupy the entire surface of the disc. However, as the phragmoplast expands centrifugally towards the cell periphery, microtubules disassemble and disappear from the disc's interior and become confined at the phragmoplast margin, so that at the orthogonal view, the phragmoplast appears as a ring. In the phragmoplast structure, microtubule plus ends converge to the midplane while minus ends locate at the daughter nuclei surface. The purpose of the phragmoplast is to deliver vesicles that contain cell wall precursors and polysaccharide synthesizing enzymes, in order to build the cell plate which is the daughter cell wall.

The assembly of the phragmoplast is regulated by coordination of microtubule nucleation and microtubule bundling (Beck et al., 2011; Murata et al., 2013) and involves the function of kinesin motor proteins for the delivery of vesicles to the cell plate (Lee et al., 2007; Liu et al., 2003).

Protein phosphorylation is important for the timely expansion of the phragmoplast, but not for the deposition of the cell plate. Of key significance to phragmoplast expansion are members of the MAPK protein kinase family. As it was shown, mutants of the MPK4 MAPK as well as of its upstream regulators ANP1, 2, 3 (MAPKKKs) and MKK6 (MAPKK), exhibit abortive cytokinesis which means that the process is terminated prematurely before the complete deposition of the cell plate and thus such mutants exhibit giant multinucleated cells with harboring many incomplete cell walls (Beck et al., 2011). Moreover activated species of plant MAPKs were found to colocalize with the expanding phragmoplast in *in situ* immunofluorescence studies (Smekalova et al., 2014; Winnicki et al., 2015). During the process of phragmoplast expansion, MAPKs are thought to phosphorylate and negatively regulate three members of the microtubule bundling protein family MAP65 and specifically MAP65-1, 2 and 3 (Beck et al., 2010; Sasabe and Machida, 2012). Particularly important in the process is

MAP65-3, which is a mitosis-specific member of the MAP65 family (Ho et al., 2011, 2012).

In accordance to the above, there has been a single report on the effects of lithium in the cytokinetic progress of dividing stamen hair cells of *Tradescantia virginiana* (Wolniak, 1987). This study revealed that lithium caused premature termination of phragmoplast expansion, reminiscent of the effects of MAPK inhibition by genetic or pharmacological means (Beck et al., 2011; Kosetsu et al., 2010), suggesting that at least GSK3s and MAPKs somehow control the centrifugal expansion of the phragmoplast.

In control Col-0 dividing root cells the telophase microtubule system and eventually the phragmoplast forms normally (Fig. 17 A-C), while the same process does not seem to be affected in lithium treated root cells (Fig. 17 D-I).

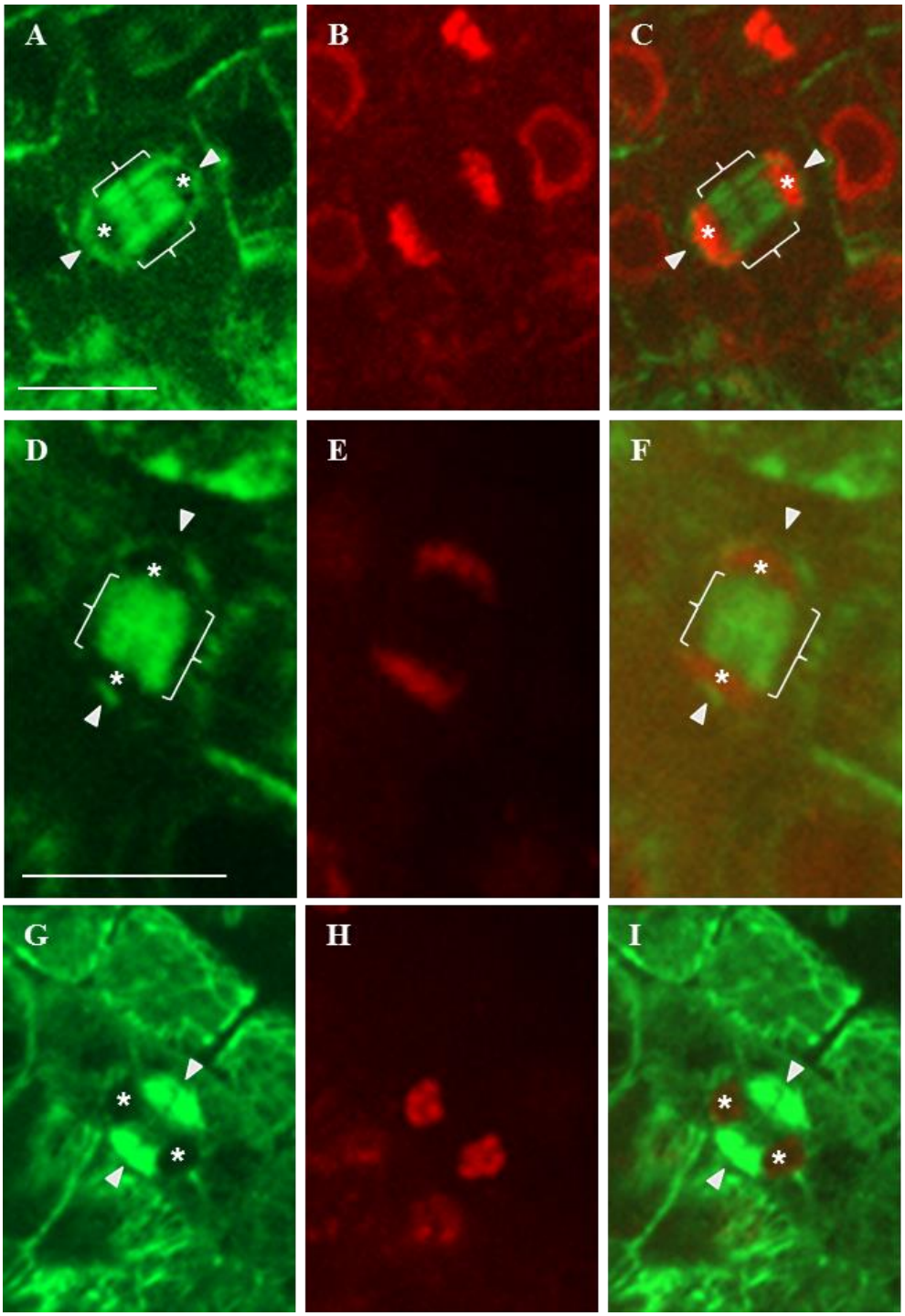

Figure 17. Telophase and cytokinetic microtubule organization in control or LiCl treated dividing cells. (A-C) Telophase microtubule organization in control Col-0, showing the organization of microtubules in the interzonal system (A, C, brackets), the position of chromosomes (B) and the overlay image. Scale bars: 10 μm

However, although phragmoplast formation was not seemingly affected in LiCl treated cells, phragmoplast centrifugal expansion was impaired showing premature signs of disassembly long before it reached the parent walls (Fig. 18 A-C).

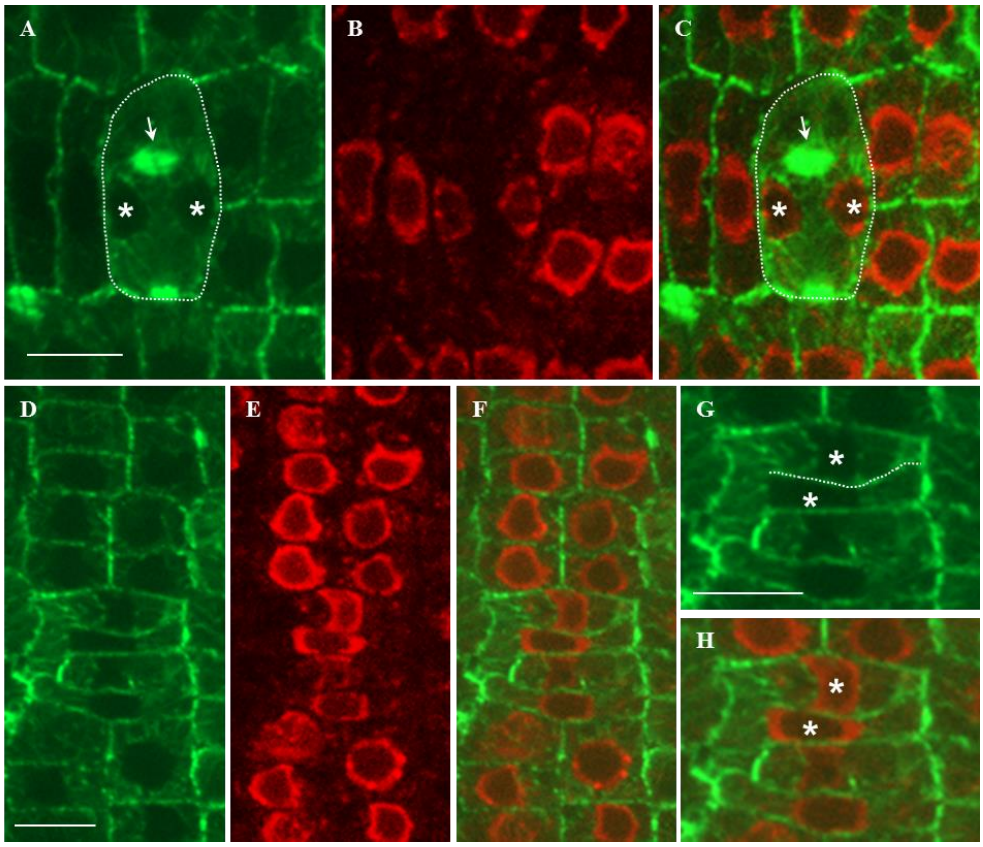

Figure 18. Examples of incomplete cytokinesis of LiCl-treated root cells. A-C. A terminated phragmoplast (arrow) that stopped progression long before reaching the parent walls (dotted line). Asterisks denote the position of daughter nuclei. D-H Example of incomplete cell plate deposition (G, dotted line) in a post mitotic cell. Scale bars: 10 μm.

4.6 Cell Division Lane Orientation

Tissue patterning and organ formation in plants largely depend on the specification of cell division plane orientation. As described before, cell division plane orientation is hallmarked by the formation of the PPB, the plane of which marks the future plane of cell plate deposition. In the root, two principal division planes are encountered. The periclinal or formative which underlies cell specification and tissue patterning and the anticlinal or proliferative, resulting in the propagation of cells of the same lineage.

Previous studies have shown that mutants of cell division plane marker proteins exhibit variably disturbed cell division planes, however, some other studies showed that disturbance of signaling proteins might affect cell division plane orientation as well. Most marked examples of the above are mutants of the YODA MAPKKK and of the MPK6 MAPK, which affect embryo development, stomatal ontogenesis and finally post-embryonic root development (Bergmann et al., 2004; Lukowitz et al., 2004; Smekalova et al., 2014; Lopez-Bucio et al., 2014).

Given the above examples it would be of interest to examine whether the exposure of roots to LiCl would result in similar defects. Indeed in such roots, cell division plane orientation defects were identified, including ectopic cell divisions (Fig. 19 A, C-G), abnormal succession of periclinal/anticlinal divisions and rare but consistent occurrence of oblique cell divisions (Fig. 19 B).

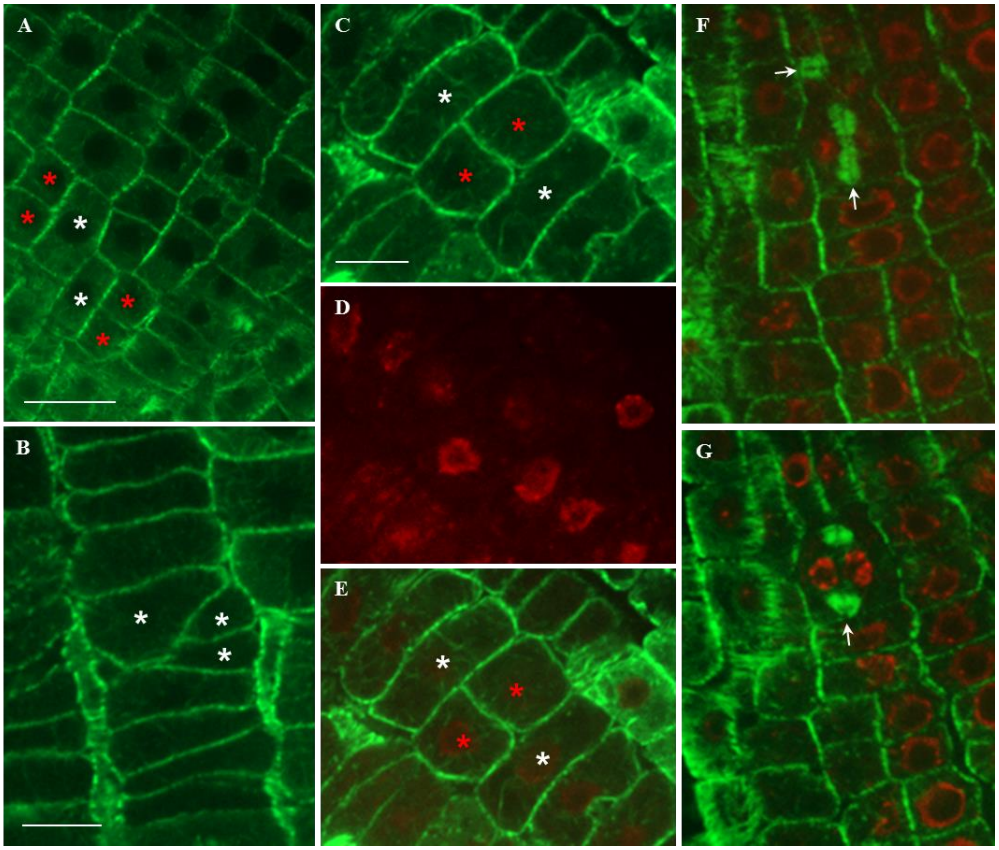

Figure 19. Cell division plane orientation defects after LiCl treatment. A-E. Abnormal succession of anticlinal – periclinal division planes (A, C-E) and occurrence of atypical oblique planes (B). Red asterisks denote periclinally divided cells and white asterisks denote anticlinally divided cells. (F, G) Top (F) and middle (G) optical sections showing a periclinal and an anticlinal phragmoplast. Scale bars: 10 μm

5 Conclusion

In the present thesis, we studied the effects of LiCl, a broad specificity inhibitor of GSK3s (Medina and Avila, 2010) and alleged inhibitor of diacylglycerol kinases (Sakane et al., 2016) on the organization of mitotic and cytokinetic microtubule arrays of dividing root cells of *Arabidopsis thaliana*. This approach was chosen due to the absence of information regarding mitotic roles of GSK3s in plants.

Our results indicate that LiCl has variable effects on dividing cells depending on the cell cycle stage examined. Although, PPB formation, metaphase, and anaphase spindle organization were considerably affected, the most consistent effects of LiCl were observed in the process of cytokinesis. In this case, it was found that in many cells it was aborted by premature termination of the centrifugal phragmoplast expansion.

The above result is reminiscent to the effects of MAPK deficiency (Beck et al., 2011) suggesting that the two classes of CMGC kinases may have overlapping functions on the regulation of phragmoplast expansion.

Such results can be considered preliminary and in the near future it would be of interest to broaden them by observation of GSK3 mutants (such as the ucu1 alleles; Perez-Perez et al., 2002) or by more narrow pharmacological treatments with inhibitors such as bikinin and its derivatives (De Rybel et al., 2009).

6 References

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7 List of Abbreviations

ASK	Arabidopsis Synthase Kinase
BIN	Brassinosteroid Insensitive
BRI1	Brassinosteroid Receptor 1
BSA	Bovine serum albumine
CAK	CDK-activating Kinase
CDK	Cyclin-dependent kinase
CDP	Cell Division Plane
CESA	Cellulose Synthase
CFP	Cyan Fluorescent Protein
CGMC	Group of Protein Kinases (CDK, MAPK, GSK3 and CLK)
CLK	CDC-like Kinase
CLSM	Confocal Laser Scanning Microscopy
Col-0	Wild Type Ecotype of *Arabidopsis thaliana*
DAPI	4',6-diamidino-2-phenylindole
dH_2O	Distilled water
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
EB1c	END BINDING protein 1c
EGTA	Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
G0 phase	Gap 0
G1 phase	Gap 1
G2 phase	Gap 2
GCP4	Gamma Tubulin Complex Protein
GSK3	Glycogen Synthase Kinase 3
Ig	Immunoglobulin
IgG	Immunoglobulin G
KCBP	Kinesin-like Calmodulin-binding Protein
KOH	Potassium Hydroxide
LiCl	Lithium chloride
M phase	Mitosis
MAPK	Mitogen Activated Protein Kinase
MAPKK (MKK)	Mitogen Activated Protein Kinase Kinase
MAPKKK	Mitogen Activated Protein Kinase Kinase Kinase
$MgSO_4 \times 7\ H_2O$	Magnesium Sulfate Heptahydrate
Milli-Q®	Ultrapure Water
MS	Murashige and Skoog
MTSB	Microtubule-stabilizing Buffer
$NaBH_4$	Sodium Borohydride
NaClO	Sodium Hypochlorite
NEB	nuclear envelope breakdown
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PIPES	Piperazine-N,N′-bis(2-ethanesulfonic acid)
POK	Phragmoplast Orienting Kinesin
PPB	Prephopase band
QC	Quiescent Center
RAM	Root Apical Meristem
RanGAP	Ran GTPase-activating Protein

RT	Room Temperature
S phase	Synthesis
SD	Spinning Disc
TUA1	Tubulin α-1
Tris	Tris(hydroxymethyl)aminomethane
UCU1	Ultracurvata
WT	Wild Type
YFP	Yellow Fluorescent Protein