Palacký University Olomouc

Faculty of Science

Department of Cell Biology and Genetics

Bachelor thesis

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Palacký University Olomouc

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Effect of inhibitor monastrol on kinesin-5 motor protein activity during mitosis in *Arabidopsis thaliana*

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Zuzana Korchanová

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Supervisor: Georgios Komis, Ph.D.

DECLARATION

I hereby declare that I elaborated this bachelor thesis independently under the supervision of Georgios Komis, Ph.D., using only information sources referred in the Literature chapter.

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SUMMARY

Mitotic spindle assembly and faithful chromosome segregation underlie growth and development of eukaryotes ensuring genetic homogeneity in the multicellular context. The above processes are driven by microtubule-based machineries and the interactions of microtubule assemblies with appropriate chromosomal territories. Structural and motor proteins associated with microtubules, coordinate spindle assembly, spindle elongation and spindle bipolarity. Kinesin-5 proteins are multitasking players involved in many aspects of mitotic cell division. Earlier high throughput screens have identified several small molecule organic inhibitors of kinesin-5 motor activity, with monastrol being a prototype. Owing to its unique non-ATP competitive nature of inhibition, monastrol appears to be a rather specific kinesin-5 inhibitors and thus it was tested for the first time as a probe of the acentrosomal mitotic spindle functions in higher plants. Treatment of *Arabidopsis thaliana* roots with monastrol and assessment of microtubule organization and chromosome arrangement showed definite defects, related to conserved kinesin-5 functions in plants. Experimental results were obtained after tubulin fluorescent immunolocalization via advanced widefield microscopy approaches and showed that under the monastrol treatment regime followed herein, the inhibitor conservatively affected distinct features of mitotic cell division and mostly chromosome and sister chromatid positioning during prophase/prometaphase, metaphase and anaphase. Cell division stages that are not dependent on the interaction of microtubule arrays and chromosomes, remained accordingly unaffected.

SOUHRN

Soustava mitotického aparátu umožňující rovnoměrnou segregaci chromozomů je základem růstu a vývoje u *Eukaryot* a zajišťuje genetickou homogenitu na mnohobuněčné úrovni. Tyto procesy jsou řízeny cytoskeletálními mechanismy a zakládají se na interakci soustav mikrotubulů s příslušnými chromozomálními oblastmi. Strukturní a motorové proteiny asociované s mikrotubuly koordinují uspořádání, bipolaritu a prodlužování mitotického vřeténka během dělení buňky. Významnou roli při formování dělícího vřeténka hrají motorové proteiny, mezi které se řadí proteiny skupiny kinesin-5. Dřívější studie napomohly identifikovat několik malých molekul organických inhibitorů aktivity kinesinu-5, přičemž monastrol byl prototypem. Vzhledem ke své jedinečné konkurenční povaze inhibice, která není závislá na ATP, se ukázalo, že monastrol je spíše specifickým inhibitorem kinesinu-5. Proto byl poprvé testován jako sonda pro pozorování uspořádání acentrosomálního mitotického vřeténka v buňkách vyšších rostlin. Působení monastrolu na kořeny *Arabidopsis thaliana* se projevuje prokazatelnými defekty v uspořádání mikrotubulů a chromozomů, které souvisí právě s funkcí kinesinu-5 u rostlin. Výsledky experimentální práce byly získány pomocí fluorescenční imunolokalizace tubulinu prostřednictvím moderních přístupů widefield mikroskopie a ukázalo se, že tento inhibitor konzervativně ovlivnil odlišná stádia mitotického dělení buněk, a to především umístění chromozomů a sesterských chromatid během profáze/prometafáze, metafáze a anafáze. Stádia buněčného dělení, která nejsou závislá na interakci svazků mikrotubulů a chromozomů, zůstaly neovlivněny.

ACKNOWLEDGMENT

I would like to thank my supervisor Georgios Komis, Ph.D. for his professional help, patience and valuable input. I am very grateful to Mgr. Pavlína Floková for sharing her expertise at the laboratory.

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TABLE OF CONTENTS

1 INTRODUCTION

The present thesis focus on the study of inhibition of kinesin-5 motor protein by monastrol and its consequences in cell division. Kinesin-5 is one of the microtubule associated protein with motor activity, involved in microtubule organization during mitosis. In previous studies monastrol has been shown as an inhibitor of kinesin-5 in animals and algae but not higher plants. Inhibition of kinesin-5 motor proteins can lead to the defective assembly of the mitotic spindle and arrest mitosis. Since kinesin-5 functions are rather understudied in higher plants, the inhibitory effects of monastrol were followed during cell division in *Arabidopsis thaliana*.

2 AIMS OF THE THESIS

- Theoretical review of the mitotic function of microtubules and microtubule-associated proteins, kinesin-5 functions in plants and pharmacological inhibitors of kinesin-5 and particularly monastrol.
- Experimental inhibition of kinesins-5 of *Arabidopsis thaliana* wild-type seedlings.
- Corroboration of the above results in root whole-mounts immunofluorescently labeled against tubulin and chromosomes after monastrol treatment.
- Imaging of immunolabeled root whole-mounts with wide-field epifluorescence microscopy and structured illumination microscopy.
- Documentation and principles of image processing.

3 THE CURRENT STATE OF THE KNOWLEDGE

3.1 Brief overview

Microtubules are tubular non-covalent polymers of the protein tubulin and are ubiquitously found in eukaryotic cells (Desai and Mitchison, 1997). They were discovered during the late 50s as a result of the successful implementation of transmission electron microscopy in biology but the term was coined independently by Slautterback working on Hydra and Ledbetter and Porter working on plants during 1963 (e.g., reviewed in Hepler *et al.*, 2013). In plants, microtubules were discovered as a constituent of the cortical cytoplasm of root epidermal cells of various plants (Ledbetter and Porter, 1963) and even in that early seminal study, the correlation between cortical microtubule and cellulose microfibrils in the overlying cell wall was noted. Ever since, microtubules were shown not only to govern cellular organization during interphase but also to regulate cell division plane orientation prior to cell division, to manage sister chromatid segregation during mitosis and to drive cytoplasmic partitioning during the process of cytokinesis.

3.2 Microtubule dynamics and methods of studying

Microtubules are formed by the end-wise self-association of the heterodimeric protein αβ-tubulin in a GTP-dependent manner. Tubulin heterodimers, assemble in linear unit structures called protofilaments and each microtubule comprises of 13 protofilaments with very few exceptions found in the animal kingdom (e.g., Chalfie and Thomson, 1982). The microtubule is inherently polar (i.e., it exhibits two structurally and functionally different ends) and comprises of a highly dynamic end, designated as plus end and an end of negligible dynamicity, termed minus end (Desai and Mitchison, 1997).

In most cases, microtubules polymerize and depolymerize through their plus ends, exhibiting a stochastic transition between growing and shrinking phases. Microtubule growth (polymerization) is supported by a plus end localized thin layer of GTP-bound dimers (plus end cap) and shrinkage (depolymerization) is triggered by activation of the GTPase activity of tubulin and hydrolysis of GTP to GDP within this plus end associated GTP cap (Desai and Mitchison, 1997). The onset of shrinkage is called catastrophe. A catastrophe event may result to the complete depolymerization of the microtubule, but in most cases, growth may be resumed after some time in a process called rescue. Catastrophe and rescue frequencies may indicate various factors, including

the accumulation of post-translationally modified tubulins, the interaction of microtubules with stabilizing or destabilizing proteins, the effects of microtubule poisons and other factors. This plus end dynamic behavior is called dynamic instability (Mitchison and Kirschner, 1984; Fig. 1).

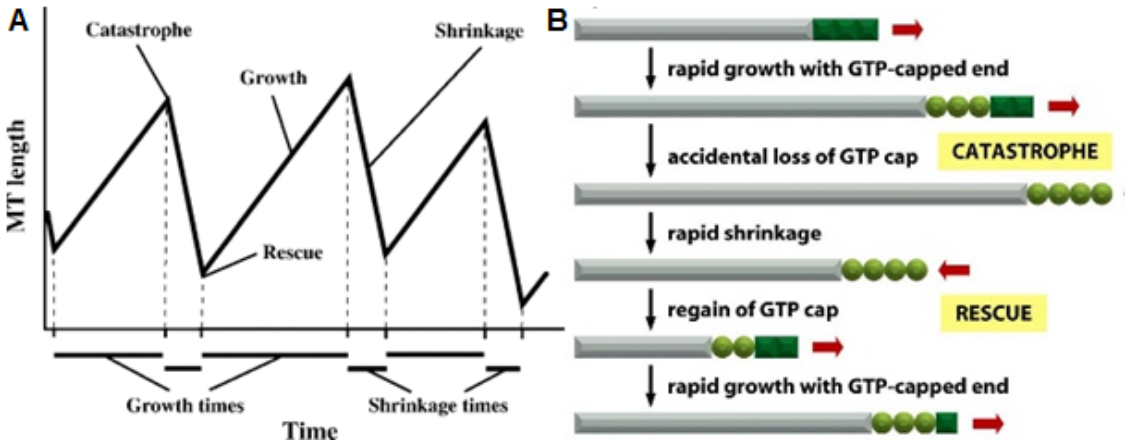

Figure 1. Microtubule polymerization and depolymerization. A life history plot of the length of a single microtubule monitored *in vitro* by video enhanced microscopy (A) and the physiological events accompanying length fluctuations over the time of observation (B). All the stages and their terms which are characterizing microtubule dynamic instability are shown (growth, shrinkage, catastrophe and rescue) can be seen (A) as well as their molecular mechanisms (B). (Compiled from Janulevicius *et al.*, 2006 and http://individual. utoronto.ca/studybuddies/[bio230]%20Lecture%2002%20-%20Cytoskeleton.html)

Another mechanism of microtubule dynamics, is called treadmilling and requires the net addition of tubulin dimers at the plus end and the net removal of tubulin dimers in the minus end (Margolis and Wilson, 1981; Fig. 2). In many instances, plant microtubules exhibit hybrid dynamics with both dynamic instability and treadmilling occurring at the same time (Fig. 2). Via treadmilling, plant cortical microtubules are seen to translocate in space (Fig. 2C). Whether microtubules will exhibit dynamic instability alone, or hybrid dynamic instability and treadmilling, maybe related to the capping of the minus end. If minus ends are capped by nucleating complexes (see later), they are protected from subunit loss and thus their dynamic length fluctuations are restricted to the plus end. However if microtubules are uncapped at their minus end, subunit loss may lead to treadmilling. Apparently this happens frequently to cortical microtubules of plant cells which are characterized by a hybrid mechanism of treadmilling and dynamic instability (Shaw *et al.*, 2003).

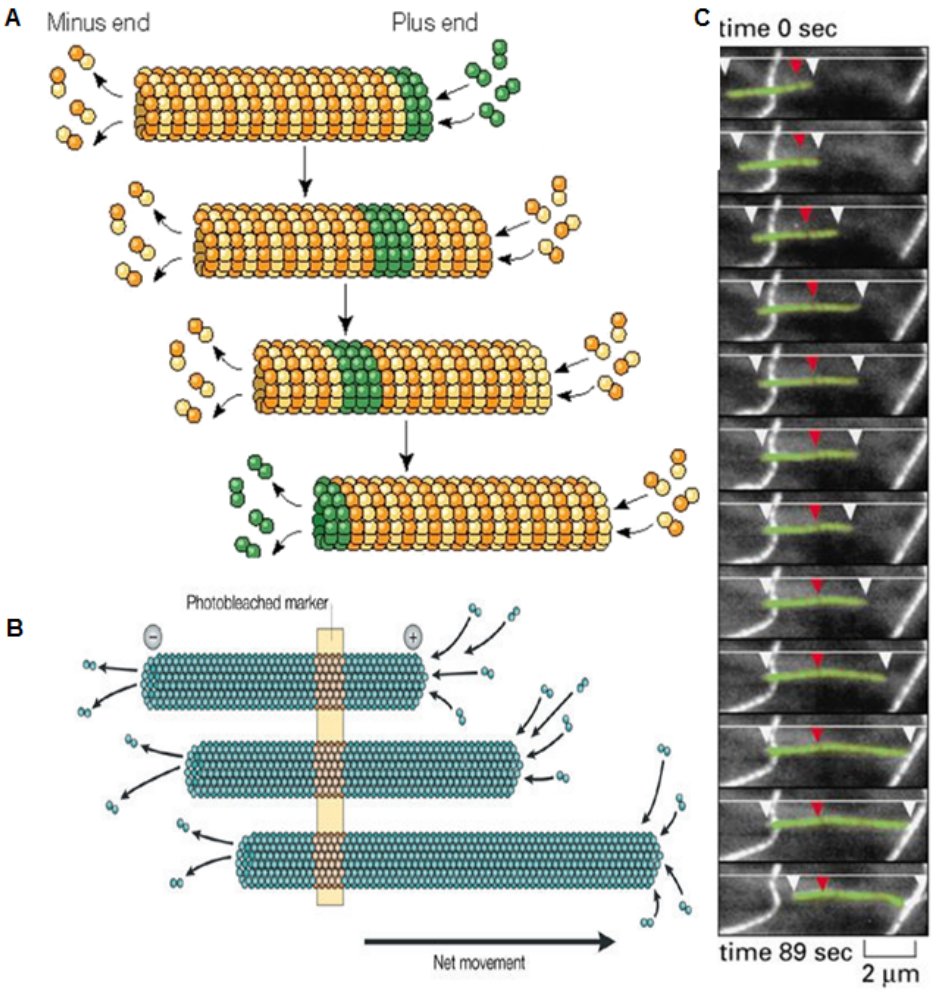

Figure 2. Theoretical and experimental demonstration of microtubule treadmilling. A. the basis of treadmilling shown as gain of subunits at the plus end and loss of subunits at the minus end at equal rates. B. Experimental setup to demonstrate treadmilling by means of fluorescence recovery after photobleaching (FRAP). C. True visualization of GFP-labeled microtubules exhibiting treadmilling showing their net linear translocation in space. (Compiled from Lloyd and Chan, 2004; http://slideplayer.com/slide/6670123/ and http://slideplayer.com/ slide/5090321/)

All the quantitative measures of the end-wise microtubule behavior can be deduced by life time analysis of individual microtubules by means of time lapsed microscopic imaging. For *in vitro* assembled microtubules, such analysis can be carried out using simple perfusion chambers for the exchange of solutions mounted under a total internal reflection microscope (TIRFM) which is ideal for studying events just beneath the coverslip (Hemmat *et al*., 2018). *In vivo* microtubule dynamics are also studied by means of microscopy, provided that the cells under study express an appropriate green fluorescent protein (GFP) based microtubule marker (Hemmat *et al*., 2018).

3.3 Microtubule organization in higher plants

3.3.1 The cortical microtubule array in cell growth and development

In growing and differentiating interphase (i.e., non-mitotic or post-mitotic) cells, microtubules are largely confined in the narrow space between the plasma membrane and the vacuolar membrane, which by definition is the cortical cytoplasm. Cortical microtubules lie in the vicinity of the overlying plasma membrane, often in contact with it and they are either randomly distributed, or arranged parallel to each other depending on cell growth directionality (Fig. 3). If the cell is committed to elongation (i.e., expansion parallel to main organ axis), cortical microtubules are arranged in a preferentially transverse fashion with respect to the cell axis. In the case the cell is prompted to expand laterally, microtubules are longitudinal, while if the growth is isotropic microtubules are randomly dispersed with no bias regarding their positioning in the cortical cytoplasm.

Cortical microtubule organization is further underlying more intricate primary cell wall patterns of very specialized cell types such as stomatal guard cells (Galatis, 1980), tracheary elements or cotyledon and leaf epidermal cells which are characterized by localized cell wall thickenings (Oda and Fukuda, 2012; Qian *et al*., 2009).

Owing to the intimate association of cortical microtubules to cell wall patterning, cell growth directionality and finally cell shaping, it is evident that they also underlie tissue patterning and eventually organ formation. To this point, the coordinated organization of cortical microtubules in shoot or root apical meristems (Uyttewaal *et al*., 2012), is a proof of concept of their importance in plant development, underlying breaking of symmetry during the emergence and subsequent growth post-meristematic structures such as leaf primordia (Uyttewaal *et al*., 2012).

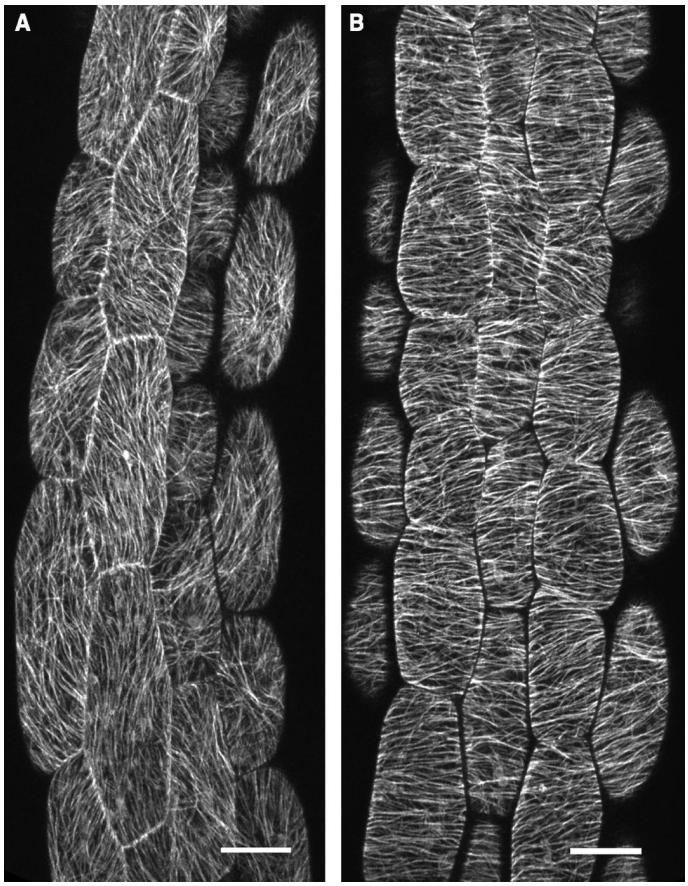

Figure 3. Cortical microtubule orientation in *Arabidopsis thaliana* hypocotyl epidermal cells stably expressing a GFP-TUA6 microtubule marker. (A) Cortical microtubules exhibit random organization in the cell cortex. (B) Cortical microtubules are largely parallel to each other and perpendicular with respect to the main cell axis. (From Elliot and Shaw, 2018)

Frequently, environmental conditions, trigger growth directionality shifts which result in cortical microtubule reorientation. Most notably such changes occur after special illumination conditions (e.g., blue light; Lindeboom *et al*., 2013) but they have been very vigorously characterized after the application of plant hormones, such as auxins and gibberellins which favor cell elongation and concomitantly a net transverse microtubule arrangement in the cortical cytoplasm, or abscisic acid and ethylene which induce cessation of cell elongation, promote cell bulging and induce a longitudinal orientation of cortical microtubules (reviewed in Shibaoka, 1994).

In special occasions, underlying the morphogenesis of particular cell types, microtubules may exhibit a clustered organization pattern. In all cases, cortical microtubules are found to be co-aligned with cellulose microfibrils, blueprinting the pattern of the cell wall. The molecular mechanism behind this coalignment is the interaction of specialized cellulose synthesizing complexes (CESAs) with microtubules

and their movement along microtubule tracks (Li *et al.*, 2015). In this way, microtubules are heavily involved in cell growth directionality and morphogenesis, since the latter is defined by the mode of cell wall deposition (Fig. 4) which is taming turgor pressure to drive cell expansion accordingly.

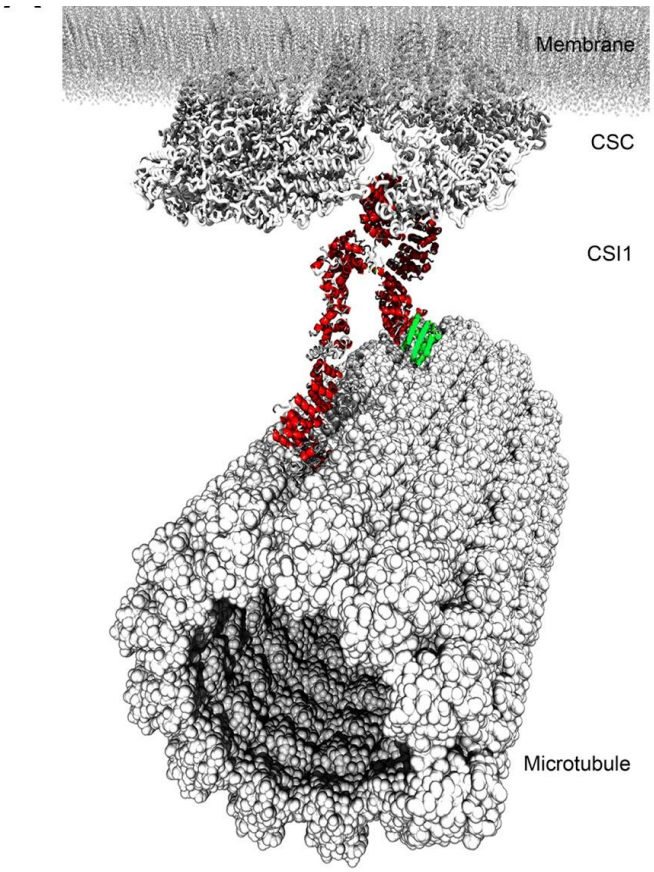

Figure 4. A molecular model showing how plant cortical microtubules associate with the plasma membrane to drive deposition of cellulose microfibrils in the overlying cell wall. The microtubule binds to cellulose synthase interacting protein 1 (CSI1) which in turn binds to the transmembrane cellulose synthesizing complex (CSC). The latter is responsible for expelling the cellulose microfibril. (Adapted with modifications from http://bmb.psu.edu/faculty/images/dr-ying-gu-research-summary-figure-1)

3.3.2 The preprophase microtubule band as a cell division plane determinant

Since plant cells are confined by the rigid cell wall, developmental processes such as pattern formation, tissue patterning and organogenesis cannot be regulated by cell migration as is largely the case in animals. Rather, the development of patterns in the plant body are regulated by the positioning of cell division and the partitioning of the daughter nuclei by the nascent daughter cell wall, the cell plate (Müller and Jürgens, 2016).

This process which is called cell division plane orientation is governed by a specialized cortical microtubular structure which is called the preprophase microtubule band (PPB; Pickett-Heaps and Northcote, 1966).

PPB is forming long before the initiation of cytokinesis and at an indeterminate time prior to mitosis. During the G2 phase, cortical microtubules start to coalesce into a cortical annular array, which is organized at the so-called cortical division zone (CDZ; Smertenko *et al.*, 2017). The early PPB is broad and loose, but progressively it narrows becoming also more robust (Fig. 5).

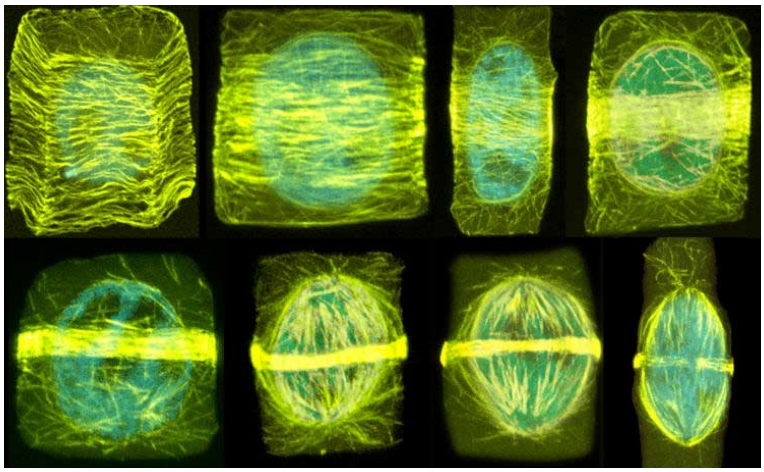

Figure 5. Progressive formation of the microtubule PPB in a tubulin immunolabeled onion root tip cell. The PPB starts as a broad microtubule band that progressively narrows and gradually disassembles concomitantly with the formation of a perinuclear prophase spindle. (From Gunning and Steer, Plant Cell Biology: Structure and Function; 1996)

Remarkably, the plane defined by the PPB coincides with the cell equator, which geometrically encompasses the center of the nucleus and later during mitosis, the equatorial plane of chromosome alignment during mitosis (Yabuuchi *et al.*, 2015). In cells committed to mitosis, the narrowed PPB progressively disassembles, allowing the formation of the prophase spindle. Remnants of PPB can be observed at the onset of mitosis and especially after nuclear envelope breakdown, when spindle microtubules encounter chromosome kinetochores. As suggested narrowing of the PPB might be related to the differential regulation of microtubule dynamics, since studies have shown its mitotic persistence in even metaphase cells either after microtubule stabilization by taxol (Panteris *et al*., 1995) or by genetic knock-out of the microtubule severing protein KATANIN (Komis *et al*., 2017).

The microtubular PPB is not the only occupant of the cortical division zone, which represents a site of accumulation of many other protein markers. Cortical actin filaments for example follow the organization of the microtubule PPB in some cases (Takeuchi *et al*., 2016), while other proteins with microtubule binding properties or not, have been found to be recruited to the cortical division zone and to persist long after the disassembly of the PPB. Such proteins include the microtubule associated proteins TANGLED (Walker *et al.*, 2007; Rasmussen *et al.*, 2011), AIR9 (Buschmann *et al*., 2006), POK1/2 (Müller *et al*., 2006), the regulatory subunit of the protein phosphatase type 2A (PP2A) FASS/TONNEAU2 (Camilleri *et al.*, 2002; Spinner *et al.,* 2013), the small GTPase effector RanGAP1 (Xu *et al*., 2008) and others. Mutants of the respective genes exhibit defective cell division plane orientation at variable extends proving that the above proteins are somehow related to cell plate guidance, without however knowledge on the precise mechanism (Fig. 6).

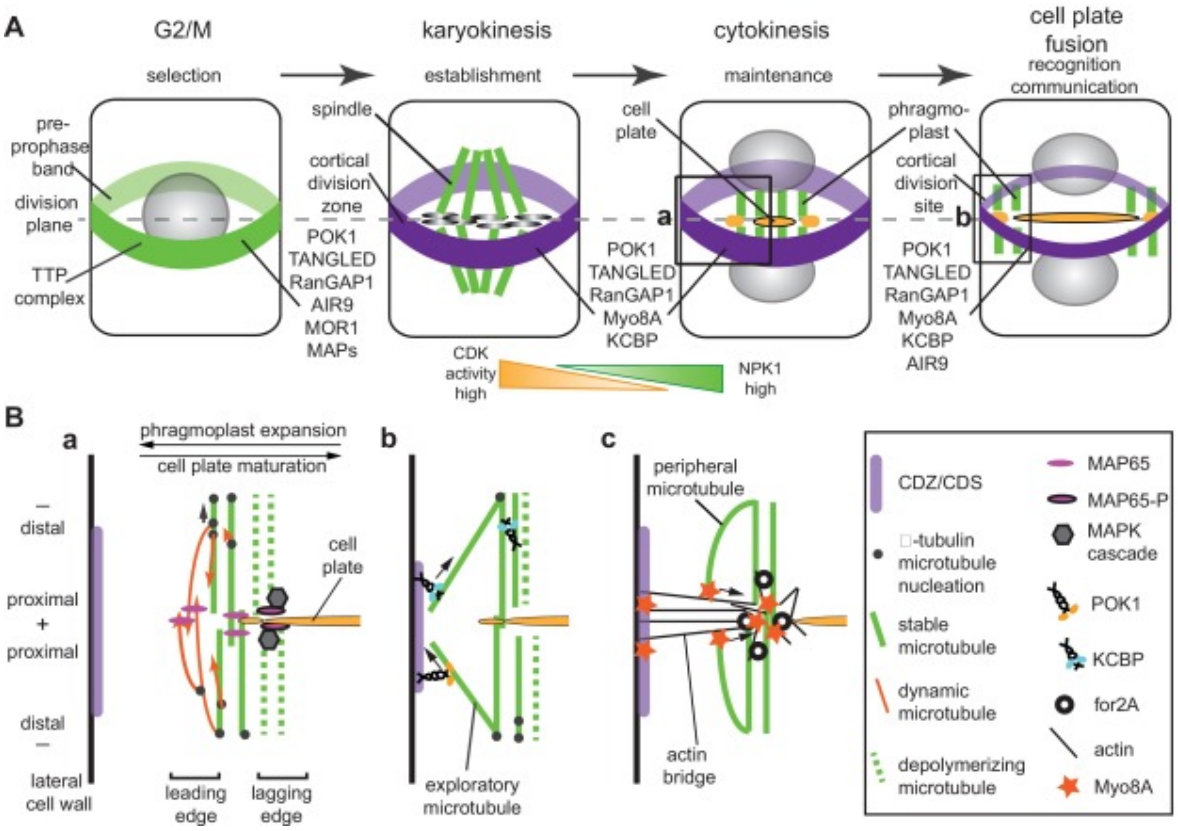

Figure 6. A model showing the localization of molecular markers of the cortical division zone at the PPB site and a putative mechanism of phragmoplast guidance to the cell periphery as guided by the demarkation of the cortical division site by the PPB and associated molecules. (From Müller and Jurgens, 2016)

3.3.3 The mitotic spindle and the phragmoplast during cell division and cytokinesis

The formation of the mitotic spindle is a central event of cell division in all eukaryotes (Yanagida, 2005). In a coarse description, the mitotic spindle is an assembly of microtubules, microtubule bundling and microtubule-based motor proteins which is responsible for interacting with chromosome kinetochores in order to faithfully separate sister chromatids to the resulting daughter cells. In mammalian and fungal cells, mitotic spindle assembly is strictly based on structurally defined microtubule organizing centers such as the centrosome and the spindle pole body, but in land plants lacking such structures, mitotic spindle assembly is likely depending on the self-organization properties of microtubules assembling in the vicinity of chromatin. In a temporal sense, the plant acentrosomal mitotic spindle undergoes large scale and distinct reorganizations which are marking respectively distinct stages of the mitotic process (Fig. 7).

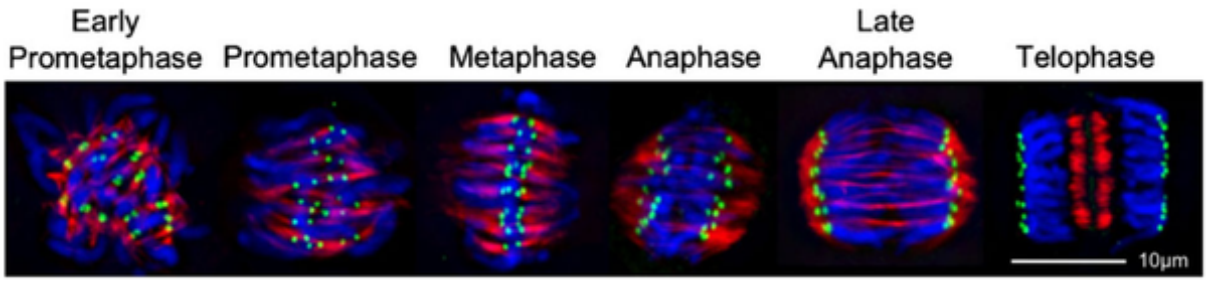

Figure 7. Plant mitotic redistribution of chromosomes (blue), kinetochores (green) and microtubules (red) showing vigorous spatial changes of the basic mitotic spindle components throughout the entire cell division process. (From https://www.quora.com/How-do-plant-cells-undergo-mitosis-without-centrosomes-How-does-the-spindle-form)

Principally, mitosis can be divided into the following stages, depending on the spindle form and the chromosome arrangement (partially shown on Fig. 7 and reviewed in Yamad and Goshima, 2017): (1) prophase: the mitotic spindle originally assembles in a tight bipolar manner around the yet intact nuclear envelope exhibiting well focused poles. Chromosomes are just starting to form while still in the nucleus, while at the end of prophase when nuclear envelope is disintegrated, chromosomes start to interact with spindle microtubules, being dispersed in the entire area occupied by the nucleus before. (2) prometaphase: the spindle consists of microtubules robustly organized in kinetochore fibers, but polar focusing is lost. Chromosomes reach their peak condensation and they fully interact with microtubule bundles from the spindles.

(3) metaphase: spindles assumes a perfectly bipolar geometry and it primarily consists of kinetochore fibers interacting with chromosome kinetochores. The latter are arranged in a straight line corresponding to the so-called metaphase plate or equatorial plane while chromosome arms extend away from the plane pointing to the spindle poles. Kinetochore fibers of the metaphase spindle are broadly focused at the poles. (4) anaphase: during anaphase, sister chromatids become disengaged at the kinetochores and are actively transported to the opposite poles. (5) telophase: sister chromatid groups have been allocated to their final position at the spindle poles and start to decondense gradually forming the daughter nuclei. The mitotic spindle is replaced by a prominent microtubule interzonal system comprising of tightly bundled antiparallel subsets of microtubules which occupies the area between the sister chromatid groups. This system will give rise to the succeeding phragmoplast. (6) cytokinesis: the phragmoplast is forming. It is consisted of two antiparallel sets of short microtubules which form between the two daughter nuclei. It appears as a disc which is expanding centrifugally towards the cell cortex. At later stages, phragmoplast microtubules disappear from the center and become confined to the margins of the expanding phragmoplast giving it the appearance of a ring. The role of the phragmoplast is to guide transport of vesicles with polysaccharidic precursors of cell wall material and thus drive the deposition of the daughter cell wall which at the early stages is called the cell plate. Once the cell plate reaches the parent walls and fuses with them, the phragmoplast is disassembled and post-mitotic microtubular networks are reinstated in the perinuclear area and the cortical cytoplasm.

Mitotic spindle and cytokinetic phragmoplast assembly are complex and temporally dynamic processes that depend on some shared features which include: (a) microtubule nucleation, (b) control of microtubule end dynamics, (c) microtubule crosslinking, (d) microtubule severing activities, (e) microtubule motor activities and (f) interactions of microtubule and actin microfilament cytoskeletal components.

3.4 Molecular components of the plant cytoskeleton

3.4.1 Microtubule nucleating proteins

Although microtubules can spontaneously form at permissive soluble tubulin concentrations (i.e., over 6 mg/ml *in vitro*), the *in vivo* formation of microtubules must be more carefully controlled. The rate limiting factor in microtubule formation is their nucleation which occurs at defined cellular sites with the aid of γ-tubulin, a member

of the tubulin superfamily (Farache *et al.*, 2018). γ-tubulin forms ring templates for microtubule assembly (Raynaud-Messina and Merdes, 2007), upon which αβ-dimers are deposited. In animal or fungal cells, γ-tubulin ring complexes (γTuRCs) are recruited to structurally defined structures such as the centrosome (Paz and Lüders, 2018) or the spindle pole body (Paz and Lüders, 2018), but in plants, devoid of discrete microtubule organizing centers (MTOCs), γTuRCs can be found in cell edges at the cortex, on the walls of preexisting microtubules and around the nuclear periphery (Nakamura, 2015). The genome of *Arabidopsis thaliana* encodes for two redundant γ-tubulin isoforms which are indispensable for microtubule nucleation. Apart from γ-tubulin, microtubule nucleation depends on other proteins as well. These include GCPs (γ-tubulin complex proteins), GIPs (γ-tubulin interacting proteins) and augmins (Nakamura, 2015).

3.4.2 Microtubule end binding proteins

Another group of microtubule-associated proteins implicated in cell division processes are plus end tracking proteins (+TIPs) which controls microtubule plus behavior and allows to microtubules to be easily assembled into diverse higher order structures. +TIPs mainly display association with microtubules on their plus ends and promote microtubule stability as well as attachment of microtubule plus ends to various cellular structures, such as the cell cortex or chromosomal kinetochores. Contrary to a number of known +TIPs in animals and fungi, only three equivalent microtubule-associated proteins have been identified to date in higher plants. These proteins which associate with microtubules in different extends include CLASP (Ambrose *et. al*, 2007), EB1 (Bisgrove *et. al*, 2008) and SPR1 (Sedbrook *et. al*, 2004). Mutations in genes of these particular proteins exhibit defects in regulation of directional cell expansion at different levels and without knowledge of the specific mechanism.

3.4.3 Microtubule bundling proteins

The formation of biased microtubule arrays in plants, either in growing or differentiating, or in dividing cells, largely relies on the crosslinking of microtubules by appropriate proteins. By definition such proteins should bear two microtubule binding domains to be able to crosslink adjacent, parallel or antiparallel microtubules. One large family of such proteins in plants is designated as the MAP65 family (microtubule associated proteins of 65 kDa MW) which are homologous to the mammalian PRC1,

the fission yeast Ase1p and the *Drosophila* feo proteins (Tolić, 2018; Loïodice *et al*., 2005). This family comprises of 9 members in *Arabidopsis thaliana*. Of these members, MAP65-1 and MAP65-2 are redundant and ubiquitous, being localized to interphase arrays and cytokinetic phragmoplasts, being excluded from the spindle, while MAP65-3 (and MAP65-4) show a more restricted localization to mitotic microtubule systems. The general structure of any given MAP65, purports to an elongated molecule with an extended aminoterminal α-helical region and a carboxyl terminus which encompasses the microtubule binding domain (Smertenko *et al*., 2004). All MAP65 proteins studied so far form obligate dimers with a coiled-coil association being the structural basis of dimerization. In this way, two microtubule binding domains are exposed to each end of the dimer and this is the principle architectural plan underlying the microtubule crosslinking properties of any MAP65 protein.

Binding of MAP65 proteins is subjected to negative regulation via phosphorylation, justifying their differential regulation during the plant cell cycle by Aurora and Mitogen Activated Protein kinases (Beck *et al*., 2010; Kosetsu *et al*., 2010; Boruc *et al*., 2017). This has been mostly studied in the case of MAP65-1, -2 and -3 members of the MAP65 family (Sasabe *et al.*, 2011). Regarding MAP65-1 and MAP65-2, phosphorylation underlies their absence from essentially all mitotic stages of spindle assembly and progression (Smertenko *et al*., 2006).

3.4.4 Microtubule motors

In eukaryotic cells movement along microtubules is in general established by the action of two families of motor proteins, dyneins and kinesins, that utilize energy derived from ATP hydrolysis to produce force and movement for intracellular transport of various cargos, including membranous complexes, protein complexes, and mRNAs (Barlan and Gelfand, 2017). Motor proteins are also extremely important for cortical microtubule organization and for cell wall assembly so they occupy almost all microtubule based structures formed during the cell cycle. In animals and yeasts dynein and kinesin translocate in the opposite and only single direction along the microtubules. Dynein moves toward the minus end and kinesin toward the plus end. Kinesins have been found as highly homologous within the wide range of eukaryotes: from fungi, animals, to the plants and human (Lee *et al*., 2015). Genome sequencing has revealed that higher plants lack minus-end-directed motor protein such as dynein (Wickstead and Gull, 2007). However, it was found that land plants encode for many more kinesins than animals (Miki *et. al*., 2014)

and some of them are predicted to be minus-end-directed motors (Yamada *et. al*, 2017) that might compensate for the lack of dynein.

Kinesins have been classified into 14 subfamilies (Lee *et al*., 2015). The structure of the kinesins in plants is similar to plus-end-directed kinesins in other species but can differ between the individual kinesin subfamilies. In general, kinesin molecule possess two heavy chains (KHC) and two light chains (KLC). Heavy chains contains globular motor domain, neck-linker and stalk domain attached to the light chain which is composed of tail domain (Fig. 8). The globular motor domain is a well conserved domain within the animals and plants also referred to as catalytic core characterized by the presence of ATP- and microtubule binding site or "head" (Fig. 8). Neck-linker is attached to the motor domain and the amino-acid sequence of the neck-linker determines the direction of motility of kinesin (Woehlke and Schliwa, 2000) (Fig. 8). Adjacent stalk domain composed of a coiled-coil structure is connected with tail domain which mediates transporting bind cargo and is highly divergent between different kinesins (Fig. 8).

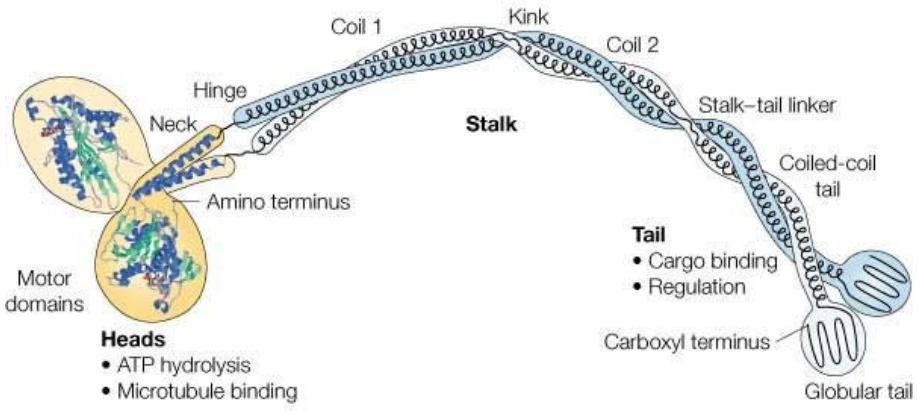

Figure 8. Structure and domain organization of the prototypical kinesin 1. (From Woehlke and Schliwa, 2000)

Kinesin movement is allowed only in presence of two motor domains capable of ATP-dependent microtubule gliding activity coupled to MT-activated ATP hydrolysis. The kinesin motility relies on precise cooperation of motor domains to avoid slipping from the microtubules after the binding. Both kinesin motor domains contain tightly bound ADP which is after the encounter with microtubules released from attached catalytic core and the empty nucleotide binding site is rapidly filled with ATP. After the ATP hydrolysis the movement is allowed (Fig. 9).

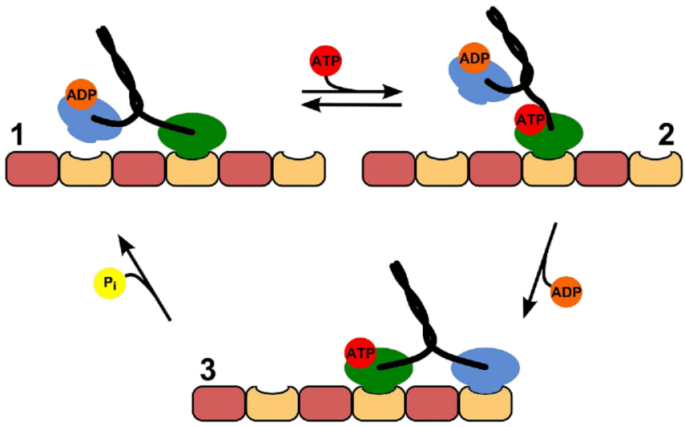

Figure 9. The mechanism of kinesin movement. Microtubule binding of one kinesin motor domain causes ADP to be released from the attached head (1) and allows to ATP to bind to this site (2). This nucleotide exchanges stabilize the bond between the kinesin catalytic core and microtubule and throws the second head forward the next binding site on the microtubule. The attached head releases phosphate during the hydrolysis ATP and is able to move forward (3). (From Sozański *et al*., 2015)

3.4.5 Importance of kinesin-5 proteins in mitotic spindle assembly

Kinesin-5 as one of the motor proteins plays an essential role in mitotic spindle assembly. Kinesin-5 molecules are N-terminal plus-end directed motors that form homotetramers allowing them to cross-link antiparallel microtubules and sliding them apart (Yukawa *et. al*, 2017; Fig. 10).

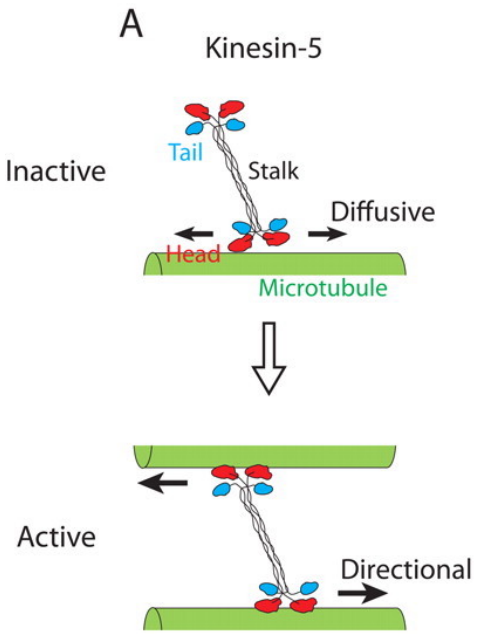

Figure 10. Microtubule motility of Kinesin-5. An inactive form of kinesin-5 is represented by diffusive motility, which is independent of ATP hydrolysis. Kinesin-5 becomes active after binding a cargo which is, in this case, another microtubule and becomes directional. (adapted with modifications from Sozański *et al*., 2015)

In the *Arabidopsis thaliana* genome, four gene sequences have been found to be related to kinesin-5 motor proteins (AtKRP125a, AtKRP125b, AtKRP125c and AtF16L2) whereas in animal genomes kinesin-5 is present usually as a single-copy gene (Bannigan *et. al*, 2017). Kinesin-5 motors are present within the nucleus during interphase until nuclear envelope breakdown and then are accumulate at higher concentrations at the midzone and spindle poles (Peters *et. al*, 2009). Perturbation of kinesin-5 activity in animals or yeast result in disruption of proper assembly of spindle characterized by the formation of monastral spindles and cell cycle arrest (Bannigan *et. al*, 2007; Peters *et. al*, 2009). In plants inhibition of kinesin-5 activity is defined mostly by a formation of multipolar spindles but the formation of monasters was also observed albeit to a lesser extend (Peters *et. al*, 2009).

Owing to their ATPase activity all members of the kinesin superfamily may be pharmacologically manipulated by either non-hydrolyzable ATP analogues that induce a rigor state, or by ATP-competitive compounds that directly inhibit their ATPase activity. Monastrol on the other hand, is a small cell permeable molecule that inhibits kinesin-5 motors via an allosteric mechanism without competing with ATP. Monastrol blocks kinesin-5 movement by binding to the motor domains. Monastrol was also tested whether affects other motor proteins and despite the fact that motor protein shares sequence similarities (Miki *et al.,* 2014) monastrol did not inhibit other kinesins (Mayer *et al.*, 1999). Monastrol phenocopies inhibition of kinesin-5 function by gene knockout which similarly leads to monaster formation during spindle assembly.

3.5 Scope of the study

3.5.1 *Arabidopsis thaliana*

Arabidopsis thaliana represents a so-called genetically tractable model for dicotyledonous plants (Meyerowitz, 1989). The genome of *Arabidopsis thaliana* is fully sequenced and extensively annotated, while central developmental processes including embryo development, vegetative growth and reproductive development have been and are still being extensively characterized. As a result, there is a core database (The *Arabidopsis* Information Resource; TAIR) which represents a huge collection of *Arabidopsis thaliana* gene annotations, enlisting additionally a large collection of allelic mutant accessions, covering most if not all the sequenced *Arabidopsis* genome.

There are many reasons why *Arabidopsis thaliana* was established as a model plant and these can be summarized as follows:

- It has a relatively small size and it can be grown very easily under standard laboratory conditions
- It has a relatively small life cycle (about 6 weeks from *germination* to mature seed)
- It produces large number of progeny allowing for the mass propagation of mutant lines etc.
- It self-pollinates but it can be very easily crossed
- There are very firm protocols for transient and stable genetic transformation of *Arabidopsis* using *Agrobacteria* species as vectors
- It has a very small number of chromosomes (i.e., 5). This is of paramount importance for genetic studies considering that in plants the large numbers of chromosomes arise mostly by polyploidy

For the above reasons methods and protocols of biochemical, biophysical, physiological, and cell biological plant research have been developed primarily on and for *Arabidopsis* and subsequently adapted to the study of other plant species. Although there is an ongoing trend to introduce and establish other plants as models (e.g., *Brachypodium distachyon* as a monocot model), *Arabidopsis* remains at the forefront of plant research.

3.5.2 Effects of monastrol on mitotic spindle assembly in *Arabidopsis thaliana*

Monastrol was identified as an non ATP-competitive allosteric inhibitor of mammalian kinesin-5 proteins in a high throughput screening of compounds for non-microtubular mitotic inhibitors (Mayer *et al*., 1999; Kwok *et al*., 2006). Specifically, monastrol has been shown to bind the kinesin-5-ADP complex and inhibit ADP release. In cellular assays, monastrol was shown to disrupt mitotic spindle bipolarity and induce its monoastral configuration (Fig. 11) hence the name.

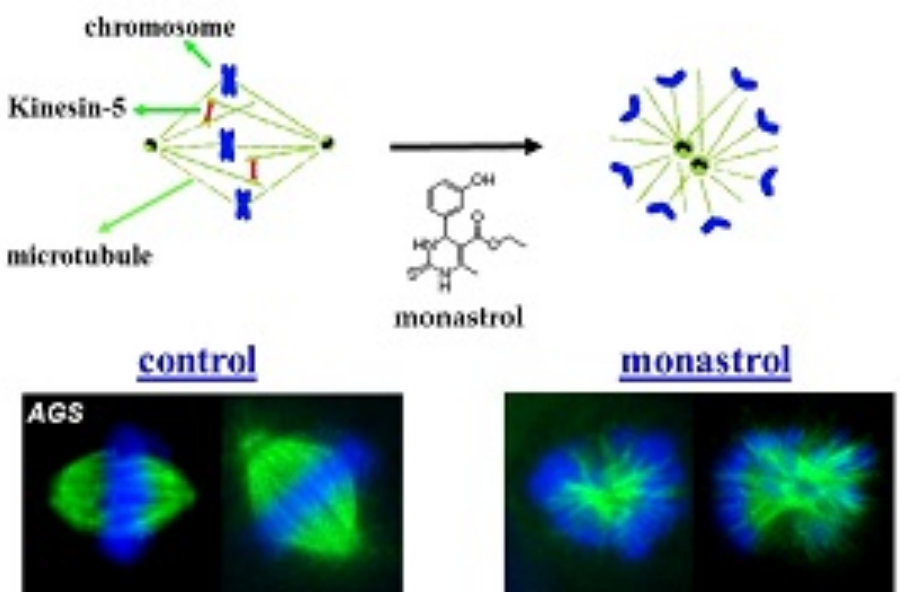

Figure 11. Effects of monastrol on mitotic spindle assembly, bipolarity and chromosome positioning. (From http://in.bgu.ac.il/en/natural_science/chem/lgheber/Pages/monastrolprototypefornewanticanceragents.aspx)

Since kinesin-5 motors were shown to be essential for mitotic spindle assembly in plants, the effects of monastrol were sought in the mitotic process, by addressing mitotic spindle form in wild type *Arabidopsis* seedlings treated with monastrol.

3.5.3 An overview of structured illumination microscopy methods

In the present thesis two versions of structured illumination (SI) were used. The first SI approach is commercially termed as Apotome and is an SI add-on for standard widefield epifluorescence microscope. This add-on contains a diffraction grid which is interfering with the beam path and can be only moved linearly. In this way, apotome allows for optical sectioning and exclusion of out-of-focus light but is not capable for high resolution studies.

Superresolution SI Microscopy (SIM) is an advanced extension of the apotome. It illuminates the sample with a diffraction generated light pattern, which may undergo 5 rotations and 5 linear phase shifts. Diffraction orders of the pattern convolve with diffraction orders of the emitting sample generating Moire patterns for each position of the grid (Fig 12).

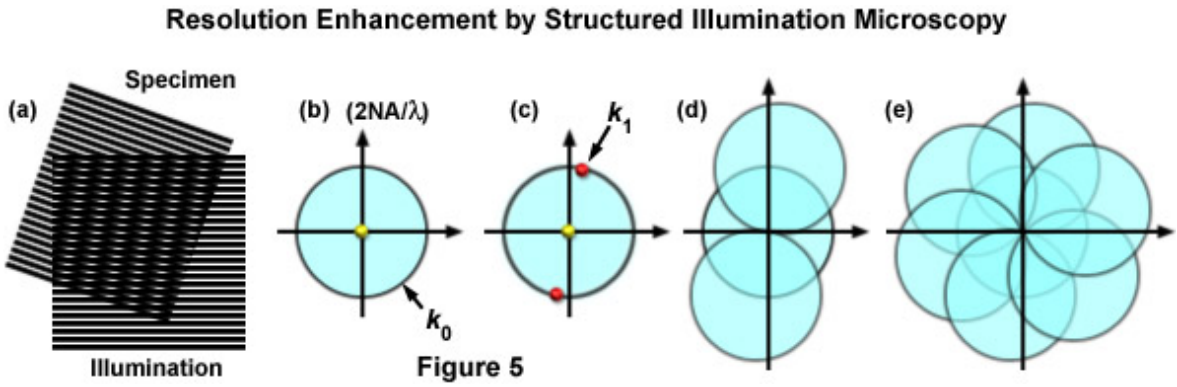

Figure 12. Principle and effects of structured illumination in the resolution of widefield microscopy. (a) generation of Moire patterns with specimen high diffraction orders and the superimposed light pattern. (b) effects of rotational and linear movements of the light pattern on the image coverage at the reciprocal space (frequency space; aka the image formed at the back focal plane of the objective). (From https://www.zeiss.com/microscopy/us/solutions/reference/superresolution/superresolution-structured-microscopy.html#sr-sim)

The final image compiles all the possible Moire patterns (5 rotations x 5 phase shifts = 25 frames for a single image), which are then deconvolved in Fourier space by means of a frequency deconvolution algorithm (typically Wiener filter and its alternatives). Optimally the final image can have a resolution doubling the resolution limit as defined by Karl Ernst Abbe ($d=\frac{\lambda}{2NA}$; $with\ d, resolution;\ \lambda, excitation\ wavelength;\ NA, numerical\ aperture\ of\ the\ objective$), which means that it can reach 100 nm (Komis *et al.*, 2014, 2015a, b, 2018).

4 MATERIAL AND METHODS

4.1 Materials

4.1.1 Chemicals and reagents

- Bovine serum albumin (BSA; Sigma-Aldrich)
- Cellulase R10 (Desert Biologicals)
- DAPI (4',6-diamidino-2-phenylindole; Sigma-Aldrich)
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich)
- Distilled water (dH_2O)
- Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA; Sigma-Aldrich)
- Aqueous glutaraldehyde 25% v/v (Electron Microscopy Sciences)
- Glycerol (Sigma-Aldrich)
- Macerozyme R10 (Desert Biologicals)
- Magnesium sulphate heptahydrate ($MgSO_4x7H_2O$; Sigma-Aldrich)
- Meicelase (Desert Biologicals)
- MES (2-(N-morpholino)ethanesulfonic acid buffer; Duchefa)
- Modified tryptone broth (MTSB; Sigma-Aldrich)
- Monastrol (Sigma-Aldrich)
- Murashige and Skoog medium (Duchefa)
- Nonidet P-40 (Sigma-Aldrich)
- Aqueous formaldehyde 16% v/v (Electron Microscopy Sciences)
- Paraphenylenediamine (Sigma-Aldrich)
- Phosphate buffered saline (PBS; Sigma-Aldrich)
- Phytagel (Duchefa)
- 1,4-Piperazinediethanesulfonic acid (PIPES; Sigma-Aldrich)
- Potassium dihydrogen phosphate (KH_2PO_4; Sigma-Aldrich)
- Potassium chloride (KCl; Sigma-Aldrich)
- Potassium hydroxide (KOH; Sigma-Aldrich)
- Rat anti-alpha tubulin monoclonal antibody (clone YOL1/34; Serotec or BioRad)
- Alexa Fluor 647-conjugated goat anti-rat IgG (Thermo Fisher Scientific)
- Sodium azide (NaN_3; Sigma-Aldrich)

- Sodium chloride (NaCl; Sigma-Aldrich)
- Sodium hypochlorite (NaOCl; Sigma-Aldrich)
- Sodium phosphate dibasic dihydrate ($Na_2HPO_4x2H_2O$; Sigma-Aldrich)
- Sucrose (Sigma-Aldrich)
- Triton X-100 (Sigma-Aldrich)
- Tris (Sigma-Aldrich)
- Tween 20 (Sigma-Aldrich)
- Ultrapure milli-Q H_2O
- Mounting medium I: VECTASHIELD Antifade Mounting Medium (Vector Laboratories)
- Mounting medium II: VECTASHIELD HardSet Antifade Mounting Medium with DAPI (Vector Laboratories)

4.1.2 Biological material

- *Arabidopsis thaliana* ecotype Columbia (Col-0)

4.1.3 List of equipment

- 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 refridgerator (ERB 34633W, Electrolux)
- Laboratory balance (S1502, BEL Engineering)
- Laminar flowbox biohazard grade (Faster, FERRARA)
- Magnetic stirrer (MSH-420, BOECO)
- Microscopes used
    - Elyra PS.1 combined SIM/PALM platform built around Axio Observer inverted microscope (ZEISS)
    - Apotome.2 add-on built in Axio Imager upright microscope (ZEISS)
- Microwave (MGE21, HITACHI)
- Freezer (Liebherr)

- Desktop pH meter (PC 2700, Eutech Instruments)
- Combi-Spin (FVL-2400N, bioSan)

4.1.4 List of solutions

Table 1. List of solutions for preparation of plant materials

Sterilization 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.	
Solid ½ MS medium without vitamins	
2,2 g . l^{-1}	Murashige and Skoog medium
10 g . l^{-1}	Sucrose
8 g . l^{-1}	Phytagel
2 g . l^{-1}	MES buffer
Supplemented with dH_2O to 1 liter, pH adjustment to 5,8 (KOH), sterilized by autoclaving.	
Inhibitor	
Monastol was prepared as a 100 mM stock solution in anhydrous DMSO.	
As a working solution, monastrol stock was diluted 1:1000 in ½ liquid MS (with a final concentration of monastrol 100 µM and DMSO at 0,1% v/v).	

Table 2. List of solutions for wholemount method

Solutions for whole mount	
Fixative solution	
1,5 ml	8% w/v Paraformaldehyde
7,5 ml	1 x MTSB
5,7 ml	Ultrapure Milli-Q H_2O
300 µl	25 % w/v Glutaraldehyde
25 µl	Triton-X

1 x MTSB	
3,775 g	PIPES
0,3075 g	$MgSO_4$ x 7 H_2O
0,475 g	EGTA
Supplemented with Milli-Q H_2O to 250 ml, pH 6,8	
0,025 g/250 ml	Sodium azide
Enzymatic solution	
0,2 g	1% w/v Meicelase
0,4 g	2% w/v Cellulase
0,2 g	1% w/v Macerozyme
20 ml	PBS (1x)
PBS (Phosphate-buffered saline)	
Stock solution (10x PBS)	
80 g	NaCl (0,8 M)
2 g	KCl (2,7 mM)
11,5 g	Na_2HPO_4 x 2 H_2O (6,5 mM)
2g	KH_2PO_4 (1,5 mM)
Supplemented to 1 l with Milli-Q, pH 7,3 (0,1 M KOH)	
Working solution (1x PBS)	
100 ml	PBS (10x)
900 ml	Ultrapure Milli-Q H_2O
Reduction solution	
20 ml	PBS (1x)
Add of aligned micro weighing spatula with $NaBH_4$ for each sample, total ca 10 mg of $NaBH_4$ for 4 samples.	
Permeabilization solution	
10 ml	PBS (10x)
10 ml	DMSO (10% v/v)
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 (1x)
Before use, store for a few minutes in a freezer (-20 °C) for better dissolution of BSA.	
Primary antibody solution	
4 ml	3% w/v Blocking solution
8 µl	Monoclonal rat anti-alfa-tubulin (clone YOL1/34; 1:500)
1 ml of antibody solution for one sample (well). Antibody solution was kept at -20 °C.	
Secondary antibody solution	
4 ml	3% w/v Blocking buffer (BSA+PBS)
16 µl	Secondary antibody: Alexa Fluor 647 goat anti-rat IgG (1:250)
1 ml of antibody solution for one sample (well).	
DAPI	
Stock solution	
10 mM 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 I	
100 mg	paraphenylenediamine
30 µl	DMSO
1 ml	Tris (1,5 M, pH 8,8)
9 ml	100% v/v Glycerol
Mounting medium II (see Chemicals and Reagents)	

4.2 Methods

4.2.1 Preparation of plant material

Arabidopsis thaliana was grown under sterile conditions *in vitro* in a growth chamber with controlled environmental conditions (temperature, humidity and light). The cultivation was carried out on square Petri dishes filled with solid Murashige and Skoog medium at half concentration of substances (½ MS) with sucrose as a carbon source, Phytagel as a solidification agent and without vitamins.

1. **Sterilization of seeds**

For experiments, the surface sterilization was made before sowing seeds on the culture medium. A 1% v/v sterilization solution was prepared by diluting a stock solution of 10% v/v sodium hypochlorite (NaClO) supplemented with 1 drop of the Tween 20 detergent. Then roughly 1 ml of sterilization solution was added to a 1,5 ml microtube containing ca. 100-150 seeds. The sterilization lasted 10 minutes with occasional vortexing to prevent seeds from clumping together.

Thereon seeds were handled under a sterile laminar flowbox with the use of only sterile instruments and solutions. After incubation, the sterilization solution was removed and replaced with 1 ml of 70% v/v aqueous ethanol. The tube was overturned few times for a total of not more than 30 seconds. Ethanol solution was removed and seeds were then washed 4 times each with 1 ml of sterile distilled water. During washing, the tube was also inverted few times to remove residues of both sterilization solution and ethanol which can prevent germination. After the last wash enough water was left in the tube to allow better sowing by pipetting.

2. **Preparation of culture and sowing**

Arabidopsis seeds cultivation was carried out under the sterile conditions *in vitro* on ½ MS medium. Sterile medium was heated in a microwave until it become boiled and moved to the sterile laminar flowbox where was poured into square Petri dishes (120x120 mm). After that, approximately 100 seeds were sown one by one on each sterile plate with help of automatic micropipette (100 µl). For easier sowing of seeds on agar plates the sterile plastic tip was cut tightly before the end. All the instruments used during the sowing were sterile or were sterilized with disinfectant solution Incidure or 70% v/v ethanol. The closed Petri dishes were sealed with parafilm and placed horizontally

in the refrigerator (4°C, dark) for one day to stratify and synchronized the germination of the seeds. The next day, they were transferred to a culture chamber (phytotron) where they were placed vertically and cultured for another 3-4 days to allow germination and seedlings growth (23°C, 16h light/8h dark).

3. **Monastrol Treatment**

For a treatment with monastrol were used 3-4 days old seedlings cultivated in phytotron. One half of them was treated with monastrol, the other one utilized for control. Seedlings treated with monastrol were transferred to a fresh twelve-wells cell culture plate with hollow plastic baskets containing 100 μM monastrol, which were made by adding small volume of a concentrated stock solution in DMSO. For control, the rest of the seedlings received the same amount of DMSO. Incubation with inhibitor took 4-6 hours at room temperature according to standard inhibitor treatment regimes (Mayer *et al*., 1999).

4.2.2 The wholemount method

This method was used for rapid and reliable localization of proteins in root tissue. The whole mount method is based on immunolocalization of proteins (Sauer *et al.*, 2006; Šamajová *et al*., 2014) by antibodies labeled with fluorescent dyes (Pasternak *et al*., 2015) in various plant tissues, including roots of *Arabidopsis thaliana* (Sauer *et. al*, 2006). After certain modifications in our laboratory which are mentioned in the text, the following procedure was followed:

1. **Fixation**

Fixation is a chemical process using immobilization of intracellular molecules to preserve plant tissue close to their native state. For this fixation procedure was used two fixative substances formaldehyde and glutaraldehyde mixed in a solution which reacts with primary amines on proteins and nucleic acids. The fixative solution is highly toxic and carcinogenic, so it is necessary to work in hood.

A fixative solution (see List of solutions - Tab. 2) prepared for the process of fixation was divided as required using Pasteur pipette into the wells in twelve-well plastic plate for cell cultures (ca. 3 ml of fixative per well). Control and monastrol-treated seedlings were transferred with the hollow plastic baskets in fixative-filled wells of the culture plate. Incubation with fixative solution took minimum of 1 hour at room temperature or was extended in some cases to overnight at 4°C. The fixative solution was

removed from the wells with help of Pasteur pipette and replaced with washing buffer (2x in ½MTSB and 2x in 1xPBS) stabilizing microtubules. The samples were washed 4 times for 10 minutes. The solutions were always removed and added to the space between the wall of the well and the plastic basket to prevent damaging of the plants. For work with a number of samples, plastic baskets were always transferred with help of tweezers and same system was also used for following steps (see below).

2. Reduction

After the post-fixation washing the fixed seedlings were treating with $NaBH_4$ to avoid antibody molecules to interact with non-specific sites providing by unreacted aldehyde groups. $NaBH_4$ reduces aldehyde to non-reactive alcohol groups.

For this purpose a small amount of $NaBH_4$ was added via a metal spoon into baskets with seedlings in 1xPBS buffer. The amount of this substances corresponded to one aligned spoon for one sample. After the 15 minutes incubation samples were washed extensively with PBS solution until the solution stopped to effervesce.

3. Cell wall digestion

To allow antibody molecules diffuse within root cells and at a considerable depth of the root it is necessary to degrade cell wall components and for this an appropriate cell wall digesting enzyme cocktail is required. Typically, such a cocktail contains activities that hydrolyze all major polysaccharidic components of the cell wall (i.e., cellulase for cellulose and pectinase for pectins).

The prepared enzymatic cocktail (see List of solutions - Tab. 2) was divided into free wells and baskets with fixed seedlings were removed from the 1xPBS solution carefully blotted on filter paper and placed into wells containing cell wall digesting enzyme cocktail. Cell wall digestion was carried out in incubator at 37°C for 30 minutes. After this, baskets with seedlings were placed back into wells with 1xPBS and washed 4 times for 10 minutes. The enzymatic cocktail was removed and returned back to the tube, and then stored at -20°C so it could be reused 5-6 more times.

4. Permeabilization

Because plants possess more than one barrier a permeabilization has to be done to enable antibody molecules diffuse through the plasma membrane. For this reason, permeabilization solution contains an organic solvent (dimethylsulfoxide; DMSO) and a non-ionic detergent (Nonidet P40) to extract the membrane lipids and allow antibody molecules enter inside.

Seedlings were incubated in permeabilization solution (see List of solutions - Tab. 2) for 15 minutes to 1 hour. The solution was removed with Pasteur pipette and the samples were washed with 1xPBS 3-5 times for 10 minutes.

5. Blocking

Blocking prevents non-specific binding of antibody molecules at non-target subcellular structures. Non-specific antibody binding may occur at highly charged or hydrophobic structures with no structural complementarity to the antibody. Such structures can be saturated with a solution of a an inert protein such as bovine serum albumin (BSA) and allow antibody molecules to be only engaged to specific antibody - antigen interactions.

In the protocol followed herein, incubation in blocking solution (see List of solutions - Tab. 2) was done for at least 1 hour and was extended occasionally to overnight at 4°C.

6. Primary antibody incubation

Immunolocalization methods require antibody labeling. For indirect labeling are used primary antibodies interacting with epitope of interest in the sample and secondary antibodies with conjugate recognizing the primary antibody. For the present work, a rat monoclonal antibody raised against yeast α-tubulin was used (clone YOL1/34; Kilmartin *et al*., 1982).

The primary antibody solution was prepared by diluting the antibody stock solution (1 mg/ml) in 3% w/v BSA in PBS at a ratio of 1:500. From the primary antibody solution, 1000 µl were pipetted as required into each well of a 24-well culture plate. Plastic baskets with seedlings were removed from the blocking solution, gently blotted on filter paper and transferred directly to the primary antibody solution. The culture plate was covered with a lid and the samples were incubated overnight at room temperature. On the following day, samples were transferred back into the wells of twelve-well plastic plate containing 1xPBS buffer. The samples were washed 9 times for 10 minutes. The diluted primary antibody was returned back to the tube and stored at -20°C so it could be reuse for 4-6 more times.

7. Secondary antibody incubation

The primary antibody was recognized by fluorophore-conjugated species specific immunoglobulins.

The secondary antibody solution was prepared by diluting the secondary antibody in 3% w/v BSA in PBS at a ratio of 1:250. In the present study, secondary antibody was a polyclonal mixture of anti-rat IgGs conjugated with AlexaFluor 647 (λexc=650 nm,

λexc=665 nm). A 1000 µl of prepared secondary antibody solution was pipetted as required into the wells of 24-well culture plate. Plastic baskets with seedlings were removed from the blocking solution gently blotted on filter paper and transferred to the secondary antibody solution. The culture plate was covered with a lid sealed with parafilm and coated with alu foil, and then the seedlings was carried out in incubator at 37°C for 3 hours. After the incubation, the samples were washed at least 6 times for 10 minutes each.

8. DAPI staining

DAPI (4',6-diamidine-2'-phenylindole dihydrochloride) is a blue emitting (λem=461 nm) fluorophore used for labelling of nucleus, chromosomes and other structures. DAPI binds to the minor groove of AT-rich stretches of DNA and exhibits a characteristic blue fluorescence after light irradiation of a certain wavelength (λexc=461 nm).

The DAPI working solution was prepared by dilution of stock solution with PBS buffer at a ratio 1:1000. Incubation took 15 minutes at a room temperature and after that, the samples were washed 3 times for 10 minutes in PBS.

9. Mounting in antifading mounting solution (medium)

The scope of using a mounting medium, is to mount the sample at a refractive index close to that of the microscope optical pathway and also to prevent photooxidative bleaching of the fluorophores under the illumination conditions used for the imaging. Under such conditions, free radicals are produce and readily attack fluorophore molecules, quenching fluorescence. Therefor mounting media for fluorescently labeled samples are routinely supplemented with antifade antioxidants such as paraphenylene diamine, n-propyl gallate and others commercially formulated (such as Prolong, FluoroGold and Vectashield; Longin *et al*., 1993). The antioxidant used here was paraphenylene diamine.

Desired amount of paraphenylene diamine is rapidly but gently dissolved in DMSO. Once dissolved Tris solution is added and then glycerol to the final desired volume. Due to the very high viscosity of the solution, mixing was done with the aid of a glass rod. Paraphenylene diamine solutions are susceptible to light- and oxygen-induced autocatalytic oxidation, therefor it is essential to prepare the solution rapidly, avoiding introduction of air and exposure to light. To improve longevity of this mounting medium it should be stored under very low temperatures (-20°C - -80°C) in an aluminium foil covered Falcon tube.

Alternatively, commercially formulated mounting media with antifading agent and DAPI was used (Chemicals and Reagents).

To prepare samples for immunolocalization two or three plants were carefully transferred to the slide and placed on a drop of the mounting medium. The slide was then covered with a cover glass and fixed on both sides with nail polish. Prepared samples were stored in boxes for slides (Heathrow Scientific) at -20°C and then observed with microscopes.

4.2.3 Image processing, data analysis and final compilation

Programs used for exporting and editing images:

ZEN Blue 2012 (Zeiss)

ImageJ (https://imagej.nih.gov/ij/)

Microsoft Powerpoint 2017

5 RESULTS

5.1 Organization of microtubules in root cells of wild type *Arabidopsis thaliana*

Root tips contain one dominant meristematic center (Root Apical Meristem or RAM; Soyars *et al.*, 2016) which is characterized by high numbers of mitotic and cytokinetic cells at different stages. Therefor it represents an ideal material to study en mass the putative effects of monastrol. Before that however it is essential to understand the principal stages of microtubule organization during mitosis and cytokinesis and become able to recognize different stages according to the form of the mitotic or cytokinetic apparatus and the configuration of chromosomes, sister chromatids or reconstituted nuclei. For this reason the microtubules were immunolabeled and chromatin was counterstained in both control and monastrol-treated roots in order to identify both components of cell division systems.

As it will be further explained, monastrol treatment brought about mitotic spindle configuration problems and disturbed chromosome alignment during metaphase and segregation of sister chromatids during anaphase (Fig. 13).

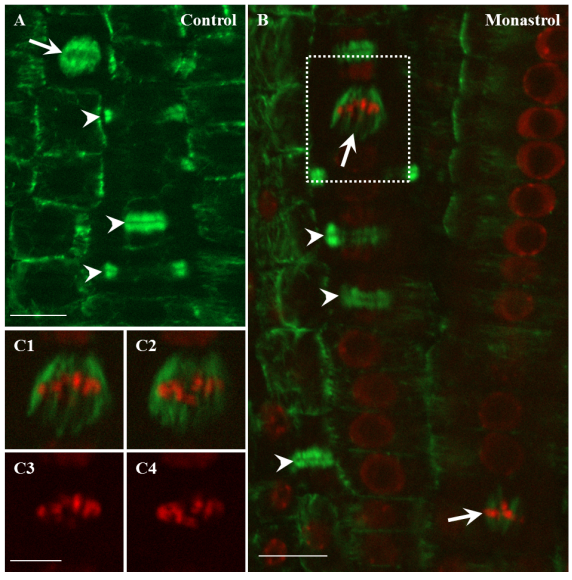

Figure 13. Overview of random mitotic stages visualized by Apotome epifluorescence imaging in root wholemounts of *Arabidopsis thaliana* before (A) or after (B) treatment with 100 µM monastrol for ca. 3 hours. C magnified view of a disturbed metaphase spindle after monastrol treatment corresponding to the outlined area of (A), showing aberrant distribution of the chromosomal metaphase plate at two different optical sections. Arrows in (A) and (B) point to mitotic spindles while arrowheads show the edges of phragmoplasts. Scale bars: 10 µm (A, B) and 5 µm (C).

5.2 Cell cycle dependent microtubule organization in wild type *Arabidopsis thaliana*

By the wholemount immunolabeling method it is possible to maintain the integrity of the root tip meristem, follow microtubule organization throughout the cell cycle and identify all microtubule arrays involved in mitosis and cytokinesis.

5.2.1 Preprophase

The first mitotic microtubule array, is the preprophase microtubule band (PPB) which is a cortical ring-like assembly of microtubules. Its formation starts with a broad band of microtubules (Fig. 14; B, D, E, G), surrounding the nucleus at the equatorial plane (Fig. 14; B, D, E, G). At later stages, the PPB becomes more narrow (Fig. 14; A), while a prominent perinuclear microtubule system is formed (Fig. 14).

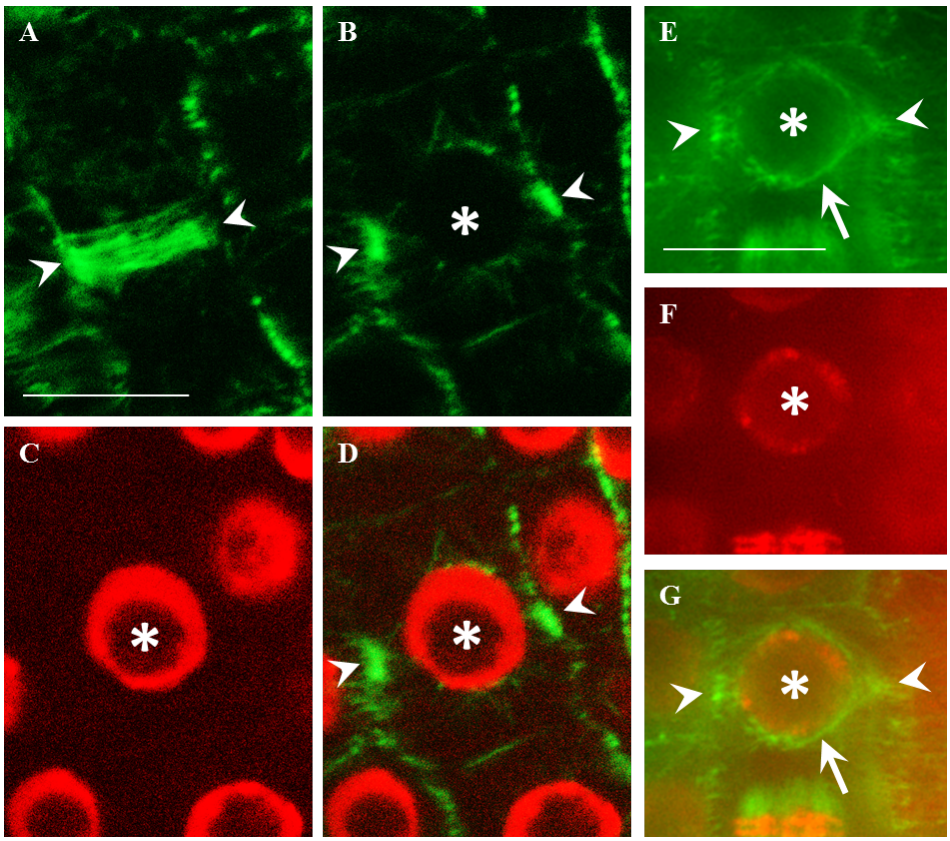

Figure 14. The different stages of the PPB assembly of *Arabidopsis thaliana* Col-0 control. In early stage, PPB starts forming along the nucleus envelope (arrowheads; B, D, E, G). Late appearance of the PPB (A) represents a well-organized microtubule band (arrowheads; A) in the cell cortex. Chromosomes remains decondensed (asteris; C, D, F, G). Scale bars: 10 μm.

5.2.2 Prophase/Prometaphase

During prophase/prometaphase the PPB disappears completely from the cell cortex, while perinuclear microtubules form a prominent but still not well focused spindle. Moreover, nuclear envelope breaks down and chromosomal kinetochores start to interact with mitotic spindle fibers (Fig. 15). During this stage, spindle - chromosome interactions intend to align kinetochores at the equatorial plane which coincides with the cell division plane previously defined by the PPB. The final alignment occurs on a stage called metaphase.

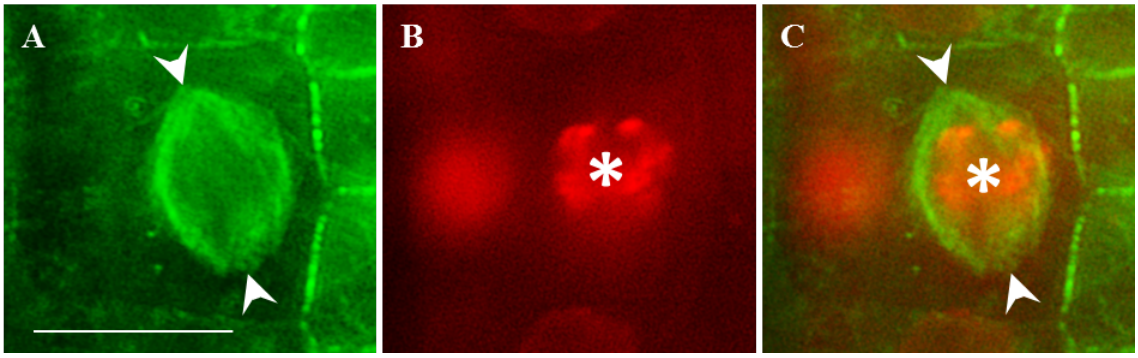

Figure 15. Prophase spindle and chromosome organisation in *Arabidopsis thaliana* Col-0 control. The spindle assembles and is nearly formed at the end of prometaphase (arrowheads; A, C) and chromosomes starts to condensate (asteris; B, C). Scale bar: 10 µm.

5.2.3 Metaphase

During metaphase, the spindle achieves perfect bipolarity and mostly comprises of kinetochore associated microtubule bundles (spindle fibers; Fig. 16; A, C). Moreover chromosomal kinetochores become aligned at the equatorial plane forming the so called metaphase plate (Fig. 16; B, C).

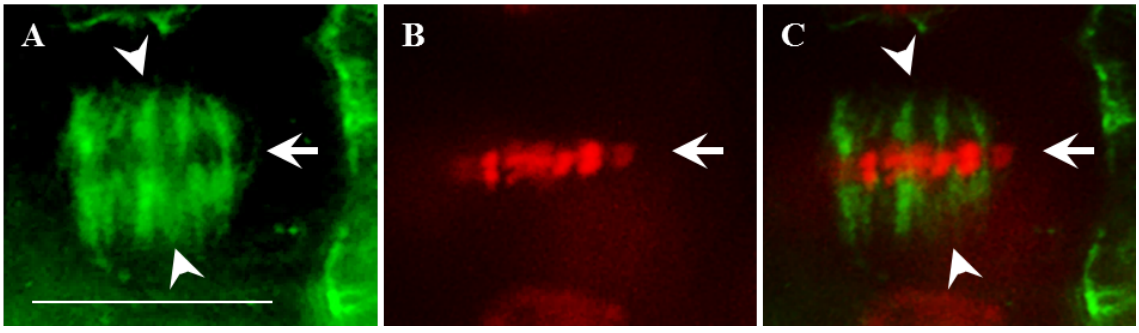

Figure 16. Metaphase spindle and chromosome organization in *Arabidopsis thaliana* Col-0 control. Theassembly of mitotic spindle is strictly bipolar with well-focused poles (arrowheads; A, C). Chromosomes are condensate and align in the middle of mitotic spindle at the equatorial plane (arrows; B, C). Scale bar: 10 µm.

5.2.4 Anaphase

Following metaphase, sister chromatids become separated into two equivalent groups. The two groups become physically separated by moving to opposite poles of the spindle during the process of anaphase (Fig. 17). This process requires both the shortening of kinetochore microtubule fibers and the activity of kinesin motor proteins.

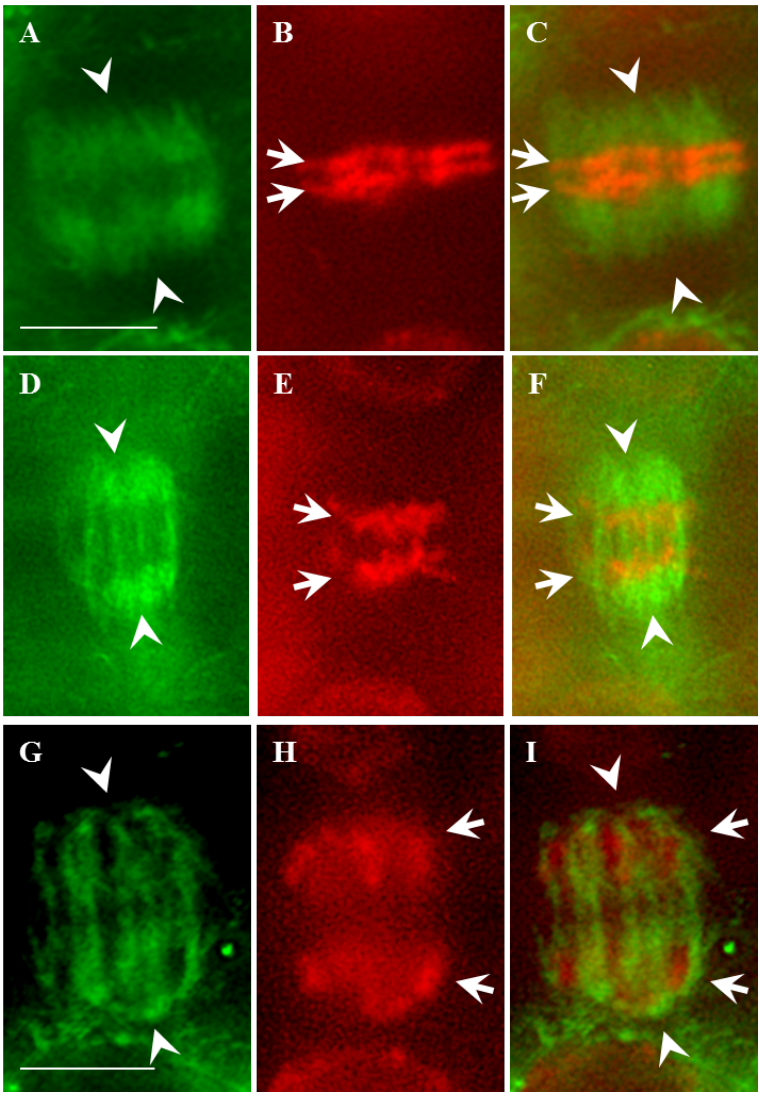

Figure 17. Anaphase spindle and chromosome organization in *Arabidopsis thaliana* Col-0 control. In early stage of anaphase (A, B, C) chromosomes form two equivalent groups (arrows; B, C) as they are separated and move further (E, F) to the pole of the spindle until they reach the spindle poles (arrows; H, I). Scale bars: 5 μm.

5.2.5 Telophase/Cytokinesis

After the groups of separated sister chromatids have established their position at the spindle poles, it is necessary to physically partition the common cytoplasm

between two daughter cells and this requires the deposition of the daughter cell wall, the cell plate (Fig. 18).

Microtubule reorganization occurs immediately after the end of anaphase. During the succeeding step of telophase, microtubules are organized in two antiparallel systems that occupy the space between the reconstituting daughter nuclei which is called interzonal system (Fig. 18). The interzonal system is the precursor of the cytokinetic phragmoplast.

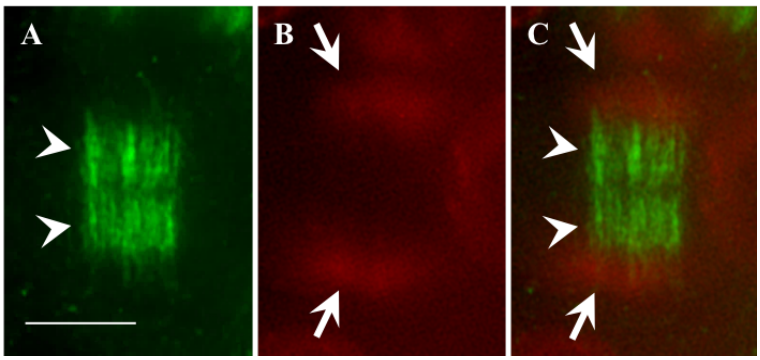

Figure 18. Telophase microtubule and chromosomes organization in *Arabidopsis thaliana* Col-0 control. Arrows show organized chromosomes at the spindle poles (B, C) and arrowheads show organized microtubules between them (A, C). Scale bar: 5 μm.

During cytokinesis, the daughter nuclei continue to be reconstituted by the gradual reinstatement of the nuclear envelope. At the same time, the antiparallel microtubules of the interzonal system shorten slightly (Fig. 19; C, D cf Fig. 18; A) and form the phragmoplast. At the onset of cytokinesis, phragmoplast microtubules form a disc whose plane coincides with the equator and the plane which was originally defined by the PPB. Subsequently, microtubules of the phragmoplast disappear from its inner part and become confined to the phragmoplast circumference.

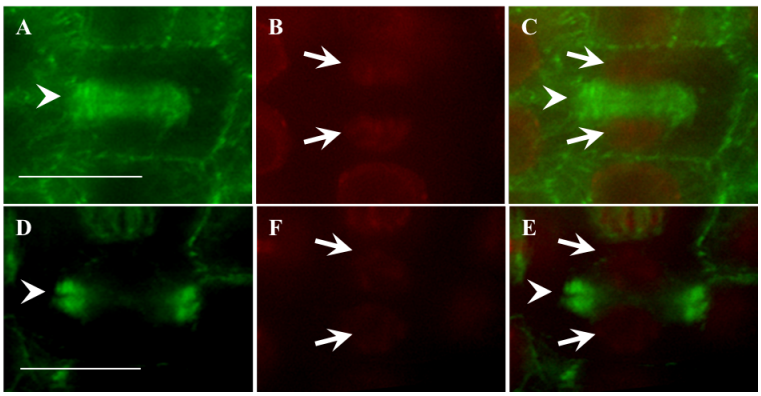

Figure 19. Cytokinetic microtubule and chromosome organization in *Arabidopsis thaliana* Col-0 control. Microtubules form phragmoplast (arrowheads; A, C, D, E) required for separating chromosomes (arrows; B, C, F, E). Scale bars: 10 μm.

5.3 Effects of monastrol on mitotic spindle assembly in wild type *Arabidopsis thaliana*

To test the utility of monastrol as an inhibitor of cell division *Arabidopsis thaliana* seedlings were treated with 100 μM monastrol for 4-6 hours. Although there was a definite effect of monastrol on distinct stages of mitosis as will be described in later sections, it was not so massive and this is probably related to the partial sequestration of the inhibitor by the cell walls of root cells.

5.3.1 Preprophase

Monastrol treatment did not affect PPB formation and hence microtubule organization in monastrol treated preprophase cells was not further examined.

5.3.2 Prophase/Prometaphase

During prophase/prometaphase it was observed variable spindle shapes ranging from quite normal (e.g., Fig. 20; A-C) to spindles with loss of bipolarity, exhibiting broader spindle pole areas compared to control (e.g., Fig. 20; D-I) sometimes accompanied by ectopic and non-uniform chromosome positioning (Fig. 20; A-C; J-L).

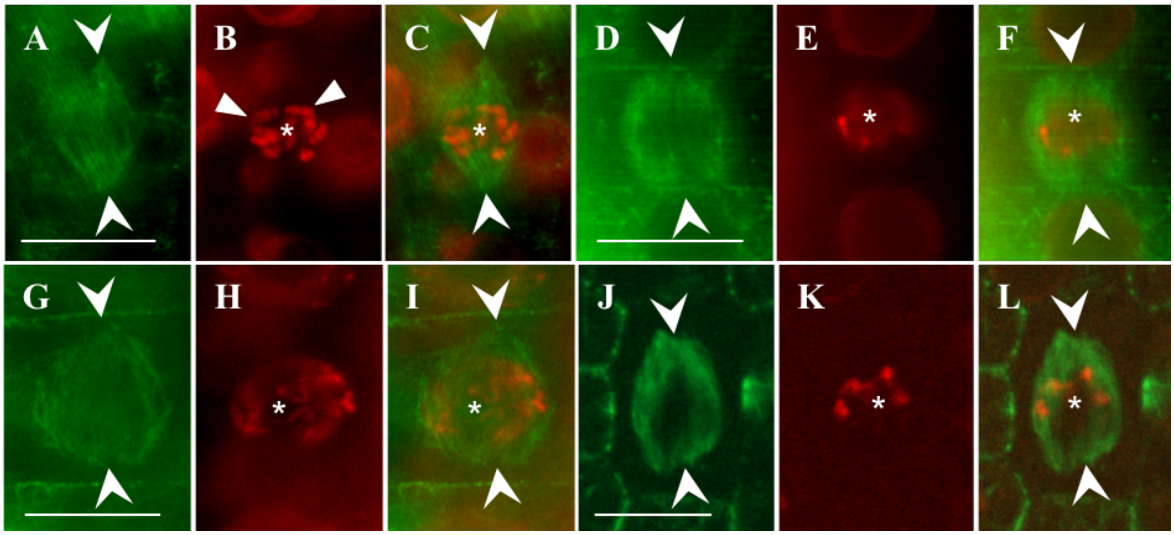

Figure 20. Effects of monastrol on mitotic spindle and chromosomes organization during prophase/prometaphase in *Arabidopsis thaliana* Col-0. Spindle shapes can resemble control (arrowheads; A-C) with acute bipolarity and well-focused poles, or exhibit broad spindle poles (arrowheads; D-L). Incorrect alignment of chromosomes shows image B (arrowheads). Asteris represents chromosomes. Scale bars: 10 μm.

5.3.3 Metaphase

The effects during metaphase were again diverse. In many cases, monastrol treatment was accompanied by misalignment of chromosomes to the metaphase spindle (Fig. 21; A-C) while in other cases, metaphase mitotic spindles and the respective chromosome positioning was unaffected (Fig. 21; D-F).

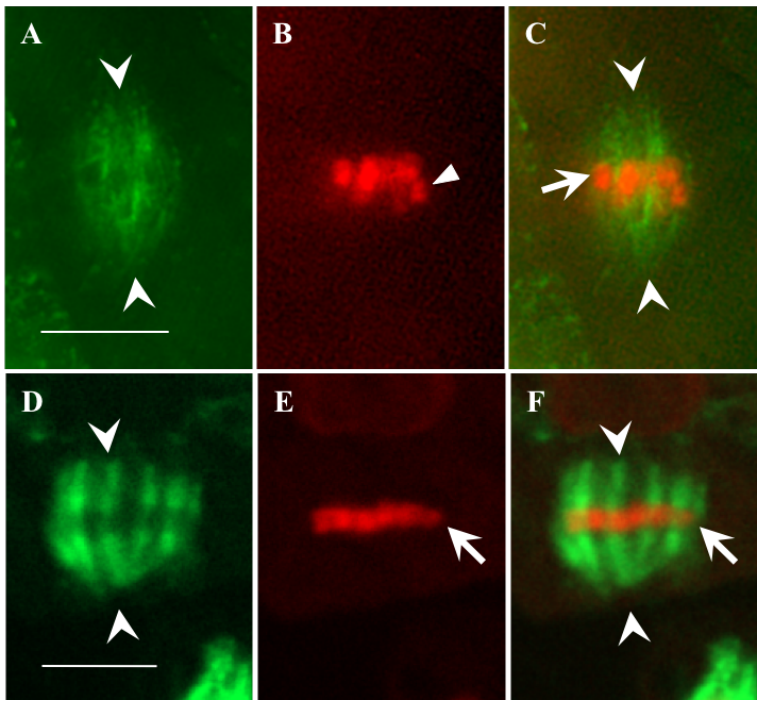

Figure 21. Effects of monastrol on mitotic spindle and microtubule organization during metaphase in *Arabidopsis thaliana* Col-0. In some cases chromosomes are misaligned (arrow; A-C) while in others the spindle (arrowheads) and the chromosomes (arrows) are indistinguishable from control (D-F). Wrong positioning of chromosomes from the equatorial plane shows image B (arrowhead). Scale bars: 10 µm.

As compared to the monoastral spindles routinely observed in mammalian metaphase cells treated with monastrol, those were extremely rarely observed in monastrol-treated roots of *Arabidopsis thaliana* (Fig. 22).

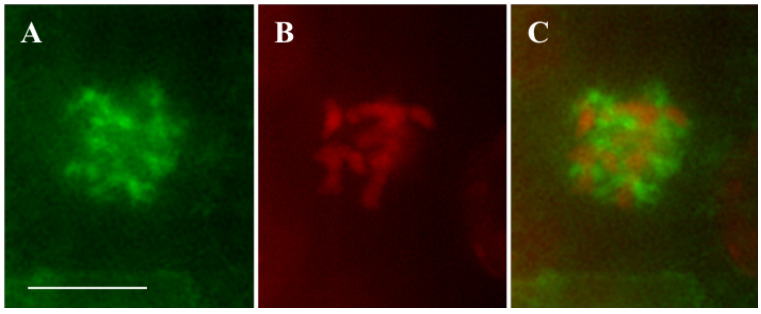

Figure 22. Monoastral spindle configuration in monastrol-treated metaphase cell. Scale bar: 5 µm.

5.3.4 Anaphase

During anaphase, monastrol treatment was shown to have the most prominent effects compared to all other cell division stages, which is a quite unique observation compared to what was published in literature. Effects were not only identified in spindle structure but also to sister chromatids positioning and presumably poleward movement. During early anaphase, slightly abnormal spindles were observed. Such spindles exhibited broad polar areas (Fig. 23; A-F) without any further defect, but the positioning of some sister chromatids was more prominently affected. Such sister chromatids were showing to be away from the rest of the group (e.g., Fig. 23; B, C and E, F).

What was quite surprising was that spindle shape disturbances were not as severe as to justify sister chromatids mispositioning and poleward movement, suggesting that monastrol rather disturbed the kinetochore machinery (see Discussion for details).

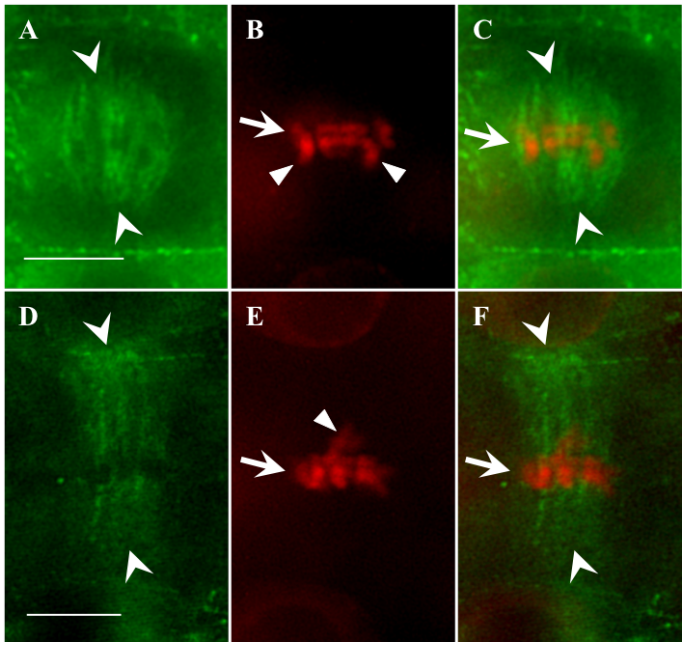

Figure 23. Effects of monastrol on mitotic spindle and microtubule organization during early anaphase in *Arabidopsis thaliana* Col-0. Spindle may exhibit slightly disturbed shape (arrowheads; A) with few sister chromatids deviating (arrowheads; B) from the rest of the group (arrow; B, C). Alternatively the early anaphase spindle might be malformed and considerably elongated with broad poles (arrowheads; D), again with deviating sister chromatids (arrowhead; E). Scale bars: 5 μm.

In more severe early anaphase cases, monastrol induced mass disarrangement of the entire sister chromatid group which appeared entangled nearby the equatorial plane. Again, this is accompanied by mild spindle malformations (Fig. 24; A-F), but the entire group of sister chromatids fails to be adequately separated (e.g., Fig. 24; B, E).

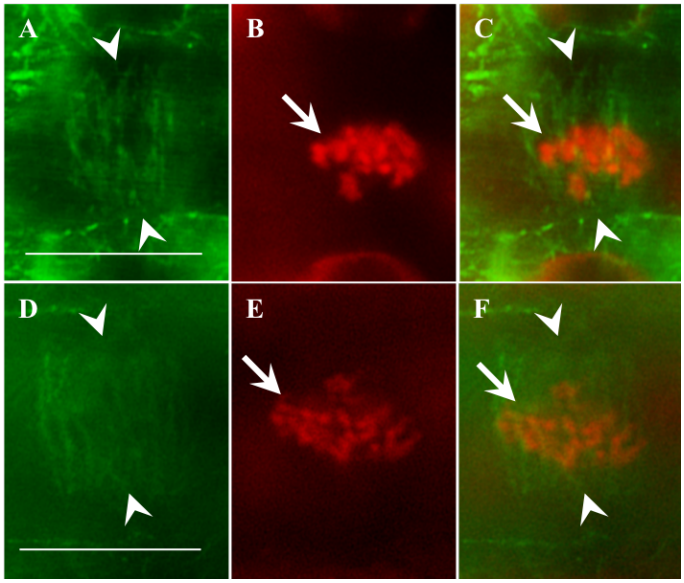

Figure 24. Effects of monastrol on mitotic spindle and microtubule organization during middle anaphase in *Arabidopsis thaliana* Col-0. During mid anaphase, spindle structure is mildly disturbed (A, C, D, F) but the spreading of separated sister chromatids is more prominent (B, C, E, F). Scale bars: 10 µm.

The effects of monastrol on the early stage of anaphase were carried over to later stages of sister chromatid separation. In this case sister chromatids were more broadly distributed within the spindle volume (Fig. 25). This observation is in favor with the hypothesis that monastrol affects the mechanism driving chromosome separation and positioning rather than spindle assembly and form in plants (see Discussion for details).

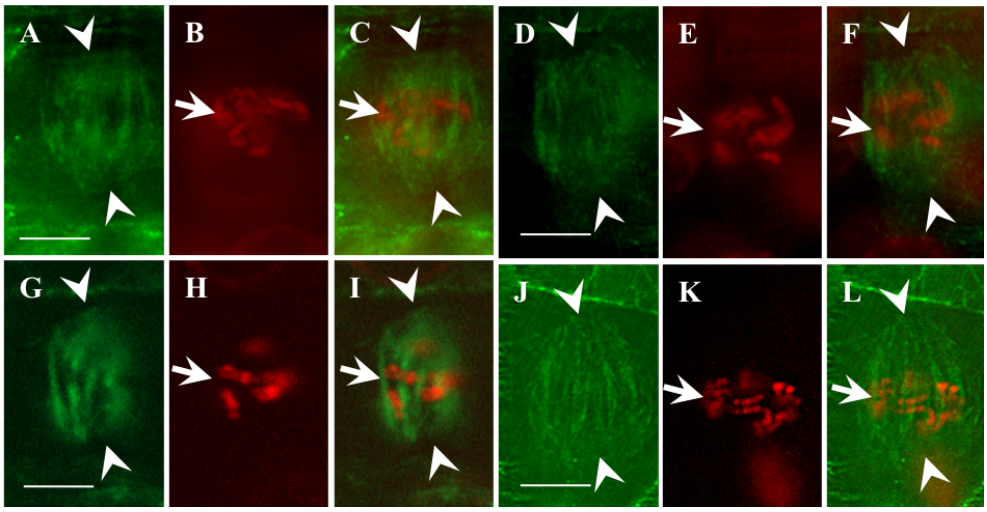

Figure 25. Effects of monastrol on mitotic spindle and chromosome organization during late anaphase in *Arabidopsis thaliana* Col-0. Spindle form appears to be affected at variable extend ranging from slightly (arrowheads; A) to progressively more severe (arrowheads; D, G and J) but without gross defects. On the other hand sister chromatids distribution considerably deviates compared to control in all cases (arrows; B, C, E, F, H, I, K, L). Scale bars: 5 µm.

In line with previous stages, it was found that not all anaphase cells were affected by monastrol and therefor normal spindle configuration and sister chromatids positioning was observed (Fig. 26).

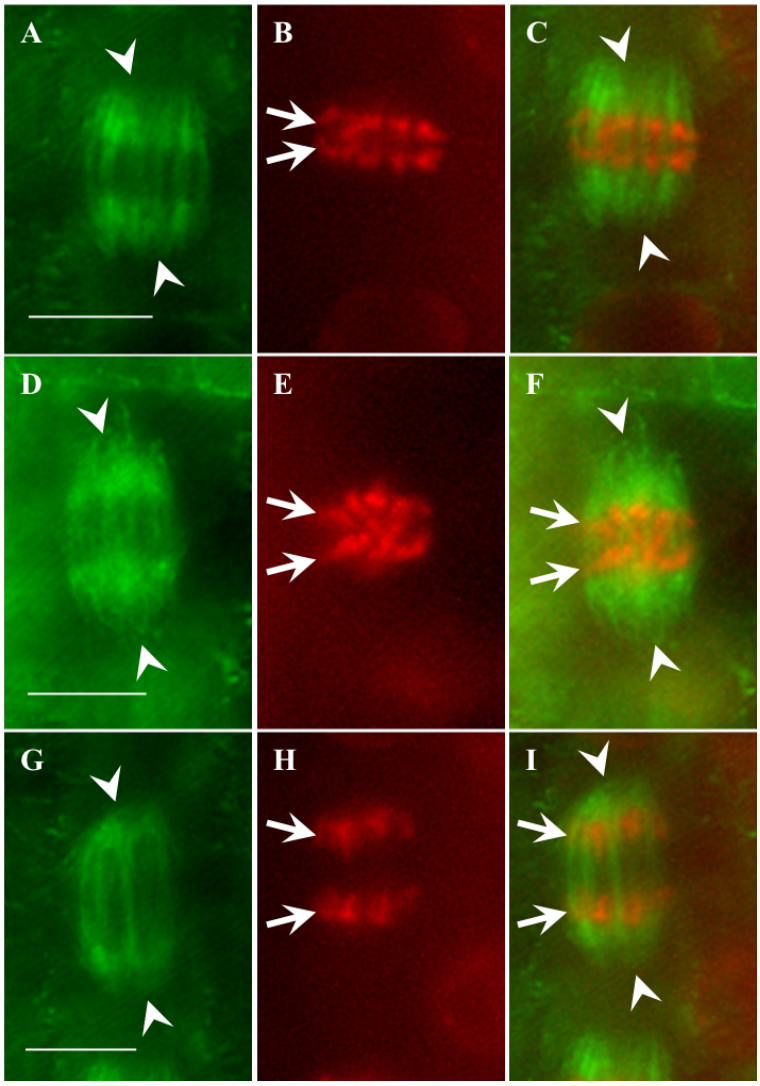

Figure 26. Examples of anaphase cells of *Arabidopsis thaliana* Col-0 that escaped monastrol effects. Early (A-C), mid (D-F) and late (G-I) anaphase stages seemingly unaffected by monastrol treatment. Spindles are represented by arrowheads and chromosomes by arrows. Scale bars: 5 µm.

5.3.5 Telophase/Cytokinesis

Telophase and cytokinesis follow anaphase, therefore it was expected that monastrol treatment would affect the formation of the telophase interzonal system and the phragmoplast either by directly inhibiting kinesin-5 processes related to such events or indirectly by having already affected the earlier anaphase stages. Surprisingly all telophase and cytokinetic systems of monastrol-treated root cells appeared to be normal and comparable to the same systems of control cells (Fig. 27).

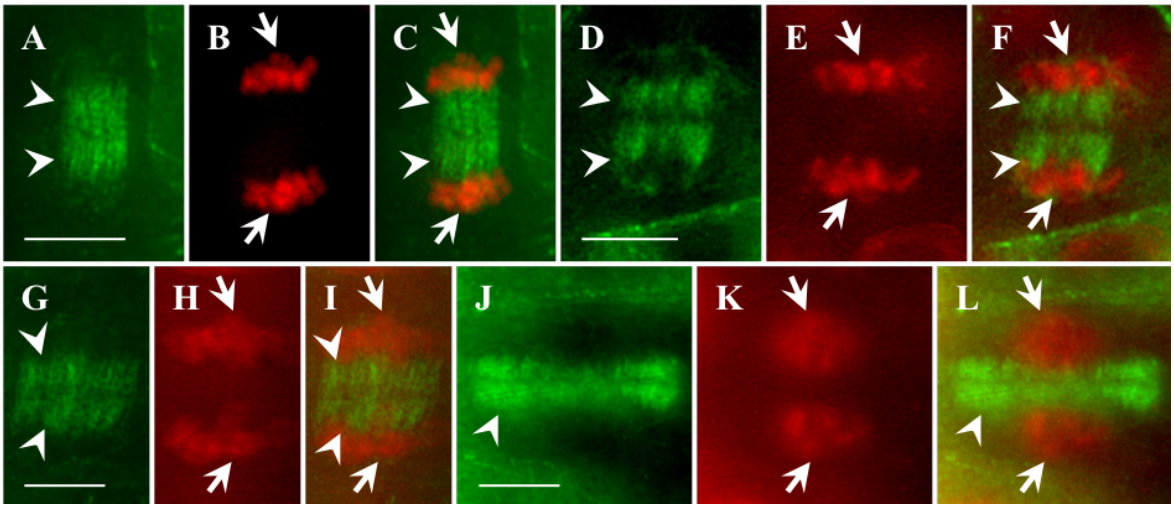

Figure 27. Effect of monastrol on phragmoplast formation and chromosome organization during telophase in *Arabidopsis thaliana* Col-0. Telophase and cytokinesis remain unaffected by monastrol treatment. Telophase interzonal system with well separated sister chromatid groups (arrows; A-C). Early (D-F), mid (G-I) and late (J-L) phragmoplasts (arrowheads) with normal microtubule configuration and gradual reinstatement of the nuclear envelope around daughter nuclei. Scale bars: 5 µm.

6 DISCUSSION

Although it was systematically documented mitotic defects during mitotic cell division after monastrol application, these defects were not massive. This suggests that monastrol was not readily permeable for the root cells of *Arabidopsis thaliana*. A second observation relates to the effects of monastrol in cells that were visibly affected and this purports to the fact that chromosome and sister chromatid positioning were more affected than spindle structure itself. Such aberrations were identified during mitotic stages of vigorous chromatin movements including prophase/prometaphase, metaphase and mostly during all stages of anaphase. Finally, monastrol treatment did not affect certain stages of the mitotic cycle, including PPB formation and maturation and telophase/cytokinetic progression.

From the very few existing studies of kinesin roles during mitosis, it can be deduced that different members of this microtubule motor superfamily act at different mitotic stages via distinct mechanisms. ATK5, a kinesin-14, seems to be responsible for the coalignment of microtubule during spindle formation therefore it can be deduced that it is necessary for the maintenance of mitotic spindle shape and integrity and possibly involved in regulation of spindle length (Ambrose and Cyr, 2007). In the same study, time lapsed observation of the mitotic process showed that although the mitotic spindle is visibly affected, the defects observed are not dramatic, while PPB and phragmoplast development are not disturbed further corroborating the absence of kinesin-5 implication in the above processes. A more relevant study, addressed mitotic spindle aberrations in a temperature sensitive kinesin-5 mutant, which shows no phenotype at permissive temperatures. When seedlings are transferred to elevated temperatures, kinesin-5 functions are disturbed resulting in the formation of multipolar, linear or radial mitotic spindles (Bannigan *et al*., 2007; Fig. 28).

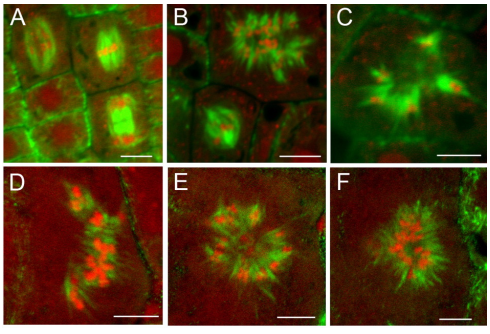

Figure 28. Mitotic spindle defects in *rsw7* mutant at restrictive temperatures. A. Wild type. B, C. Multipolar spindles. D. Linear spindle. E, F. Radial spindles. (Adapted with modifications from Bannigan *et al*., 2007)

In that study phragmoplast formation does not seem to be affected, although its expansion appears abnormal due to the malpositioning of chromosomes at the onset of mitosis. Likewise, PPB formation is also not disturbed. These results are only partially concurring with the pharmacological inhibition presented herein, since such severe defects in mitotic spindle form where very rarely observed after monastrol treatment.

As stated at the beginning of the discussion, it seems that the inhibitor is inadequately loaded within mitotic cells, and this probably results in very low cytoplasmic concentrations of monastrol that are ineffective to elicit a full-scale effect such as observed in mammalian cells treated as such.

However there are consistent defects, mostly related to chromosome and sister chromatid movements during prophase/prometaphase, metaphase and anaphase, suggesting that at the speculated low cytoplasmic concentrations of monastrol such events exhibit the highest sensitivity.

In biological models such as yeast and *Drosophila*, kinesin-5 motors have established roles at least in the congression of chromosomes to the metaphase plate (e.g., Tubman *et al*., 2018). In this case RNAi inhibition of the Klp61F in *Drosophila* S2 cells, inhibited formation of the metaphase plate (Tubman *et al*., 2018). Therefore the effects of monastrol during prophase/prometaphase reveal a conserved role of the inhibitor in chromosome alignment prior to anaphase. Since further segregation of sister chromatids does not involve kinesin-5 actions at the kinetochores it can be deduced that malpositioning of sister chromatids observed after monastrol treatment must be carried over from previous mitotic stages.

The effects of monastrol in the form of the mitotic spindle where not as dramatic as those observed in the *rsw7* mutant (Bannigan *et al*., 2007). This may owe to the inadequate diffusion of the inhibitor to the cytoplasm. Nevertheless, mild mitotic spindle aberrations were present and those mostly related to spindle bipolarity and especially to the focusing of kinetochore bundles at the spindle poles. Additionally defects were observed to the spindle size, whereby visibly affected spindles were more elongated than the control ones.

To summarize the functions of kinesin-5 motors in mitotic spindle assembly and form, they participate in merotelic chromosome spindle attachments (Choi and McCollum, 2012), they control spindle length via their microtubule depolymerase activity (Tubman *et al*., 2018) and they underlie spindle bipolarity by crosslinking and sliding of antiparallel microtubules (Shapira *et al*., 2017). The involvement of kinesin-5 in

spindle pole focusing and in the regulation of microtubule length, seems to justify the effects of monastrol observed herein.

7 CONCLUSION - FUTURE PERSPECTIVES

This is the first study reporting the effects of an allosteric kinesin-5 inhibitor, monastrol, in the mitotic progression of higher plants exemplified by the model *Arabidopsis thaliana*. Monastrol induced mild but consistent defects in mitotic spindle assembly and chromosome movements in dividing root tip cells of *Arabidopsis thaliana*. Such defects were partially concurrent to genetic depletion of kinesin-5 motors and similar although milder compared to defects observed in mammalian cells treated with the same inhibitor. In order to fully understand the function of kinesin-5 further studies will be necessary and such studies may be facilitated by either studying more thoroughly the existing *rsw7* mutant or by implementing alternative kinesin-5 inhibitors, including the ATP-competitive FCPT (Groen *et al*., 2008), Dimethylenastron (Sun *et al*., 2014), Trityl-L-cysteine (Skoufias *et al*., 2006) etc., which might have better permeability to the *Arabidopsis* root cells.

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9 LIST OF ABBREVIATIONS

+TIPS	plus end tracking proteins
ATP	adenosine triphosphate
ADP	adenosine diphosphate
BSA	bovine serum albumin
CDZ	cortical division zone
DAPI	4',6-diamidino-2-phenylindole
dH_2O	distilled water
DMSO	dimethyl sulfoxide
EGTA	ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid
GDP	guanosine diphosphate
GTP	guanosine triphosphate
MAP65	microtubule associated proteins 65
MES	2-(N-morpholino)ethanesulfonic acid buffer
MILI-Q H_2O	mili-Q water
MTSB	modified tryptone broth
MS	Murashige Skoog
PBS	phosphatase buffered saline
PIPES	1,4-Piperazinediethanesulfonic acid
PPB	preprophase microtubule band