The Enigma of Tubulin Detyrosination

Functional Evidence from Plants

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von

Dipl.-Biologin Natalie Schneider

aus

Freiburg i. Br.

Dekan: Prof. Dr. Peter Roesky

Referent: Prof. Dr. Peter Nick

Korreferent: Prof. Dr. Reinhard Fischer

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“Curiosity is always the first step to resolving a problem.”

Galileo Galilei

Die vorliegende Dissertation wurde am Botanischen Institut des Karlsruher Instituts für Technologie (KIT) von März 2011 bis Januar 2015 angefertigt.

Hiermit erkläre ich, dass ich die vorliegende Dissertation, abgesehen von der Benutzung der angegebenen Hilfsmittel, selbständig verfasst habe.

Alle Stellen, die gemäß Wortlaut oder Inhalt aus anderen Arbeiten entnommen sind, wurden durch Angabe der Quelle als Entlehnungen kenntlich gemacht.

Diese Dissertation liegt in gleicher oder ähnlicher Form keiner anderen Prüfungsbehörde vor.

Karlsruhe,

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Ich möchte mich zunächst bei Professor Peter Nick für die Ermöglichung der Bearbeitung dieses Themas an seinem Institut sowie für das in mich gesetzte Vertrauen bedanken.

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Zusammenfassung

Mikrotubuli üben als ein zentrales Element des eukaryotischen Cytoskeletts viele verschiedene Funktionen aus. Dabei reicht die Bandbreite nicht nur von struktureller Beschaffenheit wie zum Beispiel der Bildung der Mitose Spindel als auch der Regulation der Zellexpansion, sondern darüber hinaus, umfassen die Funktionen auch die Reizwahrnehmung, zum Beispiel der Schwerkraft.

Um all diese diversen Funktionen ausüben zu können, müssen Mikrotubuli markiert oder von spezifischen Proteinen erkannt werden. Daher ist eine Regulation über Mikrotubuli assoziierte Proteine essentiell. Diese assoziierten Proteine binden Mikrotubuli mit unterschiedlicher Affinität und haben dadurch einen direkten Einfluss auf die Dynamik der Mikrotubuli, weshalb sie in stabilisierende und destabilisierende assoziierte Proteine unterteilt werden können. Die Bindung an Mikrotubuli wird durch so genannte posttranslationale Modifikationen gesteuert, welche die Diversität innerhalb der Mikrotubuli erhöhen. Viele verschiedene Modifikationen sind bekannt (Hammond et al., 2008), eine bedeutende Modifikation ist dabei am C-Terminus des alpha-Tubulins lokalisiert: Die Detyrosinierung.

Ein konserviertes Tyrosin, welches als letzte Aminosäure am C-Terminus gelegen ist, wird dabei von einer bislang noch nicht identifizierten Tubulin-Tyrosin Carboxypeptidase (TTC) entfernt und kann durch eine Tubulin-Tyrosin Ligase (TTL) wieder angefügt werden. Die Detyrosinierung des alpha-Tubulins ist bei allen Eukaryoten hochkonserviert. Die biologische Funktion in Pflanzen bleibt jedoch rätselhaft. Als mögliche Ziele der Detyrosinierung wurden Kinesine vorgeschlagen. Des Weiteren weisen auch Proteine wie EB1, welche am Plus-Ende der Mikrotubuli aufzufinden sind, eine veränderte Affinität gegenüber detyrosinierten Mikrotubuli auf.

Um einen Einblick in die physiologische Funktion der Tubulin Detyrosinierung zu erhalten, wurden im Verlauf dieser Arbeit Reispflanzen untersucht, wobei neben dem Wildtyp (Oryza sativa L. ssp. japonica cv. Dongjin) ebenso Reispflanzen untersucht wurden, die das Protein TTL überexprimieren. Neben Längenmessungen wurde auch der Wurzelaufbau erforscht. Des Weiteren wurden die Wurzeln der Reiskeimlinge auf ihren Gravitropismus überprüft.

Die Auswirkungen einer TTL Überexpression auf Reiskeimlinge reichten von einer verzögerten gravitropen Antwort, verändertem Blattwinkel und einer reduzierten Anzahl an Seitenwurzeln bis zu einer verkürzten Wurzel bzw. Ligula sowie einem verlängertem Mesokotyl.

Da all diese Mechanismen in Zusammenhang mit Mikrotubuli stehen, stellte sich die Frage, ob es möglich wäre, das Ganze *in vitro* nachzustellen. Die Erkenntnis, dass Zellelongation aufgrund einer TTL Überexpression durch eine veränderte Mikrotubulidynamik gestört werden kann, konnte *in vitro* bestätigt werden.

Eine Verschiebung der Tubulingehalte hin zu tyrosinierten oder detyrosinerten Mikrotubuli führte zu einer drastischen Verlängerung der Mikrotubuli im Vergleich zu einer Kontrollpolymerisation. Es konnte ebenfalls gezeigt werden, dass das Verhalten von dem hinzugefügten Protein EB1 verändert war.

Als Schlussfolgerung liefert diese Dissertation ein Arbeitsmodell, in welchem das richtige Gleichgewicht zwischen tyrosinierten und detyrosinierten Mikrotubuli für zelluläre Prozesse wie zum Beispiel Zellelongation essentiell ist. Des Weiteren wurden neue Einblicke in den Zusammenhang zwischen Detyrosinierung und Stabilität gegeben.

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Abstract

Microtubules (MTs), a central component of the eukaryotic cytoskeleton, convey numerous different functions. These functions range from structural quality like the establishment of the mitotic spindle as well as the regulation of cell expansion, but furthermore, they can also be related to sensing, such as the sensing of gravity.

For all these kinds of different functions microtubules need to be marked or recognized by different proteins. Therefore, regulation which is performed via microtubule associated proteins is essential. The associated proteins are binding to microtubules with different affinity and have a direct influence on microtubule dynamics so that they can be classified into stabilizing and destabilizing associated proteins. Binding to microtubules is controlled by so called posttranslational modifications that increase the diversity of microtubules. Numerous different modifications are known (Hammond et al., 2008); one important modification is located at the C-terminus of alpha-tubulin: Detyrosination.

A conserved C-terminal tyrosine is removed by a still unidentified tubulin-tyrosine carboxypeptidase (TTC) and can be religated by a tubulin-tyrosine ligase (TTL). Detyrosination of α-tubulin seems to be conserved over all eukaryotes. However, its biological function in plants has remained obscure. As possible targets for the detyrosination signal, kinesin motors have been proposed and also plus end tracking proteins like EB1 show a diverse binding affinity towards detyrosinated microtubules.

In order to get insight into the physiological function of the tubulin detyrosination cycle I was working with rice plants; beside wild type rice plants (Oryza sativa L. ssp. japonica cv. Dongjin) I was using rice plants overexpressing TTL in this work. Next to length measurements root architecture was analyzed. Furthermore gravitropic response of rice seedlings was analyzed.

The effects of TTL overexpression on rice seedlings are ranging from delayed gravitropic response, altered gravitropic set point angle and less lateral roots to reduced ligule, reduced root and increased mesocotyl length.

Since all these mechanisms refer to microtubules the question arose if it is possible to simulate these conditions *in vitro*. The findings that cell elongation is disrupted by altering microtubule dynamics due to TTL overexpression could be confirmed *in vitro*.

Shifting tubulin contents towards tyrosinated or detyrosinated microtubules led to a significant increase in microtubule length compared to control polymerization. Furthermore it could be shown that the binding property of EB1 is altered.

As a conclusion this dissertation is providing a working model by which the right equilibrium of tyrosinated and detyrosinated microtubules is essential for cellular processes like cell elongation. Furthermore, new insights into the correlation between microtubule detyrosination and stability were shown.

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List of Abbreviations

+TIP	plus end tracking protein
ATP	adenosine triphosphate
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EB1	end binding protein 1
GA_3	gibberellic acid
GSA	gravitropic set point angle
GTP / GDP	guanosine triphosphate / guanosine diphosphate
KCH	kinesin with calponin homology
MAP	microtubule associated protein
MT	microtubules
PMSF	phenylmethylsulfonyl fluoride
PTM	posttranslational modification
TTC	tubulin-tyrosine carboxypeptidase
TTL	tubulin-tyrosinyl ligase
TTLox	TTL overexpressor
tyr / detyr	tyrosinated / detyrosinated

1. Introduction

Research in the field of cytoskeleton has revolutionized cell biology and biomedicine (Ramaekers & Bosman, 2004). Abnormalities of the cytoskeleton account for many diseases for example neurodegenerations like Alzheimer Disease (McMurray, 2000) or cancer invasion (Ramaekers & Bosman, 2004).

Many of these diseases are caused by abnormities of microtubules, an important component of the cytoskeleton. These abnormities may be linked with tubulin itself, or indirectly, with changes of their regulation system. Both aspects have been subject to evolutionary adaptation, and therefore the microtubular cytoskeleton differs conspicuously, if different organisms are compared. For instance, the control plant development and morphogenesis in plants is brought about by substantially different microtubular structures that are not found in animals, leading to the question, which molecular factors underlie these specific functions. To understand, which of these factors are relevant, requires a short overview about the cytoskeleton in general and the specificities of the plant cytoskeleton, in particular.

1.1. The Cytoskeleton – Not as static as indicated by its name

While animals extended their surface towards the inside and thus were able to move and avoid disadvantageous environmental conditions, plants had to extend their surface outwards to ensure photosynthesis, but thus being forced to a sessile lifestyle.

Therefore plants require different supporting mechanisms or structures than animals, where the endo- or exoskeleton is adapted to movement; in contrast plants needed an adaptation assuring a better fixation which could be reached for example via lignification and development of a mechanical support - the vascular bundle as the central element of telomes (Zimmermann, 1965).

Despite these diverse structures of external support both plant and animal cells possess an intracellular network, the cytoskeleton, which conveys many functions, such as maintaining cell shape, structuring signal pathways or driving intracellular transport.

There are three main components of the eukaryotic cytoskeleton – actin filaments, microtubules and intermediate filaments. However, intermediate filaments seem to be absent from plants. In recent years a fourth component of the cytoskeleton was identified – the septins.

Actin, a polymer consisting of monomeric G-Actin, shows a defined polarity and is highly conserved although different actin isoforms exist. F-Actin, its filamentous state, is

polymerized in interwined helical filaments. In contrast to tubulin, actin harbors an ATP binding site (Dominguez & Holmes, 2011; Mostowy & Cossart, 2012).

Intermediate filaments are sized in-between actin filaments and microtubules, which is the reason for their name. Unlike actin filaments or microtubules, intermediate filaments can be built out of different monomers and are non-polar (Mostowy & Cossart, 2012).

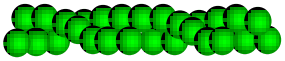
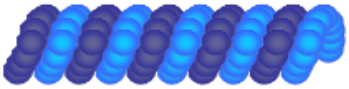
	Structure	Size	Subunit
Actin filaments		5-7 nm	actin
Intermediate filaments		8-12 nm	lamin, keratin, and others
Microtubules		25 nm	α-tubulin β-tubulin
Septins		5 nm	septin

Figure 1. Cytoskeletal components and their subunits. Figure according to Mostowy & Cossart, 2012 and Campbell & Reece, 2003.

Septins are GTP-binding proteins and can form hetero-oligomeric complexes and structures like filaments or rings. Similar to intermediate filaments they are non-polar (Mostowy & Cossart, 2012), and so far have not been detected in plants (Estey *et al.*, 2011; Ong *et al.*, 2014).

Microtubules, a central component of the eukaryotic cytoskeleton, are also central for this work and will be presented in more detail in the following chapter.

1.2. Microtubules – just hollow tubes or is there more?

Microtubules (MTs) convey numerous different functions. These functions can be of structural quality divided into two groups, such as: the establishment of the mitotic spindle, as well as the regulation of cell expansion by control of cell-wall texture are of a structural character (Nick, 2014). However, they can also be related to sensing, such as the sensing of gravity, the response to osmotic stress, or the mechanic integration of plant tissues (Nick, 2013).

1.2.1. Microtubular structure and their functions

Heterodimers composed of α- and β-tubulin are the building blocks of microtubules and can polymerize when GTP is bound. These heterodimers form a so called protofilament, 13 of

these in turn constitute the microtubule. Due to the tubular assembly of microtubules, a high mechanical stability is ensured. While α-tubulin exhibits a GTP binding site, β-tubulin possesses a GDP binding site. When polymerized, GTP is hydrolyzed to GDP. The GDP rich basal region of a microtubule is not as stable as the microtubule tip, where the GTP bound state dominates.

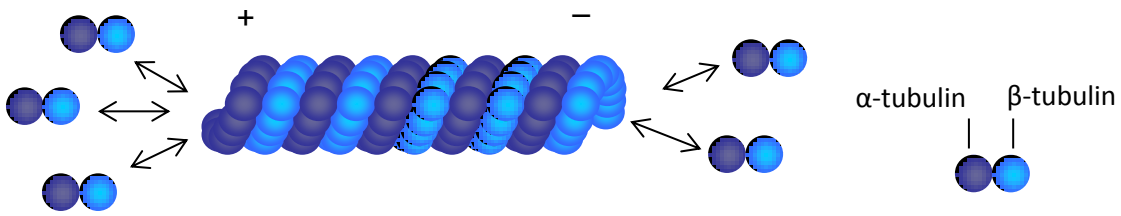


Figure 2. Schematic representation of a microtubule. Growth and shrinkage can occur on plus- and minus end of the microtubule but the plus-end is preferred.

This polarity, which is also mirrored in the alignment of the heterodimers, also underlies the polarity of the polymer with minus- and plus-end of the microtubule and also controls their characteristic dynamics. Microtubules are highly dynamic, on both sides assembly and disassembly can occur, whereby the plus end undergoes quick growth and shrinkage (Ambrose & Wasteneys, 2014), whereas the shielded minus end is protected from depolymerization (Goodwin & Vale, 2010). The velocity of this assembly and disassembly and therefore the lifespan of a single microtubule differs between different microtubules, which delineates stable and dynamic subpopulations of microtubules that can be bound and regulated by specific microtubule associated proteins (which will be described below).

At last, microtubule dynamics are complemented by a characteristic so called dynamic instability. Phases of polymerization and therefore elongation of microtubules are interrupted by sudden depolymerizations. The starting points of these depolymerization events are called “catastrophe”, the term “rescue” defines the start of a new polymerization phase.

General functions of microtubules include spindle formation and chromosome separation during mitosis, as well organelle- or vesicle trafficking, and signal transduction. Specifically for plant cells, microtubules control cell expansion through control of cell-wall formation. They also are involved in cold resistance and pathogen defense in plants. In animals, the microtubules are involved in cell movements such as the beating of cilia or flagella, as well as the growth of axons (Lodish *et al.*, 2001). In addition, microtubules act as sensors for gravitational and mechanical forces (Nick, 2008). In plants microtubules undertake some special tasks that will be mentioned separately in a later chapter.

For all kind of different functions microtubules need to be marked or recognized by different proteins. But how are these different functions regulated? One possibility is that there are different isoforms; another possibility is that microtubules are modified in different ways and

therefore bind associated proteins in a different manner. In the next two chapters the question will be, what defines these microtubule associated proteins and how can the microtubules be modified.

1.2.2. How are all different microtubular functions controlled? – Regulation of microtubules via microtubule associated proteins (MAPs)

Microtubule associated proteins (MAPs) can be distinguished into different groups and are often involved in signaling. All MAPs have a microtubule binding site at their C-terminus in common, whereas the N-terminus can bind vesicles, other microtubules or organelles. They are defined by their ability to bind microtubules *in vivo* (Morejohn, 1994; Nick *et al.*, 1995).

The associated proteins have a direct influence on microtubule dynamics and can be classified into stabilizing and destabilizing associated proteins. Examples for stabilizing MAPs are CLIP 170, Tau or EB1, whereas Katanin can be characterized as a destabilizing protein.

End binding protein 1 (EB1) belongs to the group of plus-end tracking proteins (+TIPs). +TIPs play an important role in microtubule dynamics. While they can catalyze addition of tubulin to the plus end, they are also able to induce “catastrophe” (Akhmanova & Steinmetz, 2010). End binding proteins (EB proteins) are a central component of +TIPs due to tracking growing microtubule plus ends autonomously (Akhmanova & Steinmetz, 2010; Bieling *et al.*, 2008) and targeting other +TIPs to microtubule plus ends (Akhmanova & Steinmetz, 2008). EB1, for example, can promote microtubule dynamics and growth, and suppress catastrophes in cells (Tirnauer *et al.*, 2002).

Motor proteins represent a special group of microtubule associated proteins and play a role in vesicle transport. Driving force of movement is a secession of bound ATP, leading to conformational changes. In animal cells there are two different motor proteins belonging to the group of microtubule associated proteins. On the one hand kinesins, that are moving from minus- to plus-end of a microtubule, and on the other hand dyneins, moving in the opposite direction. Because plants do not have dyneins except for spermatozoons from *Gingko biloba*, other kinesins have to be responsible for vesicle transport to the minus-end.

Within plants kinesins, so called KCH, were identified that are moving towards the minus end of the microtubules. This plant-specific member of the kinesin-14 family links microtubules and actin filaments via their calponin homology domain, and occurs in two subsets that confer two different cellular functions (Klotz & Nick, 2012) – one subset is linked with the perinuclear actin basket, and important for premitotic nuclear migration and mitosis (Frey *et al.*, 2010). The second subset is not linked to actin filaments and moves slowly with cortical microtubules and thus plays a role for cell expansion. In addition to the orientation of the cell

plate (Hiwatashi *et al.*, 2008), the premitotic positioning of the nucleus (Frey *et al.*, 2010) and a sensory microtubule function at the plasma membrane (Kühn *et al.*, 2013) have been shown to depend on these specific kinesin motors.

But how do these proteins associate to specific microtubules. In fact, microtubules are modified by so called posttranslational modifications that increase the diversity of microtubules. In the following chapters focus will be laid on these mechanisms.

1.2.3. Are all microtubules really the same?- Posttranslational modifications can increase functional diversity

Whereas the building blocks of microtubules, α- and β-tubulin, are mostly encoded by single genes in algae, they form small gene families in higher land plants (including the Bryophytes) (i.e. angiosperms). Their products, however, are relatively conserved in terms of protein sequence. Already in the mosses, the difference between the individual genes is mostly confined to the regulatory regions (Jost *et al.*, 2004) indicating that it was a regulatory rather than functional diversification that drove the formation of these gene families. In fact, microtubules were found to be composed of different isotypes (Hussey *et al.*, 1987). Thus leading to the question, how microtubules can be assigned to functionally different arrays.

MTs can be modified by so called posttranslational modifications (PTM), which seem to lead to an increase in functional diversity in the highly conserved microtubule population and also can influence the stability and structure of MTs. Numerous different modifications are known (Hammond *et al.*, 2008) and have been proposed to act as a code for functional assignment that is read out by different binding proteins (Parrotta *et al.*, 2014). Due to its external position in the microtubule, binding of regulators and other proteins occurs at the C-terminus. Thus being convenient for modifications leading to higher functional diversity and heterogeneity of tubulin (Xia *et al.*, 2000). In fact, changes of cyclic detyrosination/tyrosination as most prominent PTM has been shown to correlate with the reorientation of cortical microtubules, a key regulator of axial cell expansion in plants (Wiesler *et al.*, 2002).

Most PTMs occur on microtubules rather than on unpolymerized tubulin and it is known that stable microtubules, as compared to dynamic microtubules, accumulate more modifications (Hammond *et al.*, 2008).

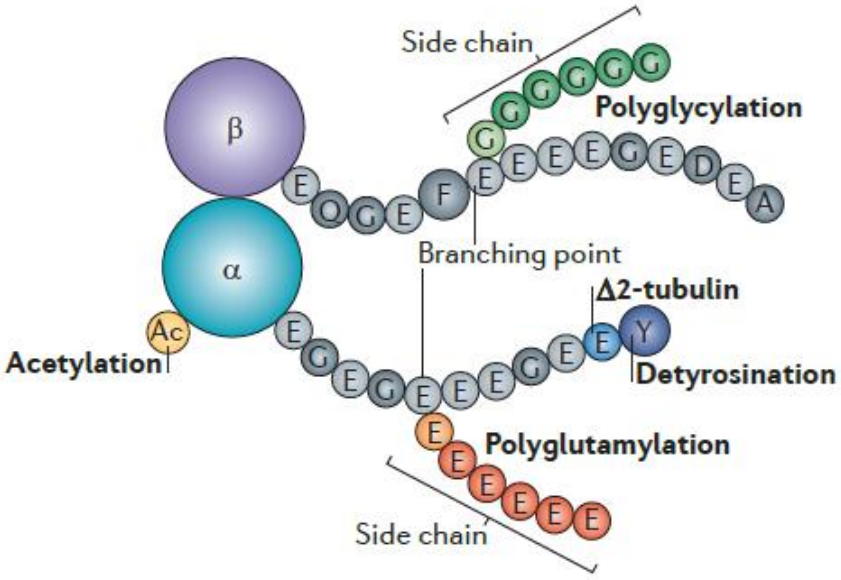


Figure 3. Schematic representation of posttranslational modifications on alpha- and beta-tubulin. The localization of diverse modifications is shown (Janke & Bulinski, 2011).

Polyglutamylation as well as polyglycylation are often summarized as polymodification, both occurring at the C-terminal region of α- or β-tubulin and being reversible (Hammond *et al.*, 2008). They function in labeling specific microtubules and thereby influencing microtubule dynamics via changing binding ability of associated proteins. Polyglutamylation for example is often found on mitotic spindle microtubules (Janke *et al.*, 2005).

Acetylation of microtubules as another posttranslational modification is special in more than one way. Next to microtubules only histones are targets for acetylation. Moreover the modification is performed, for example, at lysine 40 of the α-tubulin, which is inwards of the microtubule (Hammond *et al.*, 2008). In the last years more acetylation sites were found not only in α-tubulin but also β-tubulin (Janke & Bulinski, 2011).

Besides tubulin modifications can be linked to the regulation of microtubule dynamics via establishing a code on microtubules (Janke & Kneussel, 2010; Wloga & Gaertig, 2010).

Another important modification also involved in microtubule dynamics is located at the C-terminus of alpha-tubulin: Detyrosination. Its function is still elusive.

1.2.4. Removement of a single amino acid and its influences on a whole cell – Detyrosination

The α-tubulin of almost all eukaryotic organisms harbors a C-terminal tyrosine that can be cleaved off by a tubulin-tyrosine carboxypeptidase (TTC) (MacRae, 1997). The antagonist of this TTC is a tubulin-tyrosinyl ligase (TTL), which can religate a tyrosine to detyrosinated tubulin. While the TTL is preferentially using tubulin dimers as a substrate (Burns, 1987),

detyrosination is mainly targeted to the polymerized form (Gundersen *et al.*, 1987). *In vivo*, both, detyrosinated and tyrosinated tubulins coexist, whereby under normal conditions the tyrosinated form dominates under normal conditions (Gundersen *et al.*, 1987). The conservation of the C-terminal tyrosine throughout the eukaryotes indicates that this cycle is highly conserved (Erck *et al.*, 2005). However, little is known about its function.

Removement of tyrosine de facto influences microtubules (Peris *et al.*, 2009), visible for example in the fact that detyrosinated microtubules are more resistant to higher concentrations of MT-depolymerizing substances like nocodazol (Kreis, 1987).

In animals detyrosination cycle plays a crucial part in neural organization and generation of axons. Therefore activity of TTL is the strongest in the brain (Erck *et al.*, 2005). Also tumor genesis can be influenced by detyrosination. In tumor cells the abundance of tyrosinated tubulin is decreased, and suppression of TTL activity promotes tumor aggressiveness (Lafanechere *et al.*, 1998). Pharmacological inhibition of TTC activity represents an important target in the screening of anticancer drugs. Such inhibitors, by restoring the equilibrium between tyrosinated and detyrosinated α-tubulin, are expected to be endowed with anti-tumor activity. In fact, the sesquiterpene parthenolide, isolated from *Tanacetum parthenium* (Feverfew, Asteraceae) has been isolated as TTC inhibitor during a search for anti-cancerogenic substances (Fonrose *et al.*, 2007).

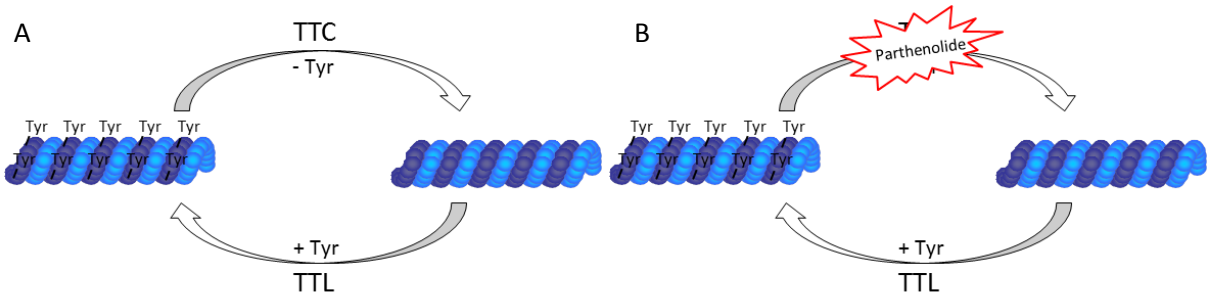


Figure 4. (A) Schematic representation of the reversible detyrosination and retyrosination cycle. (B) Schematic representation of the shifting towards tyrosinated tubulin after addition of parthenolide.

Although the TTL has been purified and cloned from porcine brain (Ersfeld *et al.*, 1993), the enzyme responsible for the TTC activity has remained enigmatic so far.

As possible targets for the detyrosination signal, kinesin motors have been proposed. However, it seems to depend on the specific type of kinesin, whether detyrosination acts as recruiting signal (Liao & Gundersen, 1998; Parrotta *et al.*, 2014; Zekert & Fischer, 2009). Some kinesins are not affected by detyrosination of their target (Zekert & Fischer, 2009), and some kinesins, such as the mammalian Mitotic Centromere-Associated Kinesin (MCAK) is even inhibited by detyrosination (Peris *et al.*, 2009).

Stable microtubules are mostly detyrosinated, leading to the hypothesis that detyrosination induces stability. This is not true in all details. Like mentioned above detyrosination is mainly targeted to the polymerized form of microtubules, therefore giving the TTC longer time in stable microtubules to remove the tyrosine (Burns, 1987). But stability and detyrosination are forming an amplifying loop. There are examples for both – detyrosination and tyrosination – and their influences on stability. If tyrosine is removed the affinity of depolymerizing motors (for example MCAK) is reduced (Peris *et al.*, 2009) and therefore stable microtubules are promoted (de Forges *et al.*, 2012). On the other side, the presence of tyrosine is important for the binding of CLIP170 (Peris *et al.*, 2006), stabilizing the plus end of the microtubule.

Detyrosination can be further edited by removing the glutamine (next amino acid after C-terminal tyrosine). This leads to the formation of Δ2-tubulin, which is considered to be irreversible and could function in stabilizing microtubules via locking them in a detyrosinated state (Janke & Bulinski, 2011).

1.3. Special edition – What makes plants unique?

Now after this general overview about microtubules and their regulation I would like to emphasize on how plants differ or rather on what is special in plants referred to microtubules.

First, plant cells differ in terms of their growth from animal cells, because they expand due to water absorption. Owing to the resulting undirected pressure a cell wall is needed to obtain cell shape. Already in 1948 Ziegenspeck recognized that the direction of cellulose is crucial for cell wall shape (Ziegenspeck, 1948), and Paul Green suspected one year before microtubules were discovered by Ledbetter and Porter (Ledbetter & Porter, 1963) that microtubules co-determine the orientation of the cellulose (Green, 1962). Two theories of the microtubule-microfibril-hypothesis resulted in this discovery both explaining the possible influence of microtubules on cellulose synthesis. On the one hand the “monorail”-model where cellulose-synthase moves along the cortical microtubules, on the other hand the “guardrail”-model with cellulose-synthase in between microtubules, acting like a fence and keeping cellulose-synthase in line. Due to simultaneous visualization of CFP-labeled tubulin and YFP-labeled cellulose synthase Paradez could confirm the “monorail”-model (Paradez *et al.*, 2006a).

Thus, by microtubule orientation the direction of cellulose microfibrils gets determined. The cell gets support, by which it can only grow in one direction namely in length and not in width. Because of this unisotropic growth, regulated by microtubules, plants can react to diverse environmental signals in their entirety. This reaction to stimuli like light or gravitation is specific (Himmelspach et al., 1999).

Gravitropism – the reaction towards gravitation – is a second characteristic of plants. Unlike animals plants have to cope with different conditions due to their sessile lifestyle.

The starch-statolith theory indicates that amyloplasts are responsible for gravisusception (Haberlandt, 1900; Nemec, 1900). Graviperception in contrast is based on pressure, which led to the hypothesis that microtubules are involved in this mechanism (Nick, 2008).

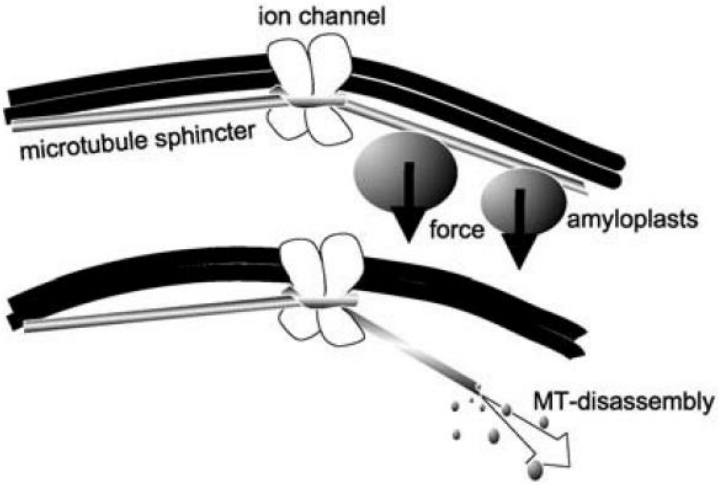


Figure 5. Model of a microtubule as primary gravity sensor (Nick, 2008). If microtubules sense a force induced by gravity, they dissemble leading towards transmission and reaction of the stimulus. Furthermore it could be that ion channels, kept closed via microtubules, are involved.

De facto, by disturbing microtubules via antimicrotubular drugs gravitropism can be blocked (Godbolé *et al.*, 2000) and gravitropic response can be even strongly inhibited when microtubule dynamics is reduced (Nick *et al.*, 1997). Treatment with acrylamide, for example, interrupts gravisensing upstream of auxin redistribution and differential growth (Gutjahr & Nick, 2006; Nick, 2008). Auxin redistribution (stimulus transmission) leads to a change in microtubule orientation (reaction towards stimulus) resulting in differential growth.

But not only must a plant react to changes in gravity. Plants also use the permanent stimulus in obtaining their morphology. The gravitropic set point angle (GSA) defines the angle of each specific plant organ towards the gravity vector. This angle can be controlled developmentally or via environmental factors (Digby & Firn, 1995), light, for example, can modify the GSA via phytochromes (Digby & Firn, 2002).

1.4. Aim of the work

What is the role of tubulin detyrosination? Answering this question is not trivial. The conservation of the C-terminal tyrosine throughout the eukaryotes indicates that this cycle is highly conserved. However, little is known about its function and here most of what is already known was examined in animal cells. In animals detyrosination cycle plays a crucial part in

neural organization and generation of axons that is why the activity of TTL is the strongest in the brain (Erck *et al.*, 2005). Also tumor genesis can be influenced by detyrosination. In tumor cells, for instance, the level of TTL and therefore also the content of tyrosinated tubulin is reduced (Hammond *et al.*, 2008). Suppression of TTL activity leads to an increase in tumor aggressiveness (Lafanechere *et al.*, 1998).

But what does this cycle mean for plant cells? Not much is known about the function of tubulin detyrosination in plants. In order to answer the question of the general function of tubulin detyrosination in plants, it would be meaningful to analyze the impact of involved enzymes separately. Since this has not been analyzed in plants so far and in order to get insight into the physiological function of the tubulin detyrosination cycle, I was working with rice plants; beside wild type rice plants (Oryza sativa L. ssp. japonica cv. Dongjin) I was using rice plants overexpressing TTL in this work.

What are the advantages of working with rice plants instead of using cell cultures for example tobacco BY-2? We have also a TTL overexpressing tobacco BY-2 cell line (for details see 2.1.2) but since the sequence of TTL is out of rice (*Oryza sativa*), working in a homogenous system is an advantage. Furthermore, plants will probably react differently compared to cell cultures, which will give insight into the physiological function of the cycle.

In addition the differences in plant cells towards animal cells in relation to growth and their environmental perception were highlighted. Following questions arise from the latter:

- ☞ Is tubulin detyrosination involved in the regulation of microtubule dynamics?
- ☞ Does the phenotype of TTL-overexpressor rice plant differ from wild type?
- ☞ What does this in total mean for function of detyrosination in plants?

In order to answer these questions I concentrated on the one hand on plant growth in different plant organs, so to speak if there is a change in growth when tubulin detyrosination is affected. On the other hand I examined if overexpression of TTL and therefore also changing in tubulin detyrosination is influencing gravitropic response. Both, growth and gravitropic response, are dependent on microtubule organization and microtubule dynamics.

Examination of plant organs was performed in rice seedlings of wild type rice plants (Oryza sativa L. ssp. japonica cv. Dongjin) and TTL overexpressor rice plants. Next to length measurements root architecture was analyzed. Furthermore rice seedlings were grown rotated by 90° in order to check influence of tubulin detyrosination on gravitropism in roots.

In a further approach since all these problems refer to microtubules the question arose if it is possible to simulate the conditions in a cell *in vitro*. To test this, a part of the field of synthetic biology was used. But what is synthetic biology? Per definition it is a “Discipline (that is)

orientated to the intentional design, modeling, construction, debugging and testing of artificial living systems" (Thomas Knight, M.I.T., Cambridge, USA) and its goal is to combine the disciplines "Life sciences" and "Engineering sciences" in order to create biological systems that do not exist in nature in that way. There are several strategies in this field. But what does this mean for this work?

Here I established an *in vitro* system for MT-polymerization. Advantage of such an *in vitro* system is that you can add only those elements that are necessary without interconnection with other proteins that are present in a cell. In this case I could analyze the reaction towards microtubule polymerization by addition of proteins to the necessary elements tubulin and GTP. Examination of following problems was performed:

- In what way does EB1 (end binding protein 1) affect microtubule polymerization *in vitro*?
- What impact does addition of detyrosinated or tyrosinated tubulin have on microtubule polymerization?
- When combinations of these proteins are added – Do the polymerized microtubules differ from microtubules with single protein additions?

2. Materials and Methods

2.1. Plant material

2.1.1. Rice material

Name	Genotype	Application
Dongjin	*Oryza sativa* L. ssp. *japonica* cv. Dongjin, wild type	Phenotyping
TTLox	*Oryza sativa* L. ssp. *japonica* cv. Dongjin, TTLox	Phenotyping

Table 1. Overview of rice lines

Figure 6. 12 weeks old rice plants. *Oryza sativa ssp. japonica* cv. Dongjin, wild type (left) and *Oryza sativa ssp. japonica* cv. Dongjin, TTLox (right). Scale bar = 10 cm

2.1.2. Tobacco cell culture

Name	Genotype	Application	Source
BY-2 TTL-RFPox	*Nicotiana tabacum* L. cv. Bright Yellow 2, OsTTL-RFPox	EPC sepharose, *in-vitro* polymerization	Jovanović et al.

Table 2. Overview of tobacco cell culture

Tobacco cell culture *Nicotiana tabacum* L. cv. Bright Yellow 2, OsTTL-RFPox was cultivated at 26°C in liquid media containing 4.3 g/l Murashige Skoog salt (Duchefa Biochemie, Haarlem, the Netherlands), 30 g/l sucrose, 200 mg/l KH_2PO_4, 100 mg/l (myo)-inositol, 1 mg/l thiamin und 0.2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), pH 5.8 (KOH) on an orbital

shaker (KS 260 basic, IKA, Staufen, Germany) at 150 rpm. Before weekly subcultivation of the cell culture 50 mg/l hygromycin (Roth, Karlsruhe, Germany) was added to the media.

In *Nicotiana tabacum* L. cv. Bright Yellow 2, OsTTL-RFPox the protein Tyrosinyl Tubulin Ligase (TTL) out of rice (*Oryza sativa*) is overexpressed. Therefore, the TTL is N-terminally coupled to a RFP sequence. The SwissProt number for osTTL-RFPox is Q10QY4.

2.2. Antibodies

All antibodies were purchased from Sigma-Aldrich (München, Germany).

	Name	Description	Reference
Primary Antibodies	ATT (TUB-1A2)	Mouse monoclonal antibody targeting tyrosinated α-tubulin epitope sequence: VEGEGEEEGEEY	Kreis (1987)
	DM1A	Mouse monoclonal antibody targeting α-tubulin epitope sequence: DMAALEK	Breitling et al. (1986), Blose et al. (1984)
Secondary Antibodies	Anti-mouse IgG, FITC-conjugated	Goat FITC-coupled, polyclonal antibody targeting mouse IgG	
	Anti-mouse IgG, alkaline phosphatase conjugated	Goat alkaline phosphatase coupled, polyclonal antibody targeting mouse IgG	

Table 3. Overview of used antibodies

2.3. Phenotyping methods

2.3.1. Rice sterilization

The dehusked seeds were covered with ethanol (70% v/v) and incubated for one minute. After discarding the ethanol (Roth, Karlsruhe, Germany) the seeds were washed two times with water before they were sterilized in 6% (v/v) sodium hypochlorite (Roth, Karlsruhe, Germany) for 20 minutes while gently shaking. Before sowing, the seeds were washed again four times with autoclaved water under the clean bench.

2.3.2. Measuring mesocotyl length

Sterilized seeds were sown into magenta boxes containing 0.4% (w/v) phytoagar (Duchefa Biochemie, Haarlem, the Netherlands). Depending on the type of experiment the phytoagar was either without further addition or supplemented with 0.5 µM or 1 µM gibberellic acid (GA_3; Fluka, Taufkirchen, Germany), respectively. Rice seedlings were grown in darkness for seven days. Measuring of the mesocotyl length was performed via the length measurement tool of ImageJ (NIH, Bethesda, USA). In addition, coleoptile length was analyzed as well.

2.3.3. Measuring gravitropic response in rice seedlings

Sterilized seeds were sown into square petri dishes containing for one half 1.3% (w/v) phytoagar (Duchefa Biochemie, Haarlem, the Netherlands) and grown at 25°C under continuous white light (20 µmol/m^2s) for four days. Before starting the experiment plates were oriented in a 90° angle towards the bottom. Pictures were taken at following different time points: 0, 30, 45, 60, 75, 90, 105, 120, 135, 165, 195, 225, 255, 285, 315, 375 minutes, and after 24 hours. Measuring of the gravitropic response of the roots was performed via the angle measurement tool of ImageJ.

2.3.4. Measuring root growth

Sterilized seeds were sown into magenta boxes containing 0.4% (w/v) phytoagar and grown under light conditions (continuous white light, 20 µmol/m^2s) for five days. The root length was analyzed using the length measurement tool of ImageJ. In a second experimental setup sterilized seeds were sown into square petri dishes containing for one half 1.3% (w/v) phytoagar and grown under light conditions for seven days. Each day the root length was marked on the plate. After seven days the root length was analyzed using the length measurement tool of ImageJ.

2.3.5. Measuring leaf angle

Sterilized seeds were sown into magenta boxes containing 0.4% (w/v) phytoagar and grown under light conditions (continuous white light, 20 µmol/m^2s) for twelve or eighteen days, respectively. The leaf angle of the first leaf of the rice seedling was analyzed via the angle measurement tool of ImageJ (NIH, Bethesda, USA).

2.3.6. Measuring ligule length

Sterilized seeds were sown into magenta boxes containing 0.4% (w/v) phytoagar (Duchefa Biochemie, Haarlem, the Netherlands) and grown under light conditions (continuous white light, 20 µmol/m^2s) for 14 days. Afterwards rice seedlings were transferred into sand and grown at 28°C. After additional five weeks ligule length was measured.

2.3.7. Counting number of lateral roots

Sterilized seeds were sown into magenta boxes containing 0.4% (w/v) phytoagar (Duchefa Biochemie, Haarlem, the Netherlands) and grown under light conditions (continuous white light, 20 µmol/m^2s) for 5, 6 or 7 days. Lateral roots were counted using a binocular microscope (Leica S6d; Leica Microsystems, Wetzlar, Germany).

2.4. Cell biological methods

2.4.1. Microtubule staining of the rice mesocotyl

Rice seedlings were grown in darkness for seven days. Before cutting sections out of the mesocotyl with a razor blade the mesocotyl was fixed for 60 minutes in 3.7% (w/v) paraformaldehyde (PFA; Sigma-Aldrich, München, Germany) in microtubule-stabilizing buffer (MSB; 50 mM PIPES, 5 mM EGTA, 1 mM $MgSO_4$, 1% w/v glycerol, 0.1% v/v Triton-X100, pH 6.9) at room temperature. The sections were then collected in MSB and washed three times for 10 minutes with MSB on a coverslip. To block unspecific interactions the sections were transferred in 5% (w/v) bovine serum albumin (BSA; Sigma-Aldrich, München, Germany) in Tris-buffered saline (TBS; 150 mM NaCl, 20 mM Tris-HCl, 0.25% v/v Triton-X100, pH 7.4) for 20 minutes. Thereafter the sections were incubated with the primary antibody ATT in TBS (1:400) for 2-4 hours at 4°C. Following another three washing steps in TBS each for 5 minutes, the sections were incubated over night at 4°C with the secondary antibody FITC in TBS (1:250). On the next day the sections were washed five times thoroughly in TBS for five minutes. For microscopy the sections were transferred in MSB and confocal z-stacks were recorded using a AxioObserver Z1 (Zeiss, Jena, Germany) inverted microscope equipped with a laser dual spinning disk scan head from Yokogawa (Yokogawa CSU-X1 Spinning Disk Unit, Yokogawa Electric Corporation, Tokyo, Japan), a cooled digital CCD camera (AxioCam MRm; Zeiss) and two laser lines (488 and 561nm, Zeiss, Jena, Germany) attached to the spinning disk confocal scan head. Images were taken using a Plan-Apochromat 63x/1.44 DIC oil objective operated via the Zen 2012 (Zeiss, Jena, Germany) software platform and analyzed using the angle measurement tool of the AxioVision Software 4.8 (Zeiss, Jena, Germany).

2.5. Biochemical methods

2.5.1. Sodiumdodecylsulfate polyacrylamide gel electrophoresis (SDS-Page)

Separation of proteins by size was carried out using discontinuous SDS-polyacrylamide gels (Laemmli, 1970) consisting of stacking and separation gels.

Gels were run in a Atto mini Page system (Atto, Tokyo, Japan) containing electrophoresis buffer (25 mM Tris, 192 mM glycin, 0.15% w/v SDS) closing the current circuit at 25 mA per gel over 90 minutes. Samples supplemented with sample loading buffer (30% v/v glycerol, 300 mM dithiothreitol (DTT), 6% w/v SDS, 48% v/v stacking gel buffer (Stac GB), 0.01% w/v bromphenol blue) were denatured by heat at 95°C for 10 minutes and loaded onto the gels instantly.

Component	Separation gel (10%)	Stacking gel (4%)
Acrylamid/Bisacrylamid (30:1)	5.3 ml	1 ml
Sepa GB (1.5 M Tris/HCl, 0.6% w/v SDS, pH 8.8)	4 ml	-
Stac GB (0.5 M Tris/HCl, 0.6% w/v SDS, pH 6.8)	-	1.8 ml
dd H_2O	6.7 ml	4.7 ml
Temed	70 µl	40 µl
APS (10%)	140 µl	80 µl

Table 4. Composition of a SDS-polyacrylamide gel. The amount is sufficient for three gels.

In order to evaluate the size of the proteins a protein marker (SDS6H2, Sigma-Aldrich, München, Germany; see table 5) was run as well.

Protein	Source	Approx. Molecular Mass (Da)
Myosin	Porcine	200.000
β-Galactosidase	*E. coli*	116.000
Phosphorylase	Rabbit muscle	97.000
Albumin	Bovine	66.000
Albumin (Ovalbumin)	Chicken egg white	45.000
Carbonic anhydrase	Bovine erythrocytes	29.000

Table 5. Composition of protein marker SDS6H2 (Sigma-Aldrich, München, Germany) used in SDS-Page

To visualize the proteins the gels were stained immediately in Coomassie staining solution (0.04% w/v Brilliant Blue R, 40% v/v methanol, 10% v/v acetic acid) for 60 minutes and afterwards destained in 30% [v/v] ethanol supplemented with 10% [v/v] acetic acid. The destaining solution was renewed several times until sufficient destaining. Gels were stored in between watered cellophane foils which were dried for two days. Gels used for western blotting were run with a prestained marker (P7709v, New England Biolabs, Frankfurt, Germany; see table 6).

Protein	Source	Approx. Molecular Mass (Da)
MBP-β-galactosidase	*E.coli*	175.000
MBP -paramyosin	*E.coli*	80.000
MBP-CBD	*E.coli*	58.000
Aldolase	Rabbit muscle	46.000
Triosephosphate isomerase	*E.coli*	30.000
CBD-BmFKBP13	*E.coli*	25.000
Lysozyme	Chicken egg white	17.000
Aprotinin	Bovine lung	7.000

Table 6. Composition of prestained protein marker P7709v (New England Biolabs, Frankfurt, Germany) used for western blotting

2.5.2. Western blotting

Western blotting was performed using a semi-dry transfer system (Trans-Blot SD cell, Bio-Rad, München, Germany). The experimental set-up from anode onwards was built in the following way: Two layers of blotting paper soaked in transfer buffer (1.4% w/v glycine, 1.2% w/v Tris, 20% v/v methanol) were covered with a polyvinylidene fluoride (PVDF) membrane (Roth, Karlsruhe, Germany), which was activated in methanol, next the protein gel, and finally again two layers of blotting paper. Blotting occurred at a constant current of 100 mA per gel for 60 minutes. Subsequent to blotting the membrane was dried and afterwards incubated overnight with the primary antibodies ATT or DM1A, respectively. After three washing steps over 15 minutes each with TBST-buffer (20 mM Tris/HCl, 150 mM NaCl, 10% Triton, pH 7.4) to remove unspecific bound primary antibodies, the membrane was incubated for two hours with the secondary antibody anti-mouse IgG conjugated with alkaline phosphatase. Before signal development the membrane was washed twice with TBST-buffer for five minutes to remove the secondary antibody. Signal development was performed with following steps: The membrane was incubated for 15 minutes in staining buffer (100 mM Tris/HCl, 100 mM NaCl, pH 9.7) freshly supplemented with 1:10 v/v magnesium stock (500 mM $MgCl_2$) followed by incubation in developer buffer (66 µl nitrobluetetrazolium (NBT); 75 mg/ml in 75% [v/v] dimethylformamide (Roche, Mannheim, Germany) and 33 µl 5-bromo-4-chloro-3-indoxylphosphate-p-tuloidin (BCIP); 50 mg/ml in 75% [v/v] dimethylformamide (Roche, Mannheim, Germany) in 5 ml staining buffer with 1:10 v/v magnesium stock solution) until bands appeared. The signal development was stopped by washing the membrane with water.

2.5.3. Tubulin purification out of pig brain

The purification of pig brain tubulin took place in two cycles of polymerization and depolymerization. Pig brains were freshly collected at the local slaughterhouse and directly put on ice. Connective tissue and blood vessels were removed and subsequently 700 g brains were shredded with 700 ml buffer A (100mM PIPES, 2 mM EGTA, 1 mM $MgSO_4$, 100 µM ATP, 1 mM DTT, pH 6.9) in a blender (Type 708A, Krups, Offenbach, Germany). After clarifying at 13.000 rpm at 4°C for 70 minutes (Sorvall RC2-B; Sorvall Inc., Newtown CT, USA), the tubulin-containing supernatant was collected. After addition of 25% glycerol and 2 mM adenosine triphosphate (ATP; Sigma-Aldrich, München, Germany) it was incubated in a waterbath at 35°C for 45 minutes while gently shaking letting the tubulin polymerize. Polymerized tubulin was harvested by centrifugation for 50 minutes at 40.000 rpm in a rotor 45Ti (Beckman L8-70M; Beckman Coulter GmbH, Krefeld, Germany) at 32°C. Depolymerization was achieved by using a dounce tissue grinder (Bruno Kummer GmbH, Freiburg i.Br., Germany) and incubating the tubulin with an appropriate volume of buffer C

(100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, 1 mM ATP, 1 mM DTT, pH 6.94) for 25 minutes on ice. Before further polymerization in the waterbath (35°C, gently shaking) for 30 minutes, the homogenized tubulin was centrifuged in a TLA 100.2 rotor (Beckman TL100, 53.000 rpm, 10 minutes, 4°C) and 2 mM ATP was added to the supernatant. The second depolymerization step was fulfilled after centrifugation for 19 minutes at 49.000 rpm and 35°C by using a dounce tissue grinder and an appropriate volume of buffer D (100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, 50 mM ATP, 1 mM DTT, pH 6.94) on ice for 25 minutes. The tubulin was clarified by centrifuging (53.000 rpm, 10 minutes, 4°C, TLA 100.2 rotor). Till further experiments the tubulin was stored in the -80°C freezer.

2.5.4. ATTO488-labeling of tubulin

1 ml tubulin (10 mg/ml) was thawn, 1 mM GTP (Roth, Karlsruhe, Germany) was added, and incubated for 5 minutes on ice. To support polymerization, DMSO (Roth, Karlsruhe, Germany) in a final volume of 10% (v/v) was added. After polymerization for 45 minutes at 37°C the polymer was layered on an adequate volume high pH cushion (100 mM NaHEPES, 1 mM $MgCl_2$, 1 mM EGTA, 60% v/v glycerol, pH 8.6) and centrifuged for 6 minutes at 80.000 rpm and 25°C in a TLA 100.2 rotor (Beckman TL100; Beckman Coulter GmbH, Krefeld, Germany). Following the removement of the cushion the sediment was resuspended in 1 ml labeling buffer (100 mM NaHEPES, 1 mM $MgCl_2$, 1 mM EGTA, 40% v/v glycerol, pH 8.6) and supplemented with 10 µl ATTO488 (Atto-Tec, Siegen, Germany). Thereby it had to be ensured to keep microtubules polymerized during the labeling process which was carried out during incubation for 60 minutes at 37°C in a waterbath. Labeling was completed by addition of the same volume Quench (100 mM potassium glutamate, 40% v/v glycerol in 2x BRB80 (160 mM PIPES, 2 mM EGTA, 2 mM $MgSO_4$, pH 6.9) and incubation for another 5 minutes at 37°C. To guarantee the functionality of the labeled tubulin several cycles of depolymerization and polymerization were performed after labeling. Therefore, polymer was layered on low pH cushion (60% v/v glycerol in BRB80 (80 mM PIPES, 1 mM EGTA, 1 mM MgSO4, pH 6.9)) and after centrifugation (80.000 rpm, 10 minutes, 25°C, TLA 100.2) the supernatant was removed and the sediment was resuspended in 1 ml ice-cold IB buffer (50 mM potassium glutamate, 500 µM $MgCl_2$, pH 7.0). To promote depolymerization incubation for 30 minutes on ice was supported using a dounce tissue grinder. Following an additional centrifugation step (80.000 rpm, 10 minutes, 2°C, TLA 100.2) 5x BRB80 (resulting in a final concentration of 1x) and 1 mM GTP was added and incubated for 3 minutes on ice. The mixture was supplemented with 33% (v/v) glycerol and polymerized for another 60 minutes at 37°C in a waterbath. Before centrifugation for 15 minutes at 80.000 rpm and 25°C the polymer was layered on low pH cushion. Afterwards the sediment was resuspended in an adequate volume (50-100 µl) IB buffer and incubated for 25 minutes on ice. In a final step the mixture

was again centrifuged for 10 minutes at 80.000 rpm and 2°C. Till further experiments the labeled tubulin was stored as small aliquots in the -80°C freezer.

2.5.5. EB1 protein expression and purification

The expression plasmid pET28b was transferred into the *E. coli* strain ER2566. 100 ml LB medium (Roth, Karlsruhe, Germany) supplemented with 50 µg/ml kanamycin (Roth, Karlsruhe, Germany) was inoculated with *E. coli* ER2566 containing the expression vector pET28b. Cells were grown until an OD_{600} of 1 was gained. Afterwards 900 ml LB medium supplemented with kanamycin were added and cells were grown at 37°C until an OD_{600} of 0.35 was reached. Expression was then induced using 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG, AppliChem, Darmstadt, Germany). After incubation of the cells for five hours at 37°C while constantly shaking, cells were harvested via centrifugation at 5000 rpm for 10 minutes (Sorvall RC-2). The sediment was washed in ground buffer (50 mM Tris, 5 mM EDTA, 300 mM NaCl, pH 7.8) and after another centrifugation for 10 minutes at 5000 rpm and 4°C resuspended in ground buffer with 10% (v/v) glycerol (Roth, Karlsruhe, Germany). Extraction of EB1 was performed using a French Press (Aminco french pressure cell press (French & Milner, 1955)). For removing of cell debris a centrifugation for 30 minutes at 11.000 rpm and 4°C (Sorvall RC-2.) was carried out. In order to concentrate the protein solution an ammonium sulfate precipitation was performed followed by a centrifugation step (11.000 rpm, 30 minutes, 4°C). Afterwards the sediment was resuspended in ground buffer without EDTA closing with an additional centrifugation step (11.000 rpm, 20 minutes, 4°C). The supernatant was then transferred on an affinity column of 25 ml Ni^{2+}-NTA agarose matrix, equilibrated with washing buffer (50 mM Tris, 10 mM imidazole, 300 mM NaCl, pH 7.8). For elution a 2.5x higher concentration of imidazole was used (50 mM Tris, 250 mM imidazole, 300 mM NaCl, pH 7.8). Fractions were selected and E280, referring to protein content, was determined. A second ammonium sulfate precipitation was carried out ending with two further centrifugation steps (30 minutes, 4°C, first 11.000 rpm, secondly 15.000 rpm), in between the sediment was resuspended in ground buffer. To secure that the protein EB1 is in the supernatant a SDS-Page was performed including samples from different time points of the extraction.

2.5.6. EPC-Sepharose

Tyrosinated and detyrosinated tubulin can be distinguished by utilizing their different binding affinity to EPC (Ethyl N-phenylcarbamate) (Wiesler *et al.*, 2002). In order to answer the question if the polymerizing ability changes due to different amounts of detyrosinated or tyrosinated tubulin in the *in vitro*-polymerization assay, a tubulin extract was run in an EPC affinity chromatography. Therefore several different steps were needed.

Production of a soluble tubulin extract from BY-2 tobacco cells

Three days-old cell cultures were harvested via centrifugation (3000 rpm, 10 min), the supernatant was removed, and the sediment was resuspended in the same volume extraction buffer (25 mM MES, 5 mM EGTA, 5 mM $MgCl_2$, pH 6.9) supplemented with 1 mM DTT (Roth, Karlsruhe, Germany) and 1 mM PMSF (Sigma-Aldrich, München, Germany). Extraction was performed on ice using a dounce tissue grinder. In order to remove cell debris a centrifugation step at 13.000 rpm for 5 minutes was carried out followed by an ultracentrifugation in a TLA100.2 rotor (50.000 rpm for 15 minutes at 4°C). Until further usage the supernatant was stored at -80°C.

Production of EPC-sepharose

For production of EPC-sepharose carboxy-EPC is linked to aminoethyl-sepharose. In order to make carboxy-EPC 20 g 3-aminobenzoic acid was dissolved in 250 ml 1N NaOH and pH was adjusted to 7.0. 25 g ethylchlorocarbonate were added under constantly stirring. During this exothermic reaction a carbamate bond is formed. N-carboxyl-ethyl N-phenylcarbamate (N-carboxyl-EPC) precipitated and was filtrated. Aminobenzoic acid which did not participate in the reaction is hardly soluble in ethanol. Therefore ethanol is used for crystalline transformation of N-carboxyl-EPC, which is dissolved in 250 ml absolute ethanol, filtrated and then stored under a fume hood until the formation of crystals.

Aminoethyl-sepharose was produced referring to (Cuatrecasas, 1970)). 15 g CNBr-activated sepharose 4B was soaked in 50 ml 1 N HCl. After addition of 75 ml binding buffer (100 mM $NaHCO_3$, 500 mM NaCl, pH 8.3) supplemented with 100 mM ethylendiamine it was incubated overnight at 4°C in an overhead rotator followed by several washing steps. First, the sepharose was washed for two hours at room temperature in washing buffer (100 mM Tris/HCl, pH 8.0), afterwards three alternating washing steps with washing buffer with salt (500 mM NaCl) and acid washing buffer (100 mM sodium acetate, 500 mM NaCl, pH 2) were performed.

Finally, 1 g N-carboxyl-EPC was dissolved in 50 ml absolute ethanol, mixed with 50 ml aminoethyl-sepharose and 150 mg carbodiimid was added. Linking occurred via incubating in an overhead rotator for three days at room temperature. Different washing steps were attached afterwards starting with washing with water, followed by washing with ethanol (96%) and closing with a washing step with 1 M NaCl. In the end EPC-sepharose was stored in 3 M KCl supplemented with 0.1% sodium azide (Mizuno *et al.*, 1985).

Ethyl N-phenylcarbamate (EPC) affinity chromatography

EPC affinity chromatography was performed at 4°C. At first EPC-sepharose was washed with extraction buffer (25 mM MES, 5 mM EGTA, 5 mM $MgCl_2$, 1 M glycerol, pH 6.9) to

remove salt of the storage buffer. One volume of the EPC-sepharose was mixed with the soluble tubulin extract and incubated in an overhead rotator for one hour to promote binding of tubulin to the EPC-sepharose. Afterwards the mixture was transferred to the column and washed with extraction buffer. The flow-through was collected and elution of proteins occurred with KCl in ascending order of concentrations starting with 0.05 M KCl, eluting tyrosinated tubulin and ending with 3 M KCl eluting detyrosinated tubulin. One part of the fractions was concentrated via ammonium sulfate precipitation and checked using SDS-page and western blotting.

2.5.7. *In vitro*-polymerization of microtubules

6 µl unlabeled and 2 µl labeled tubulin (10 mg/ml) were mixed together with 0.4 µl GTP (100 mM). To start polymerization the reaction tube was incubated at 37°C for 45 minutes. Depending on the type of experiment 2 µl of different protein solutions (1.5 mg/ml; EB1, tyrosinated and detyrosinated tubulin, BSA) were added before polymerization. The polymer was diluted in a mixture of BRB80 (80 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, pH 6.9) and 100 µM taxol (Sigma-Aldrich, München, Germany) prior to microscopy. Images were taken using an AxioImager.Z1 Apotome microscope (Zeiss, Jena, Germany) with a 63x objective and digital image acquisition controlled by the AxioVision Software 4.8 (Zeiss, Jena, Germany). Length of polymerized microtubules was measured using the length measuring tool of AxioVision Software 4.8 (Zeiss, Jena, Germany).

3. Results

What is the role of detyrosination? Answering this question is not trivial. The α-tubulin of almost all eukaryotic organisms harbors a C-terminal tyrosine that can be cleaved off by a tubulin-tyrosine carboxypeptidase (TTC) (MacRae, 1997). The antagonist of this TTC is a tubulin-tyrosinyl ligase (TTL), which can religate a tyrosine to detyrosinated tubulin. The conservation of the C-terminal tyrosine throughout the eukaryotes indicates that this cycle is highly conserved. However, little is known about its function and here most of what is already known was examined in animal cells. But what does this cycle mean for plant cells?

In order to get a clue to answer this question in the first part of my work I took a closer look on the phenotype of rice seedlings and rice plants that are overexpressing TTL. Does the phenotype change due to this overexpression? Special focus was placed on plant growth (here especially root growth, mesocotyl growth and ligule growth) and furthermore focus was placed on the influence of TTL overexpression on gravitropic response of the rice plants.

Especially examination of mesocotyl growth and particularly staining of the cortical microtubules showed that TTL overexpression influences microtubule structure. To examine these observations more closely in a second approach I established an *in vitro* system for MT-polymerization. Advantage of such an *in vitro* system is that you can add only those elements that are necessary for the polymerization like tubulin and GTP but also add those proteins or protein solutions in a defined way that you want to analyze without interconnection with other proteins. In this work I took a closer look on the influence of EB1 (end binding protein 1), detyrosinated tubulin, tyrosinated tubulin or combinations of these on microtubule polymerization.

3.1. Overexpression of TTL and its influence on gravitropic response

Gravity defines life on earth. But unlike animals plants have to cope with this environmental condition in another way due to their sessile lifestyle. As gravity is a constant stimulus plant organs of each species respond in an evolutionary defined way and plants must be capable to react quickly to any change of gravitation in order to survive.

In the next two chapters different gravitropic responses were analyzed. While in the first part the gravitropic response in reference to time was examined, the second part refers to the gravitropic set point angle (GSA).

3.1.1. Overexpression of TTL in rice leads to a delayed gravitropic response

Dependent on changing the angle of the roots towards the ground plants are susceptible for this stimulus and are adapting their growth towards the gravity vector. Following perception of the stimulus in the root tip it is transmitted towards the elongation zone where the actual growth occurs.

Especially the early events are important when dealing with changed gravity conditions. To see if there is a difference in the gravitropic response between wild type and the TTL overexpressor line four days old rice seedlings were tested on their gravitropic response. Therefore the angles of eight rice seedlings each were measured in three independent experiments.

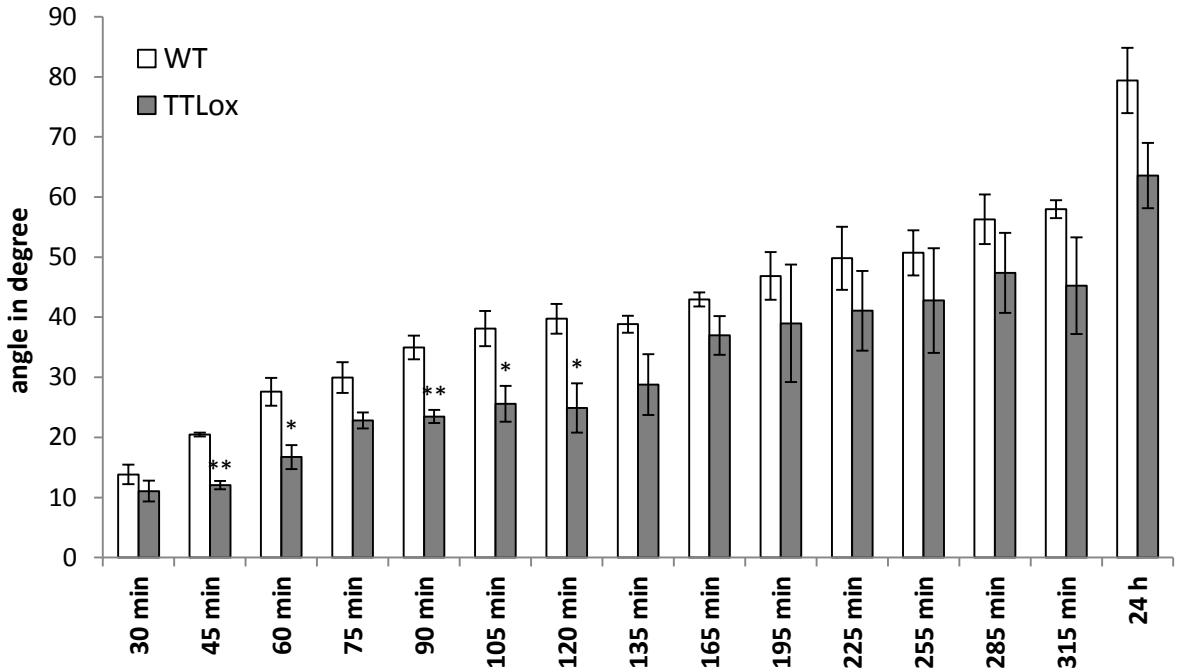


Figure 7. Gravitropic response of wild type (WT, white) and TTL overexpressor (TTLox, grey) rice seedlings. Data show mean and standard error from three independent experimental series; in each series eight rice seedlings were analyzed. Statistically significant differences compared to the wild type are illustrated by one or two asterisks (Student's t-test, $p < 0.05$ or $p < 0.01$, respectively).

While the wild type was adapting continuously and a 20° angle was already reached after 45 minutes, the TTL overexpressor line (TTLox) was reorienting more slowly. To gain the same angle of 20° it took almost 30 minutes longer compared to the overexpressor line. A second difference in the behavior towards a changed gravity between wild type and TTLox was the way both lines were adapting. Whereas the bending of the wild type root occurred continuously it was rather stepwise with constant phases and abrupt increase of bending in

the overexpressor line. This can be seen in Figure 2 after the time points 45 minutes and 135 minutes.

3.1.2. Leaf angle is changed due to TTL overexpression

Like the root, all other plant organs also must respond to gravity. The gravitropic set point angle (GSA) defines the specific way and angle each species bends towards the ground. With regard to leaves it also defines how effective light can be absorbed and used for photosynthesis. The efficiency is higher when leaves are more bent.

For this experimental setup the leaf angles of ten rice seedlings each were measured on three independent time points, 12 days and 18 days after sawing.

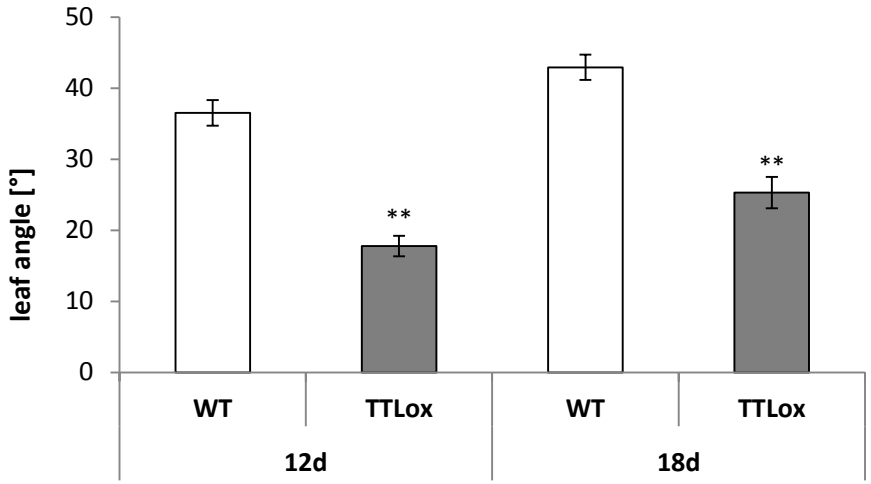


Figure 8. Reduced leaf angle due to TTL overexpression in 12 and 18 days old rice seedlings. Data show mean and standard error from three independent experimental series with 10 wild type (WT, white) and 10 TTL overexpressor (TTLox, grey) rice seedlings. Statistically significant differences to the wild type are illustrated by two asterisks (Student's t-test, $p < 0.01$).

Figure 3 shows that the angle was reduced and differs significantly due to TTL overexpression. In 12 days old rice seedlings the average leaf angle of the TTL overexpressor line was only half the angle compared to the wild type. In 18 days old rice seedlings the leaf angle of TTLox was almost 60% of the wild type angle, but it was still significantly reduced.

3.2. Overexpression of TTL and its influence on growth

Like every organism plants are dependent on growth. In the following chapters the influence of TTL overexpression on growth was analyzed with main focus on different rice organs, namely roots and lateral roots, ligule and mesocotyl.

3.2.1. Reduced root growth in TTL overexpressor rice line

While performing different experimental series it became obvious, that the root of TTLox is clearly shorter than the wild type root. Therefore the roots of 20 rice seedlings were measured in three independent experimental series.

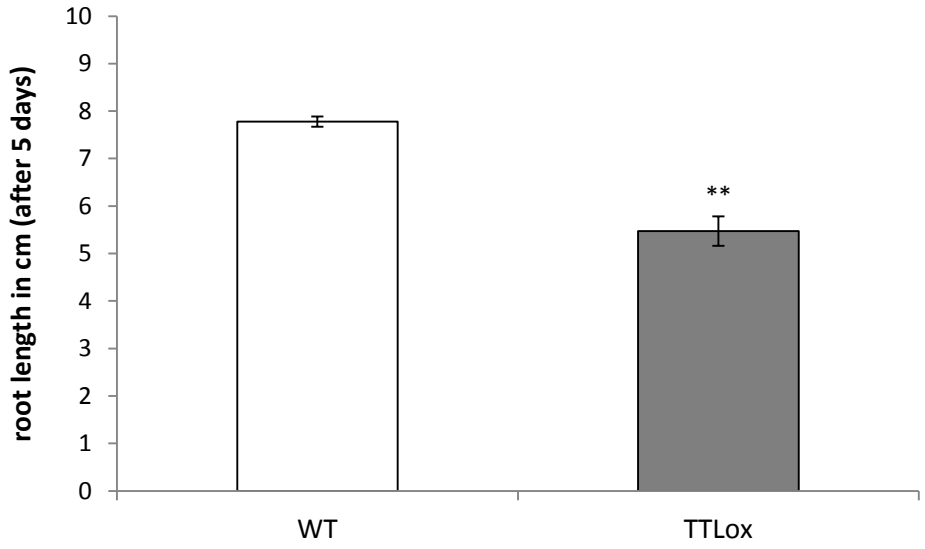


Figure 9. Reduced root length due to TTL overexpression in 5 day old seedlings. Data show mean and standard error from three independent experimental series with 20 wild type (WT, white) and 20 TTL overexpressor (TTLox, grey) rice seedlings. Statistically significant differences to the wild type are illustrated by two asterisks (Student's t-test, $p < 0.01$).

The results confirmed our observation. The root length was reduced at about 30%, which can be seen in Figure 4.

In order to conclude if decrease of growth is a consequence of a shift in growth rate, a time series over 7 days was performed. During this time the increase in root length and therefore also the growth rate was measured daily.

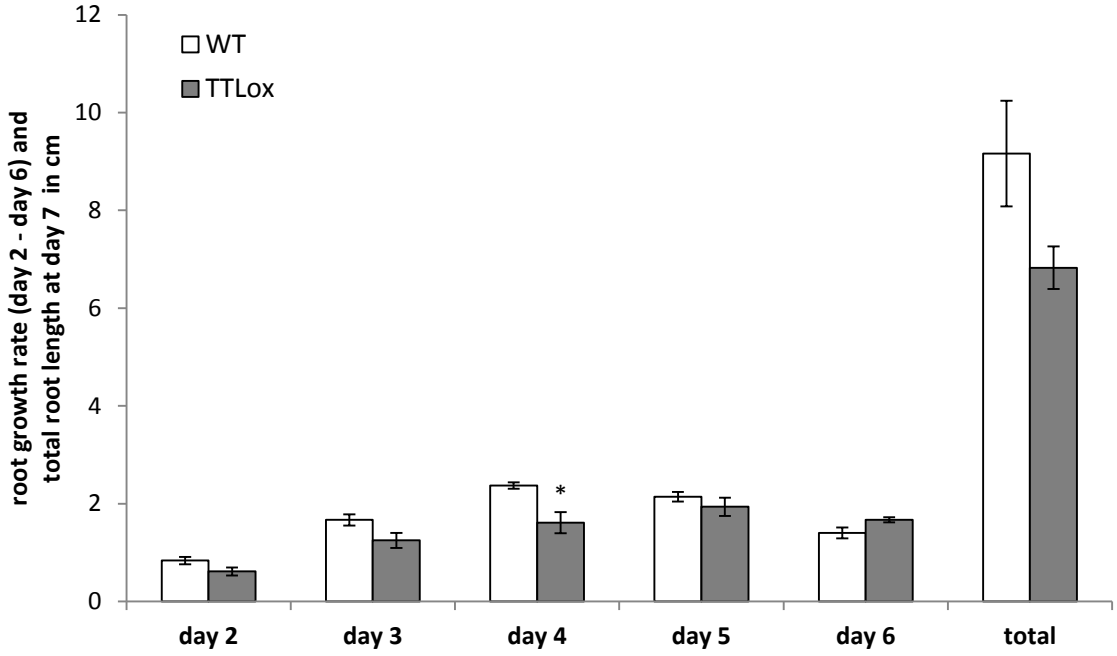


Figure 10. Growth rate of wild type (white) and TTLox (grey) over a one week time period and total root length at day 7. Data show mean and standard error from three independent experimental series. Statistically significant differences to the wild type are illustrated by one asterisk (Student's t-test, $p < 0.05$).

Figure 5 shows the results of three independent time series. The TTL overexpressor line tended to be shorter than the wild type. Especially on day 4 a significant reduced root growth was visible.

3.2.2. Amount of lateral roots of TTL overexpressing rice seedlings is significantly reduced in comparison to the wild type

Lateral roots emerge out of pericycle cells which form a lateral root meristem due to their high dividing activity. These specific pericycle cells are found at the end of xylem rays in the vascular tissue.

Lateral roots were counted using ten rice seedlings at the age of 5 to 7 days which were examined in three independent experimental series using a binocular microscope. The results are shown in Figure 6.

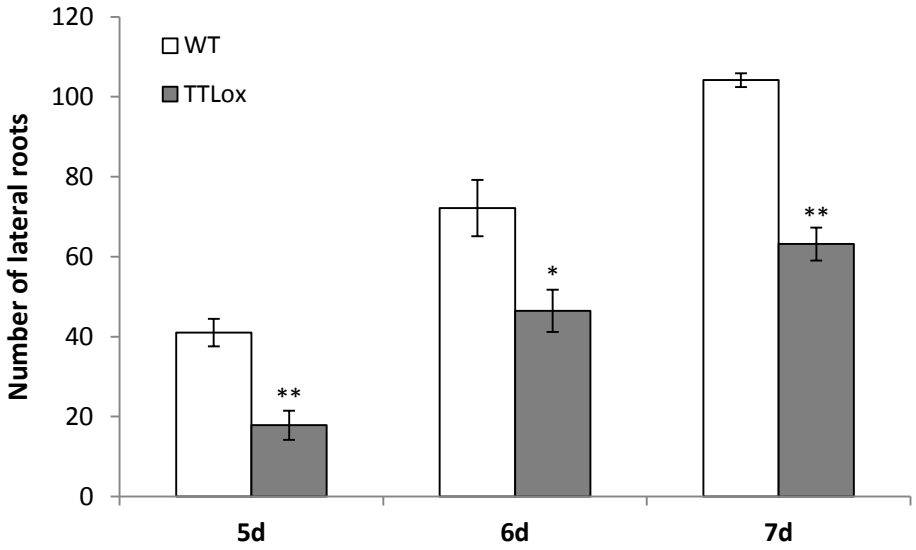


Figure 11. Reduced number of lateral roots due to TTL-overexpressing rice seedlings (grey) compared to wild type seedlings (white). Data show mean and standard error from three independent experimental series (n=10). Statistically significant differences to the wild type are illustrated by one or two asterisks (Student's t-test, $p < 0.05$ or $p < 0.01$, respectively).

The number of lateral roots in the mutant was extensively reduced. While after 5 days TTLox exhibited only 43.5% of lateral roots compared to the wild type, more lateral roots were formed in after 6 and 7 days. Percentage rate of number of lateral roots was increased to almost 60% in these days, but still a significant difference was existent.

3.2.3. TTL overexpression leads to a reduced ligule length

The ligule is often described as a thin outgrowth of the upper epidermis of leafs. It has several functions such as protection from the entry of water, dust or harmful spores. In some species the ligule has an even more active function and plays a role as a secretory tissue (Chaffey, 2000). Unlike membranous ligules of many grass species *Oryza* ligules are vascularized (Chaffey, 1983). But no matter whether membranous or vascularized the ligule design is an important distinctive feature in the family of Poaceae.

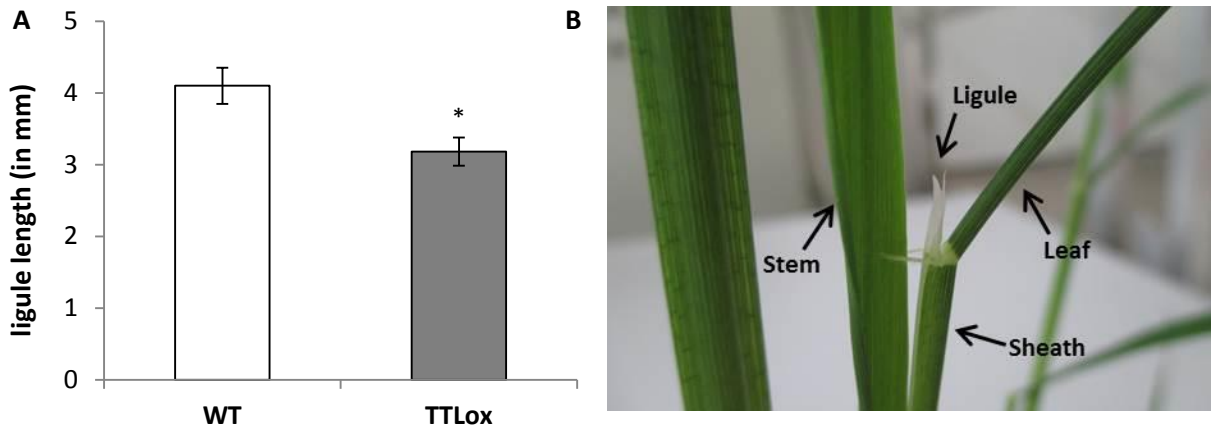


Figure 12. (A) Ligule length in 7 week old wild type (white) and TTLox (grey) rice plants. For wild type as well as TTLox ligules of seven rice plants were measured (n=70). Statistically significant differences to the wild type are illustrated by one asterisk (Student's t-test, $p < 0.05$) (B) Composition of a rice leaf with special focus on the ligule. The ligule can be found between the sheath which surrounds the stem of the rice plant and the leaf which is often also called blade.

Although a distinctive feature the ligule of the TTL overexpressor line was reduced in length for nearly 20% compared to wild type rice plants. The mean ligule length can be seen in Figure 7.

3.2.4. Mesocotyl length can be influenced by TTL overexpression

The region between the root and the crown roots is called mesocotyl. It expands the shoot towards the light when the seed is below ground. Due to this mechanism the mesocotyl is elongated when seedlings are grown in darkness. Often problems with light perceiving can be seen at the length of the mesocotyl.

For the following three independent experimental series nine wild type and TTL overexpressing rice seedlings were grown in darkness for seven days and mesocotyl and coleoptile length was measured.

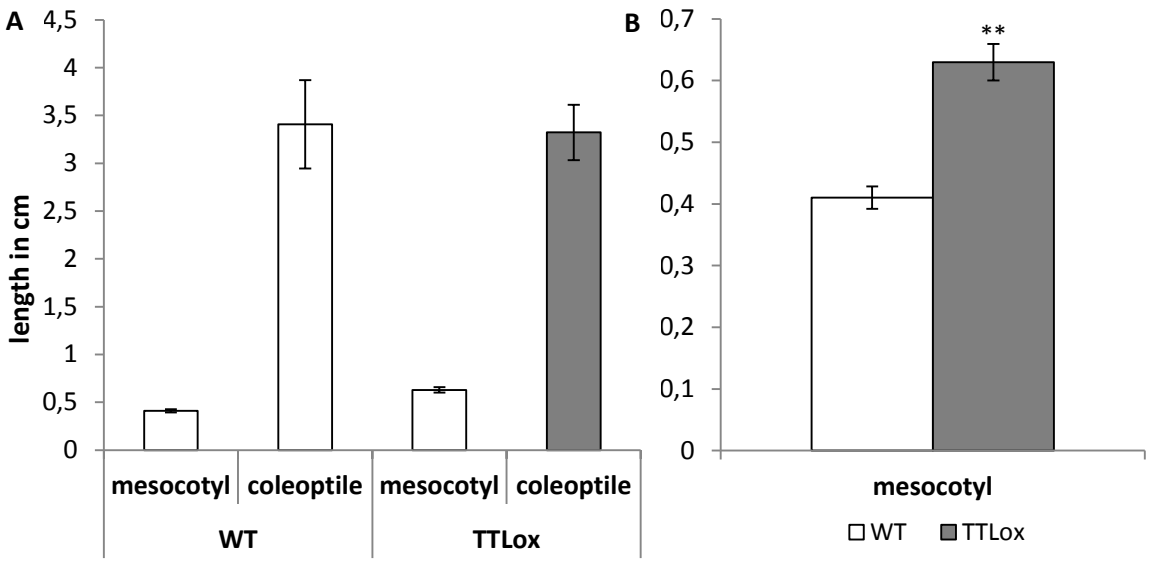


Figure 13. (A) Mesocotyl and coleoptile length in WT (white) and TTLox (grey) rice seedlings. The mesocotyl is enlarged in the mutant, whereas the coleoptile is not altered. Data show mean and standard error from three independent experimental series (n=9). (B) Closer look on mesocotyl length in WT (white) and TTLox (grey) rice seedlings. Data show mean and standard error from three independent experimental series (n=9). Statistically significant differences to the wild type are illustrated by two asterisks (Student's t-test, $p < 0.01$).

Without any further treatment the mesocotyl of the TTL overexpressing rice seedling was elongated. Figure 8 shows that the mesocotyl length of the TTL-overexpressing rice seedling was around 50% increased compared to the wild type. The coleoptile instead was not altered. It was almost the same size in wild type and TTL overexpressing rice seedlings.

Using the phytohormone gibberellic acid (GA_3) the mesocotyl can be enlarged. Gibberellic acid first was isolated out of *Giberella fujikuroi*, a fungus that is attacking rice plants and leading to an abnormal elongation growth. There are various gibberellins in higher plants and their biosynthesis occurs from trans-geranylgeranyl diphosphate. Beside elongation growth gibberellins influence developmental processes like germination, dormancy and flowering. Furthermore gibberellins can increase dividing activity of a cell.

In order to see if the mesocotyl of wild type rice seedlings can gain the length of TTL-overexpressing rice the seedlings were treated with two different concentrations of gibberellic acid (0.5 µM, 1µM) and mesocotyl length was measured (Figure 9). Treatment of TTLox rice seedlings with GA_3 was performed in order to check if mesocotyl growth could be even more increased or if it was limited.

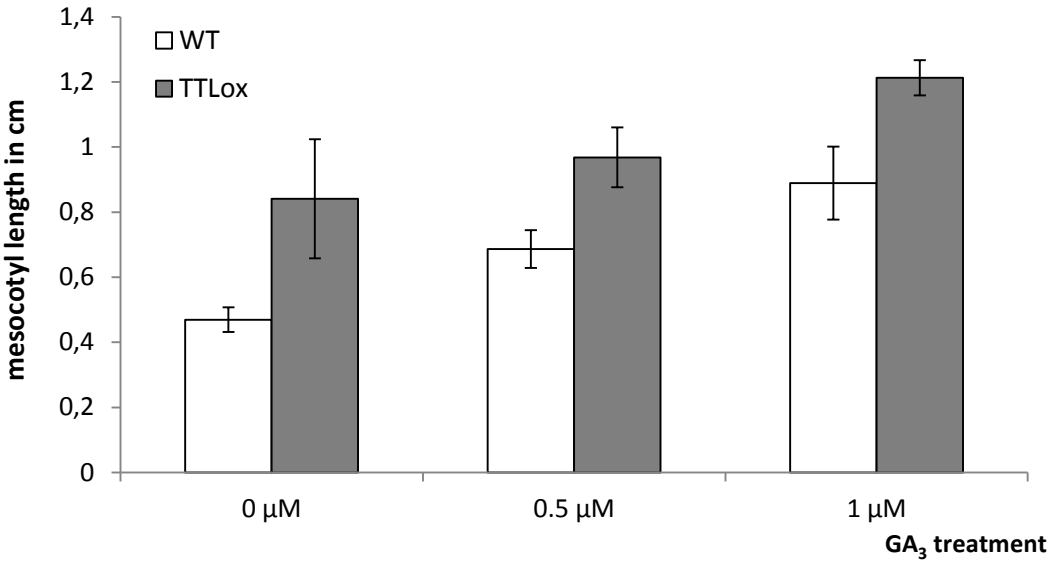


Figure 14. Mesocotyl length in WT (white) and TTLox (grey) rice seedlings after treatment with either 0 µM, 0.5 µM or 1 µM gibberellic acid (GA_3). Data show mean and standard error from three independent experimental series (n=10).

Like in untreated rice seedlings mesocotyl of TTL overexpressing rice was elongated referring to wild type mesocotyl. Table 7 shows the percentaged increase of mesocotyl length. Calculations were done assuming wild type control as 0%.

	WT	**TTLox**
control	-	79%
0.5 µM GA_3	46%	106%
1 µM GA_3	89%	158%

Table 7. Increase of mesocotyl length under gibberellic acid ($GA_{3)}$ treatment. Wild type control was set to 0%.

After treatment with 1 µM gibberellic acid wild type mesocotyl was almost doubled in length (89%). This is roughly the increase (79%) of untreated mesocotyl of TTL overexpressing rice compared to untreated wild type mesocotyl (see Figure 9).

The mesocotyl of TTLox rice seedlings could also be enlarged by gibberellic acid but the increase occurred more slowly. While the mesocotyl of wild type rice seedlings was enlarged at around 50% after treatment with 0.5 µM gibberellic acid compared to the control, the mesocotyl growth in TTLox rice was increased by 15% compared to its control (Figure 10).

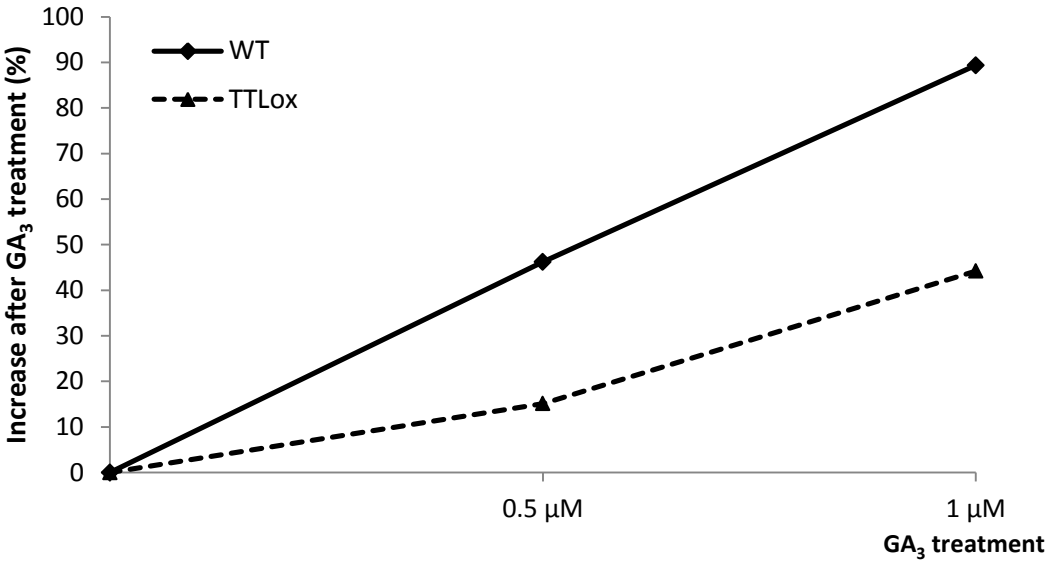


Figure 15. Increase of mesocotyl length of WT (white) and TTLox (grey) rice seedlings under gibberellic acid treatment compared to its control. Each control was set as 0%.

In terms of their growth plant cells have a special feature compared to animal cells. They grow due to water absorption. The resulting pressure is undirected, the plant cell therefore requires a cell wall, which opposes the pressure and maintains the cell shape. Already in 1948 Ziegenspeck recognized that the direction of cellulose is crucial for the shape of the cell wall (Ziegenspeck, 1948), Paul Green suspected that microtubules co-determine the orientation of the cellulose (Green, 1962). The result was the monorail-model, according to which the cellulose-synthase moves along the cortical microtubules, later confirmed by Paradez due to simultaneous visualization of CFP-labeled tubulin and YFP-labeled cellulose synthase (Paradez *et al.*, 2006b). Thus, by microtubule orientation the direction of cellulose microfibrils gets determined. The cell gets a kind of support, by which the cell can only grow in one direction namely in length and not in width.

To examine if the increase of mesocotyl length is a result of different arrangement of cortical microtubules in TTLox mesocotyl cells, microtubules of mesocotyl cells were visualized using immunofluorescence (Figure 11).

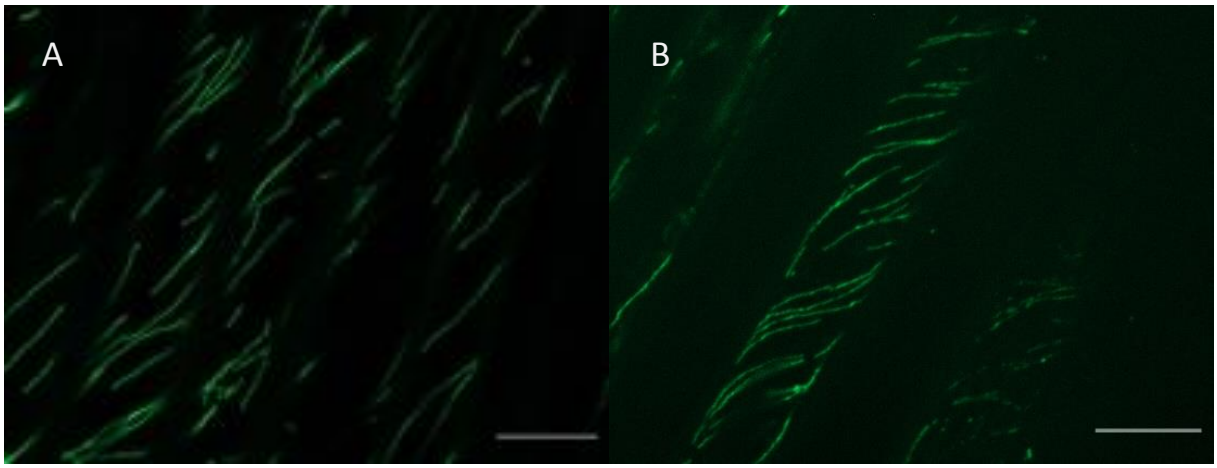


Figure 16. Microtubules of mesocotyl cells visualized using immunofluorescence. Staining occurred with primary antibody ATT against tyrosinated alpha-tubulin and was completed with secondary antibody FITC. (A) wild type mesocotyl cells (B) TTL overexpressing rice mesocotyl cells. Scale bars = 10 µm.

Afterwards the angle between microtubule and cell wall was measured (Figure 12).

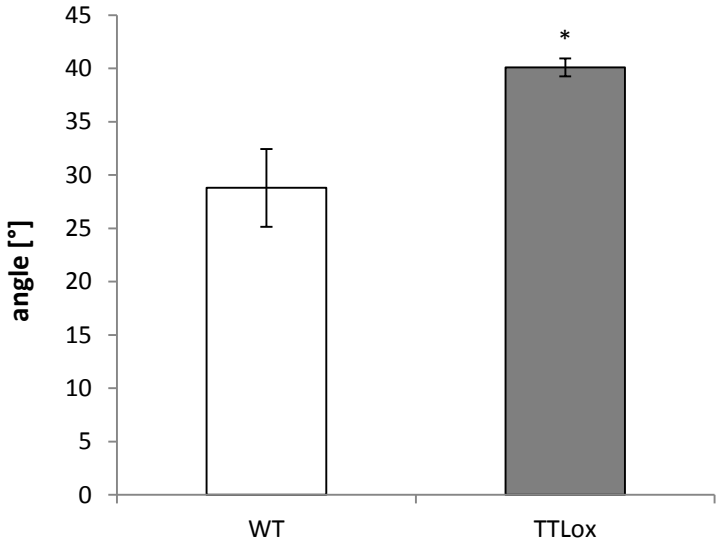


Figure 17. Microtubular orientation of mesocotyl cells. Angle between microtubules and cell wall of mesocotyl cells of wild type (white) and TTLox (grey) mesocotyl cells are shown. Data show mean and standard error from four independent experimental series (n=300). Statistically significant differences to the wild type are illustrated by one asterisk (Student's t-test, $p < 0.05$).

The angle of microtubules in cells of the TTLox mesocotyl was larger than the angle of microtubules in cells of the wild type mesocotyl. While the mean angle of wild type mesocotyl microtubules was at around 30° the angle of TTLox microtubules is 40°.

To analyze in which amount the angle is changed a frequency distribution over the microtubular orientation of mesocotyl cells was constructed (Figure 13).

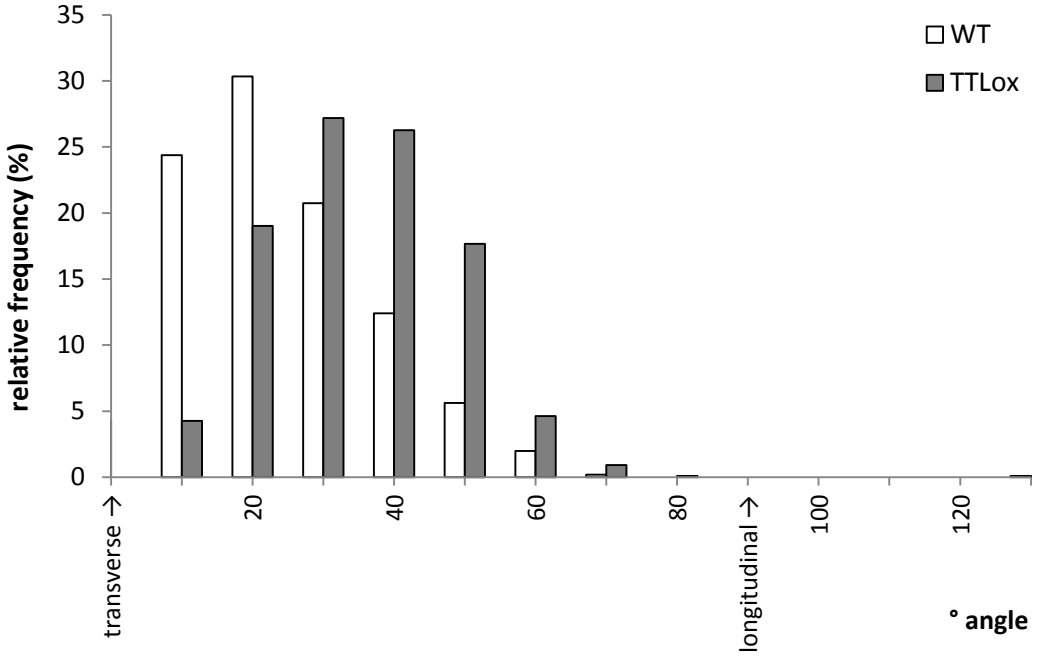


Figure 18. Frequency distribution of microtubule orientations at cells of the outer mesocotyl of wild type (white) and TTLox (grey) rice seedlings.

In the mutant a shift towards longitudinal orientation of the microtubules was visible. While 43% of the wild type microtubules were oriented between 10 and 20 degrees, only 20% of the mutant microtubules were found in this orientation. On the other hand the percentage of 50° and higher oriented microtubules in the mutant was 23% whereas only 10% of wild type microtubules were oriented this way.

3.3. Microtubule polymerization *in vitro*

Staining of cortical microtubules of mesocotyl cells, results are shown in the last chapter, confirmed in a cell biological manner that TTL overexpression influences the microtubule structure. What influences microtubule polymerization more – detyrosination or tyrosination? Is there a way to examine this question in a biochemical approach outside a cell? In a cell many components interact together building the cytoskeleton. Due to this the advantage of an *in vitro* system is that only defined elements can be added in order to analyze their specific function without any further interactions.

I established an *in vitro* system for MT-polymerization and could examine whether it is possible to change the polymerization by adding defined combinations of proteins.

In the first step plane surfaces like a cover slip were used for tubulin polymerization. The microtubules were polymerized in a way that is similar to the polymerization of the control (Figure 14 A). Addition of EB1 (end binding protein 1) instead was able to change polymerization. While addition of EB1 resulted in less but therefore longer microtubules due

to its stabilizing effect on microtubules when added before incubation (Figure 14 B) it shortened the microtubules when added afterwards (Figure 14 C). BSA acted as a negative control in order to check if addition of a protein solution that is naturally not involved in MT polymerization was capable to trigger a microtubule response, which was not the case (Figure 14 D).

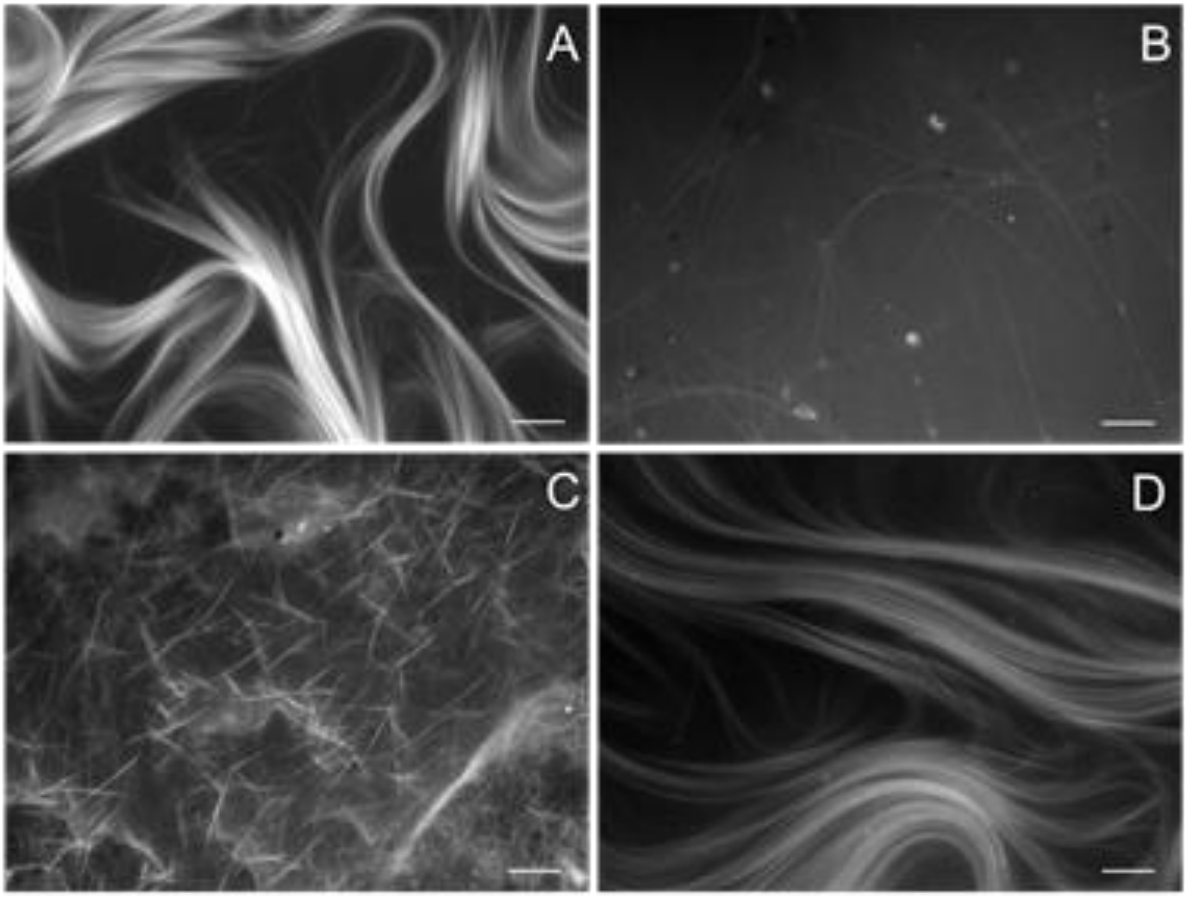


Figure 19. *In vitro* polymerization of microtubules on a cover slip under influence of different treatments. (A) control without treatment, (B) addition of EB1 before polymerization, (C) addition of EB1 after polymerization, and (D) addition of BSA. Scale bars = 20 µm.

For further analyses MT polymerization was performed in a tube, which led to the advantage of being able to dilute the sample in a reproducible way. Microtubules were diluted in a mixture of BRB80 and the microtubule stabilizing drug taxol.

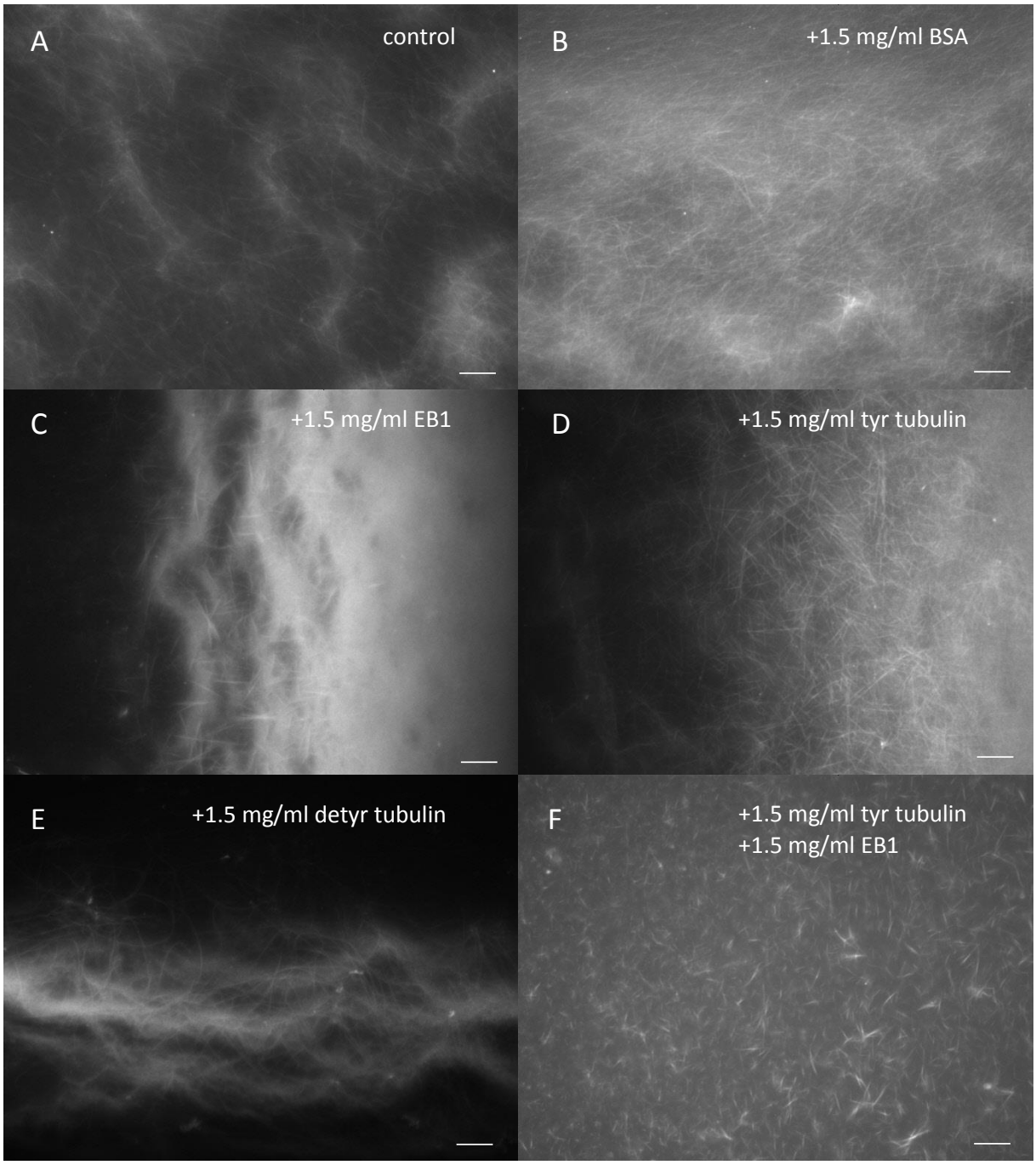


Figure 20. *In vitro* polymerization of microtubules in a tube under influence of different treatments (concentration 1.5 mg/ml) before polymerization. Afterwards microtubules were diluted. (A) control, (B) addition of BSA, (C) addition of EB1, (D) addition of tyrosinated tubulin, (E) addition of detyrosinated tubulin, and (F) addition of a combination of tyrosinated tubulin and EB1. Scale bars = 10 µm.

In order to check if microtubule length can be influenced by addition of tyrosinated or detyrosinated tubulin, protein solutions were added before incubation at 37°C which resulted in shorter or longer microtubules (Figure 15). For calculations of the mean length of microtubules each treatment was repeated in three independent experimental series and for each experimental series 500 microtubules were measured.

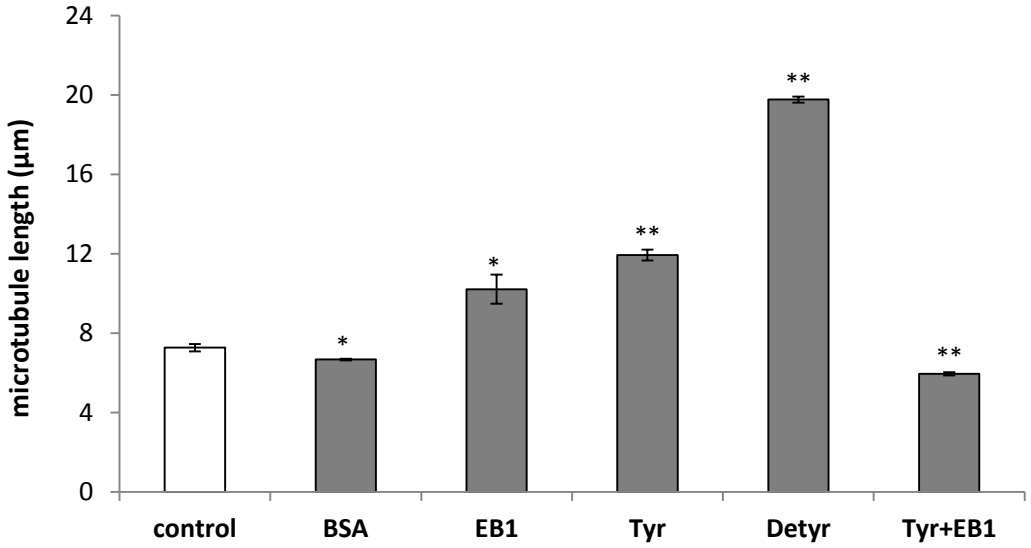


Figure 21. Quantification of microtubule length after *in vitro* polymerization under influence of different protein solutions (BSA, EB1, tyrosinated tubulin, detyrosinated tubulin and a combination of tyrosinated tubulin and EB1; concentration 1.5 mg/ml). Data show mean and standard error from three independent experimental series (n=500). Statistically significant differences to the wild type are illustrated by one or two asterisks (Student's t-test, $p < 0.05$ or $p < 0.01$, respectively).

Figure 16 shows clearly an influence on microtubule polymerization depending on the type of protein that was added. Microtubule length was either increased or shortened. While addition of EB1 was actually elongating polymerized microtubules, the effect of adding tyrosinated or detyrosinated tubulin was even more distinct. The strongest effect on microtubule polymerization could be achieved when detyrosinated tubulin was added, which resulted in more than a doubling of microtubule length. Combination of tyrosinated tubulin and EB1, when added alone both elongate the microtubules, did not result in longer microtubules. In contrast, the microtubules were almost 20% shorter than the control microtubules without treatment.

3.4. Summary

In order to answer the question "What role does detyrosination play?" and "What is it good for in plants?" my work was organized in two different parts. In the first part I took a closer look on the phenotype of rice seedlings and rice plants that are overexpressing TTL. During phenotyping special focus was placed on plant growth and gravisensing of plants. Gravisensing is disturbed due to TTL overexpression. While gravitropic response is delayed in TTLox rice roots, gravitropic set point angle (GSA) also is altered as well compared to wild type. Rice seedlings that are overexpressing TTL show a significantly smaller leaf angle which is typically determined by the gravitropic set point angle than wild type rice seedlings.

Furthermore it became evident that plants that are overexpressing TTL have 30% shorter roots, 20% reduced ligule length and that the amount of lateral roots is also significantly reduced. Mesocotyl length of TTL overexpressing rice seedlings instead is increased around 50% compared to the wild type. The elongation could be further increased by GA_3 treatment. After treatment with 1 µM GA_3 wild type mesocotyl length was similar to the length of an untreated TTLox mesocotyl. In plant cells the direction of cellulose microfibrils gets determined by microtubule orientation. By this the cell gets a kind of support, by which it can only grow in one direction. In order to analyze if the increase in mesocotyl length was a result of a changed pattern of cortical microtubules in TTLox mesocotyl cells, the microtubules were visualized using immunofluorescence. The results showed a clear shifting towards longitudinal microtubules in TTLox cells.

Because especially examination of mesocotyl growth and staining of the cortical microtubules showed that TTL overexpression influences microtubule structure, in the second part of my work I established an *in vitro* system for MT-polymerization. By addition of only those solutions that you want to analyze, you do not have to deal with possible interconnections with other proteins which can be seen as the biggest advantage of this *in vitro* system compared to examination of microtubules in living cells. In this work I took a closer look on the influence of EB1 (end binding protein 1), detyrosinated tubulin, tyrosinated tubulin or combinations of these on microtubule polymerization. Due to its stabilizing effect on microtubules I expected that EB1 could elongate the polymerizing microtubules. Therefore addition of EB1 was my positive control. BSA instead acted as a negative control in order to check if addition of a protein solution that is naturally not involved in MT polymerization was capable to trigger a microtubule response. Both controls were consistent with my expectations. Microtubules, that were treated with EB1 while polymerization, were longer than untreated microtubules, microtubules that were treated with BSA during polymerization did not show much difference in length. Addition of tyrosinated tubulin showed an increase in microtubule length. This effect was even more obvious when detyrosinated tubulin was added before polymerization. This resulted in an almost doubling of microtubule length. Combination of tyrosinated tubulin and EB1 instead did not result in longer microtubules. In contrast, the microtubules were almost 20% shorter than the control microtubules without treatment.

4. Discussion

The function of tubulin detyrosination is still elusive. Yet most of what is already known was analyzed in animal cells. In animals detyrosination cycle plays a crucial part in neural organization and generation of axons (Erck *et al.*, 2005). Since tubulin detyrosination can also influence tumor genesis (Hammond *et al.*, 2008) as well as tumor aggressiveness (Lafanechere *et al.*, 1998), the question about the role of this highly conserved cycle is important.

But tubulin detyrosination is also present in plant cells. What is the role of it in these cells? The answer could possibly also provide an indication of the function of tubulin detyrosination in animals. For examination I analyzed characteristics of plants using a TTL overexpressing rice line in terms of increasing the level of tyrosinated tubulin.

These characteristics including plant specific gravitropism and growth of different plant organs are known to be primarily or at least partly affected by microtubules. Microtubule dynamics is essential in all these mechanisms since regulation is performed via microtubule associated proteins. These have different affinities towards modified microtubules. While some stabilizing MAPs were found to bind more easily to detyrosinated microtubules (de Forges *et al.*, 2012), others like EB1 are preferentially binding tyrosinated microtubules (Cai, 2010). Tubulin detyrosination can even inhibit binding to microtubules, like binding of the Mitotic Centromere-Associated Kinesin (MCAK) of mammalian cells (Peris *et al.*, 2009).

If tubulin detyrosination influences mechanisms like gravitropism and growth via altering microtubule dynamics, it would be very likely that differences between wild type and TTL overexpressing rice plants would be visible being generated from disruptions either in mitosis or cell elongation. Expectations were complied with. Next to shorter roots and less lateral roots the gravitropic response was also delayed, to mention some examples that were shown in the results.

Microtubule polymerization *in vitro* gave a further insight into the correlation between tubulin detyrosination and stability. Moreover, the analyses confirmed the findings that cell elongation is disrupted by altering microtubule dynamics due to TTL overexpression.

In the following chapters I will focus first on each finding itself, afterwards a conclusion will summarize these findings by giving a model on how tubulin detyrosination is influencing plant growth.

4.1. Influence of detyrosination on gravisensing

4.1.1. Gravitropic response is delayed in TTL overexpressing rice plants

Generally, gravitropic response can be distinguished into four steps – susception, perception, transmission and reaction or effection towards a stimulus. While amyloplasts are responsible for stimulus susception postulated in the starch-statolith theory (Haberlandt, 1900; Nemec, 1900) and stimulus transmission is generated via auxin redistribution like stated in the Cholodny-Went theory (Cholodny, 1927; Went, 1928), microtubules are involved in perception (Nick, 2008) as well as in the reaction towards the gravity vector.

The latter is induced by auxin redistribution, which results in a changed microtubule orientation (Blancaflor & Hasenstein, 1993; Nick & Schäfer, 1991) and therefore differential growth. Signal perception is based on pressure, which led to the hypothesis that microtubules are involved in graviperception (Nick, 2008) confirmed by studies showing that gravitropism can be blocked by antimicrotubular drugs at concentrations that do not influence the growth machinery (Godbolé *et al.*, 2000; Gutjahr & Nick, 2006; Nick & Schäfer, 1991).

Changing microtubule dynamics leads to an inhibition of gravitropic responses (Godbolé *et al.*, 2000; Gutjahr & Nick, 2006; Nick *et al.*, 1994; Nick, 2008). In TTL overexpressing rice seedlings gravitropic response is delayed, especially the early events in TTLox occur slower than in wild type rice seedlings. As detyrosination and tyrosination are involved in microtubule dynamics, the result could indicate that overexpression of TTL is influencing microtubule dynamics and therefore also reducing gravitropic responses.

Detyrosinated α-tubulin is preferentially found in stable microtubules leading to the hypothesis that detyrosination might confer stability to a given microtubule (Schulze *et al.*, 1987). Alternatively, detyrosination might just be the consequence of stability (Khawaja *et al.*, 1988). Since microtubules assemble and disassemble in the manner of a treadmill, a tubulin heterodimer integrated at the plus end of the microtubule will progressively travel towards the minus end and eventually leave the microtubule. The duration of this journey depends on the velocity of the treadmill, which can be quite diverse in different microtubule populations. Since TTL preferentially binds non-assembled heterodimers of tubulin (Burns, 1987), whereas TTC prefers assembled microtubules (Gundersen *et al.*, 1987), stable microtubules would be detyrosinated in preference, whereas dynamic microtubules would escape the activity of TTC. This would create a code, recruiting stable microtubules as targets for the binding of microtubule associated proteins (Janke & Kneussel, 2010; Wloga & Gaertig, 2010) with higher affinity for modified microtubules (Verhey & Gaertig, 2007). In fact, several kinesins have been shown to bind predominantly to detyrosinated microtubules (Liao & Gundersen, 1998; Zekert & Fischer, 2009). However, the link between detyrosination and

stability might also be bidirectional: Some stabilizing MAPs were found to bind more easily to detyrosinated microtubules (de Forges *et al.*, 2012), and binding of the Mitotic Centromere-Associated Kinesin (MCAK) of mammalian cells is even inhibited by detyrosination (Peris *et al.*, 2009).

End binding protein 1 (EB1) is preferentially binding tyrosinated microtubules (Cai, 2010), which is meaningful since the plus end is rather tyrosinated due to its constant growth. Another correlation between gravity response and detyrosination is given in loss-of-function mutants for EB1 in *Arabidopsis,* that are strongly impaired in the sensing of touch and gravity stimuli (Bisgrove *et al.*, 2008).

4.1.2. Gravitropic set point angle is altered in TTL overexpressing rice plants

The gravitropic set point angle (GSA) defines the angle each organ of a plant bends towards the gravity vector. It is important that plants can obtain a stable gravitropic position of their organs that can be regulated via environmental parameters or by developmental age (Firn & Digby, 1997). Depending on environmental conditions like the availability of light they can adopt to the most suitable angle controlled by GSA.

Different studies have shown, that the GSA can be of course influenced on the one hand by phytohormones like auxin (Roychoudhry *et al.*, 2013) and ethylene (Edelmann *et al.*, 2002), on the other hand it can be regulated by light via phytochromes (Digby & Firn, 2002), both leading to differential growth. But furthermore gravitropic set point angle can also be influenced by nutrients like phosphate (Bai *et al.*, 2013) or nutrient availability. Concerning particularly roots this is meaningful as roots have to adapt in their growth in situations where nutrients are running short. Concerning leaves, GSA is especially used to perform photosynthesis in a most efficient manner due to best usage of incident light (Digby & Firn, 2002).

Different hypothesis exist on the mechanisms that can influence the GSA of an organ. Long time it was assumed that two opposing mechanisms are working together forming the resulting angle. Firn and colleagues proposed a concept in 1997 by which a single mechanism is sufficient for all forms of gravitropic responses including the gravitropic set point angle (Firn & Digby, 1997).

In summary it is very likely that the GSA is regulated by the same mechanisms as the control mechanisms of gravitropic response.

TTL overexpressing rice seedlings show a delayed gravitropic response as mentioned above. Accordingly, leaf angle of TTLox rice seedlings and therefore GSA is also altered. The leaf angle is significantly reduced compared to the wild type and reaches only 50% of the value measured in wild-type seedlings. Therefore, the data might also indicate that by

manipulation of detyrosination via overexpression of TTL, resulting in changed microtubule dynamics, the gravitropic set point angle is modified.

4.2. Influence of detyrosination on growth

4.2.1. Root length is reduced in TTLox rice seedlings

Root growth occurs due to two different mechanisms: (1) cell proliferation via mitosis and (2) cell elongation. In TTL overexpressing rice seedlings root length is significantly reduced, providing several possible explanations:

First it could be a result of fewer cells because mitosis is disordered due to changed detyrosination level. The second reason could be that cell elongation is decelerated or due to TTL overexpression disrupted. Finally it could also be a combination of both possibilities.

In order to get a hint to these possibilities, mitotic index was measured (Supplemental data Figure 1). To conclude if decreased growth is a consequence of a shift in growth rate, a time series over 7 days was performed.

Measurement of the mitotic index in the meristematic zone revealed no significant difference between wild type and TTL overexpressing rice seedlings, although TTLox rice seedlings showed a slightly reduced mitotic index.

In the time series experiment it could be detected that only at day 4 a significant difference between wild type and TTLox rice seedlings is visible. On day 1, 2, 3 and 5 the increase in root length of the TTLox rice seedling is just slightly reduced, whereas on day 6 the increase in TTLox rice seedlings is higher compared to the wild type.

What makes day four special that in the end the total root length is clearly reduced?

The root can be divided into the following different zones: meristem, where new cells divide, transition zone, elongation zone and at last differentiation zone (Ubeda-Tomas *et al.*, 2012), whereby cell elongation in the elongation zone resulted due to microtubule redistribution and therefore also changed cellulose microfibril synthesis.

The findings of the time series experiment indicate, that on day four, the time point where cell elongation is becoming stronger, elongation in rice seedlings that are overexpressing TTL is disrupted. Thus, the roots of TTLox rice seedlings are shorter in the end. But further experiments have to be done to confirm this hypothesis, for example treatment with interacting phytohormones like auxin and ethylene.

4.2.2. TTL overexpression leads to less lateral roots

The amount of lateral roots of TTL overexpressing rice seedlings is significantly reduced in comparison to the wild type. In five days old rice seedlings less than half of the number of lateral roots are visible in the overexpressing rice line.

Hence the question arises if it takes more time for the lateral roots in the mutant to emerge or if the lateral roots are not even generated in the mutant. To answer this question it is necessary to become aware of how lateral roots are formed.

Lateral root growth is similar to primary root growth (Tian *et al.*, 2014) – lateral roots also have a meristem, but are responding plagiotropic towards gravity. The initiation of lateral roots requires asymmetric cell division of a limited number of pericycle cells (De Smet, 2012). This in turn needs a tightly controlled cell cycle so that the pericycle cells can divide in a way that can build a new organ and do not start to proliferate in an uncontrolled way (De Smet, 2012; Himanen *et al.*, 2002). All together the process of lateral root formation out of pericycle cells next to xylem poles (Laskowski *et al.*, 1995) is very complex and therefore several growth control points exist (Dubrovsky *et al.*, 2011) in cell cycle progression, asymmetric cell division and differentiation (De Smet, 2012). Here the findings come full cycle. Microtubules are involved in all of these control points. Disturbance of the equilibrium of tyrosinated and detyrosinated tubulin leads to disruption in lateral root formation resulting in less lateral roots in TTL overexpressing rice seedlings.

But still the questions if the lateral roots just take longer time to emerge or if they rather are not established in the root are not answered.

Analysis of differences in primordia (supplemental data figure 2) formation did not indicate that lateral roots were not generated leading to the hypothesis that lateral roots emerge more slowly either by disruption during mitosis or in elongation. Although measurement of mitotic index in rice root meristem cells (supplemental data figure 1) showed no significant difference, in BY-2 tobacco cells I could show that detyrosination is influencing mitosis (supplemental data figure 3, (Schneider, 2010)). These experiments were performed using parthenolide, a TTC inhibitor, thus shifting tubulin to the tyrosinated form. But as lateral roots are growing like primary roots I rather suggest that due to TTL overexpression cell elongation is affected resulting from disordered microtubule dynamics.

4.2.3. Ligule size is altered due to TTL overexpression

The ligule is often described as a thin outgrowth of the upper epidermis of leafs. It prevents rainwater from entering the leaf sheath (Chaffey, 2000; Lee *et al.*, 2007). Although not much is known about the molecular mechanisms in rice that controls the development of the ligule, ligule formation is started by periclinal division of the adaxial side in a leaf primordium (Lee *et*

al., 2007). Mutants of genes that regulate ligule development like LGN (liguleless narrow – a kinase) also show defects in lateral growth showing a clear linkage between proximal-distal patterning and medial-lateral growth (Moon *et al.*, 2013).

Since asymmetric distribution and divisions seem to be important for ligule development (Moon *et al.*, 2013) the three stages of normal leaf development (Sylvester *et al.*, 1990) are noteworthy:

1. In the preligule stage the primordium is dividing throughout its length until a row of cells is dividing more rapidly. The primordium itself is undifferentiated at that time.
2. The ligule is forming, the blade is growing faster than the sheath and differentiation starts.
3. A rapid increase in sheath length is visible.

When TTL is overexpressed in rice seedlings and therefore the equilibrium of tyrosinated and detyrosinated tubulin is changed, the ligule, although a distinctive feature, was significantly reduced in length. This could either be a result of a challenging mitosis or it could indicate that microtubule distribution leading to cell elongation is not working properly. Congruent with the other findings I would suggest that due to cell elongation ligule length is reduced.

4.2.4. Mesocotyl length is induced in mutant rice seedlings

The mesocotyl is a part of the plant which is located between the seed and the plumule. It is considered to be partly hypocotyl and partly cotyledon and functions in pushing the coleoptile towards the soil surface while extending. Therefore elongation of the mesocotyl is controlled by several environmental and developmental signals (Hu *et al.*, 2010). Mesocotyl growth is inhibited by red light absorption (Loercher, 1966), endogenous absisic acid (Saab *et al.*, 1992) and by endogenous jasmonate (Riemann *et al.*, 2003), whereas elongation is enhanced by auxin (Jones *et al.*, 1991) or gibberellins (Carpita & Kanabus, 1988; Gao *et al.*, 2012). In general there are two control mechanisms for mesocotyl growth: on the one hand it is the lengthwise elongation of each cell, but on the other hand the increase in the number of cells by cell division (Hu *et al.*, 2010).

Comparison of mesocotyl length in seven days old wild type and TTL overexpressing rice seedlings showed a significant increase in the overexpressor line. Since experiments visualizing microtubules of mesocotyl cells using immunofluorescence revealed that redistribution towards longitudinal microtubules in the overexpressor line is visible, it can be concluded, that cell elongation is affected. Plant cells elongate due to water absorption. The cell wall opposes the pressure and maintains the cell shape. The cellulose-synthase moves

along the cortical microtubules (Paradez *et al.*, 2006a). Thus, the microtubule orientation determines the direction of cellulose microfibrils arrangement.

As gibberellins are enhancing mesocotyl elongation I analyzed, if the mesocotyl of wild type rice seedlings can gain the length of TTL-overexpressing rice seedlings. In fact, the increase in mesocotyl length in untreated TTLox rice is almost the same as wild type rice mesocotyl which was treated with 1 µM GA_3. The mesocotyl of TTLox rice seedlings could also be enlarged by gibberellic acid but the increase occurred more slowly. Why is this the case in TTL ox rice seedlings? GA_3 does not only increase growth rate, but as well alters the pattern of expansion – tissues are narrower and longer (Carpita & Kanabus, 1988). In mutants that are lacking gibberellic acid, the alignment of cellulose is inclined because of altered cortical microtubule orientation (Wenzel *et al.*, 2000). Since mesocotyl length in TTL overexpressing rice seedlings is already increased due to diverse microtubule orientation, the effect of GA_3 on TTL overexpressing rice seedlings is not that distinct than the effect of GA_3 on wild type seedlings.

4.3. Influence of detyrosination on microtubules *in vitro*

Changing tyrosination-detyrosination equilibrium of tubulin is influencing microtubule structure as shown in plant's reaction to TTL overexpression in the last chapters, confirmed in a cell biological manner when staining cortical microtubules. In a cell many components interact together building the cytoskeleton. Therefore, for examination if microtubule structure is more influenced by tubulin tyrosination or tubulin detyrosination, the first aim was to establish an *in vitro* system for MT-polymerization in order to analyze this question in a biochemical approach outside a cell when only defined proteins are added.

Microtubule polymerization is very fragile. If concentration of tubulin or GTP is either below or beyond a certain point, no microtubules will polymerize. The same is valid for temperature changes. Therefore, an effect of slightly diluted tubulin concentration when adding protein solutions was avoided by addition of equivalent amount of buffer in the control polymerization.

Bovine serum albumin (BSA) acted as a negative control in order to check if addition of a protein solution that is naturally not involved in MT polymerization was capable to trigger a microtubule response, which was not the case.

End binding protein 1 (EB1) was used as a positive control since it is already known that it has a stabilizing effect on microtubules inside a cell (Akhmanova & Steinmetz, 2010). Addition of EB1 before incubation resulted in less but therefore longer microtubules, whereas addition of EB1 after incubation led to shortened microtubules. Since tubulin content stays the same, less microtubules will polymerize when the already existing microtubules are

stabilized. Therefore, EB1 has also a stabilizing effect on microtubules *in vitro* (de Forges *et al.*, 2012). When microtubules are not stabilized while polymerization, they can break and addition of EB1 after incubation can then lead to a situation, where EB1 proteins are competing for binding sites, resulting in many shorter microtubules.

Furthermore I was interested in what happens if tyrosinated or detyrosinated tubulin is added to the tubulin-polymerization mix. Inside a cell microtubule dynamics are controlled by microtubule associated proteins (MAPs). The associated proteins have a direct influence on microtubule dynamics and can be classified into stabilizing and destabilizing associated proteins. Examples for stabilizing MAPs are CLIP 170, Tau or EB1, whereas Katanin is a destabilizing protein. Some stabilizing MAPs were found to bind more easily to detyrosinated microtubules (de Forges *et al.*, 2012), and binding of the Mitotic Centromere-Associated Kinesin (MCAK) of mammalian cells is even inhibited by detyrosination (Peris *et al.*, 2009). Other MAPs like kinesins are important for cellular processes like mitosis or transport. Thus, the specific decoration of a given microtubule by a kinesin will depend on the type of kinesin. The observation that the punctate decoration of cortical microtubules by KCH is transformed into a more continuous label after treatment with parthenolide, a TTC inhibitor, (Supplemental data figure 4) indicates that this plant-specific kinesin is of the type preferring tyrosinated microtubules. But what happens *in vitro*, when there are no interacting proteins?

When tyrosinated tubulin is added to the polymerization mix, longer microtubules are generated. The microtubule length is elongated referred to control polymerizations or EB1 addition. This effect is even more distinct when detyrosinated tubulin is added before polymerization. Microtubules are almost three times longer referred to control without further protein addition. Both types of tubulin seem to have a stabilizing effect. Since detyrosinated tubulin has an even stronger effect, a possible explanation could be that detyrosinated tubulin is bundling tubulin and that due to this there are less nucleation sites which would explain longer microtubules.

In a last approach I combined these treatments in order to investigate the reaction of the microtubules. When tyrosinated tubulin and EB1, both elongating microtubule length, when added alone, are combined in the polymerization mix, developing microtubules are significantly shorter than control microtubules. Since EB1 as a plus-end tracking protein is preferably binding tyrosinated tubulin, this is explainable. Excessive supply of tyrosinated tubulin results consequently in shorter microtubules since EB1 is binding many tyrosinated tubulin dimers, stabilizing them and due to this giving the opportunity for many nucleation sites.

4.4. Conclusion

To sum up, the last chapters showed the effects of TTL overexpression on rice plants which are ranging from delayed gravitropic response, altered gravitropic set point angle and less lateral roots to reduced ligule and increased mesocotyl length.

All these processes can be linked with cell elongation and cell division – the two central events in plant cell growth. Microtubules are involved in both processes. In plants especially cell elongation is based on microtubules as plant cells elongate due to water absorption. The cell wall opposes the pressure and maintains the cell shape. The cellulose-synthase however moves along the cortical microtubules (Paradez *et al.*, 2006a). Thus, the direction of cellulose microfibrils gets determined by microtubule orientation. *Arabidopsis thaliana* mutant *botero* can be seen as another proof for microtubule participation in cell growth. This mutant exhibits swollen organs and deranged microtubules. The source for this impairment is inactivation of Katanin, a microtubule associated protein, which is destabilizing microtubules and functions like a molecular scissor. The malfunctioning shape of the mutant is thus a result of a disturbed MT organization (Bichet *et al.*, 2001; Burk *et al.*, 2001).

Furthermore, it turned out that it is not microtubules by themselves that are involved in growth but moreover microtubules are regulated or controlled by associated proteins that can influence microtubule dynamics. These proteins are recognizing specific microtubules due to posttranslational modifications that create a binding code for MAPs (Janke & Kneussel, 2010; Wloga & Gaertig, 2010).

Since TTL overexpressing rice seedlings showed a significant diverse growth reaction compared to wild type rice seedlings in all tested organs it can be postulated that detyrosination is involved in control of microtubule dynamics and therefore also in processes like cell elongation. Congruent with this hypothesis, microtubule dynamics was also altered *in vitro*, when level of tyrosinated or detyrosinated tubulin was increased.

In addition I suggest that not the detyrosination alone is controlling MT dynamics. Moreover it’s the equilibrium of tyrosinated and detyrosinated tubulin which is essential for cellular growth functions.

In vivo, both, detyrosinated and tyrosinated tubulins coexist, whereby the tyrosinated form dominates under normal conditions (Gundersen *et al.*, 1987). This is supported by microtubule associated proteins, as some of them preferably bind to detyrosinated tubulin and others like EB1 have a higher affinity to tyrosinated tubulin. Nevertheless, in this composition the coexisting tyrosinated and detyrosinated tubulins form kind of a balance with dominating tyrosinated and less detyrosinated tubulins. The findings of this work now

indicate that when one part of this balance is disturbed, it results in a chain reaction leading to changed growth responses in plants.

In summary, I suppose following working models: A general overview is given in Figure A which is uniting all different aspects of growth related processes. Afterwards a more detailed working model (Figure B) is shown, explaining the processes of cell elongation under the aspect of TTL overexpression.

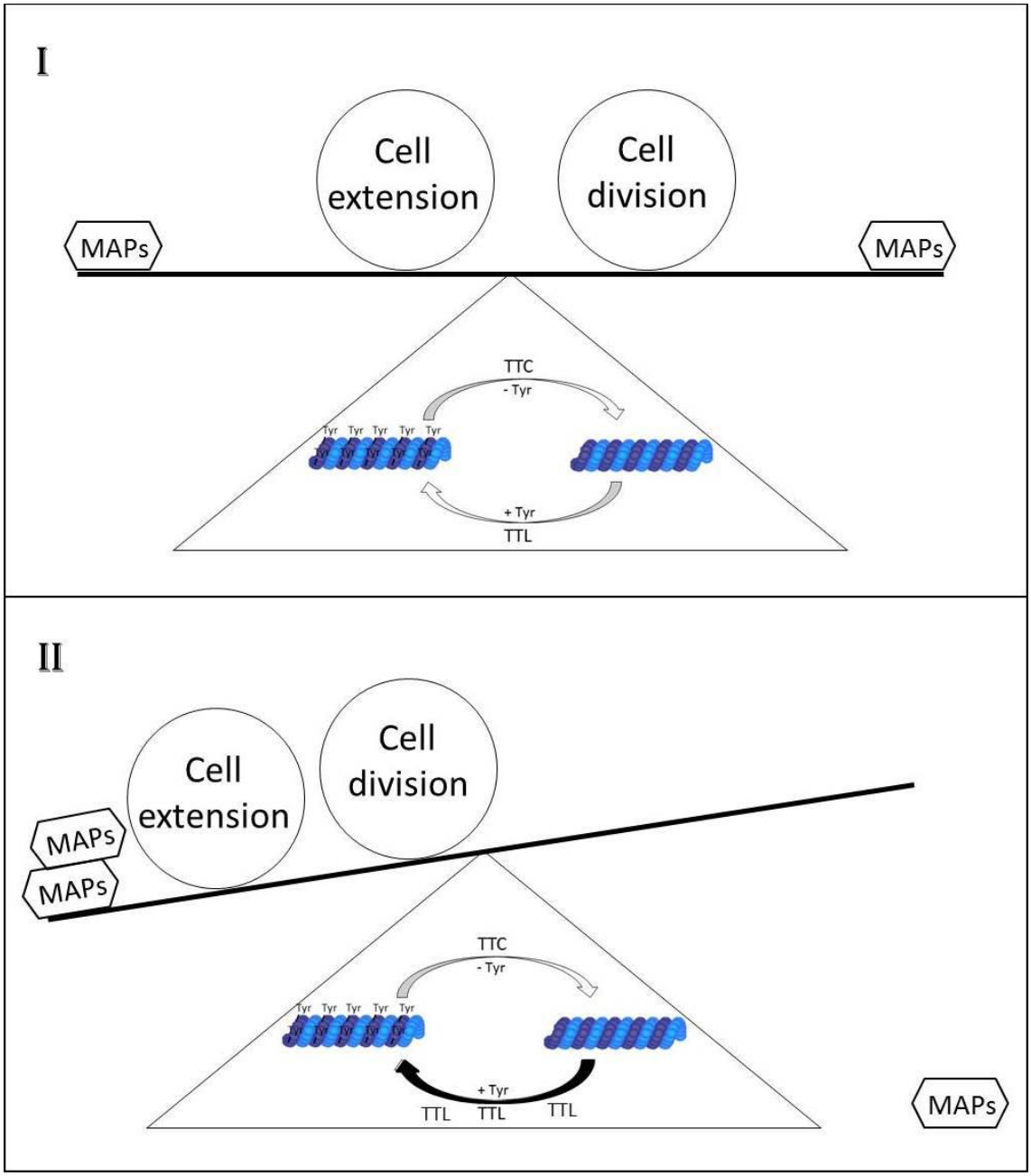


Figure A: Illustration of the relationship between tubulin detyrosination, microtubule associated proteins and growth related processes like cell extension and cell division. I: wild type; II: TTLox.

In wild type cells (I) the equilibrium of tyrosinated and detyrosinated tubulin is intact. Diverse MAPs are stabilizing the microtubules depending on their tyrosination state. Growth related processes like cell extension and cell division operate in a normal way.

In cells that are overexpressing TTL (II) an abundance of tyrosinated tubulin is existent, which leads to diverse binding properties of associated proteins and therefore challenges in growth processes. Although these growth processes are not arrested, the extent of their impact on plant growth is changed.

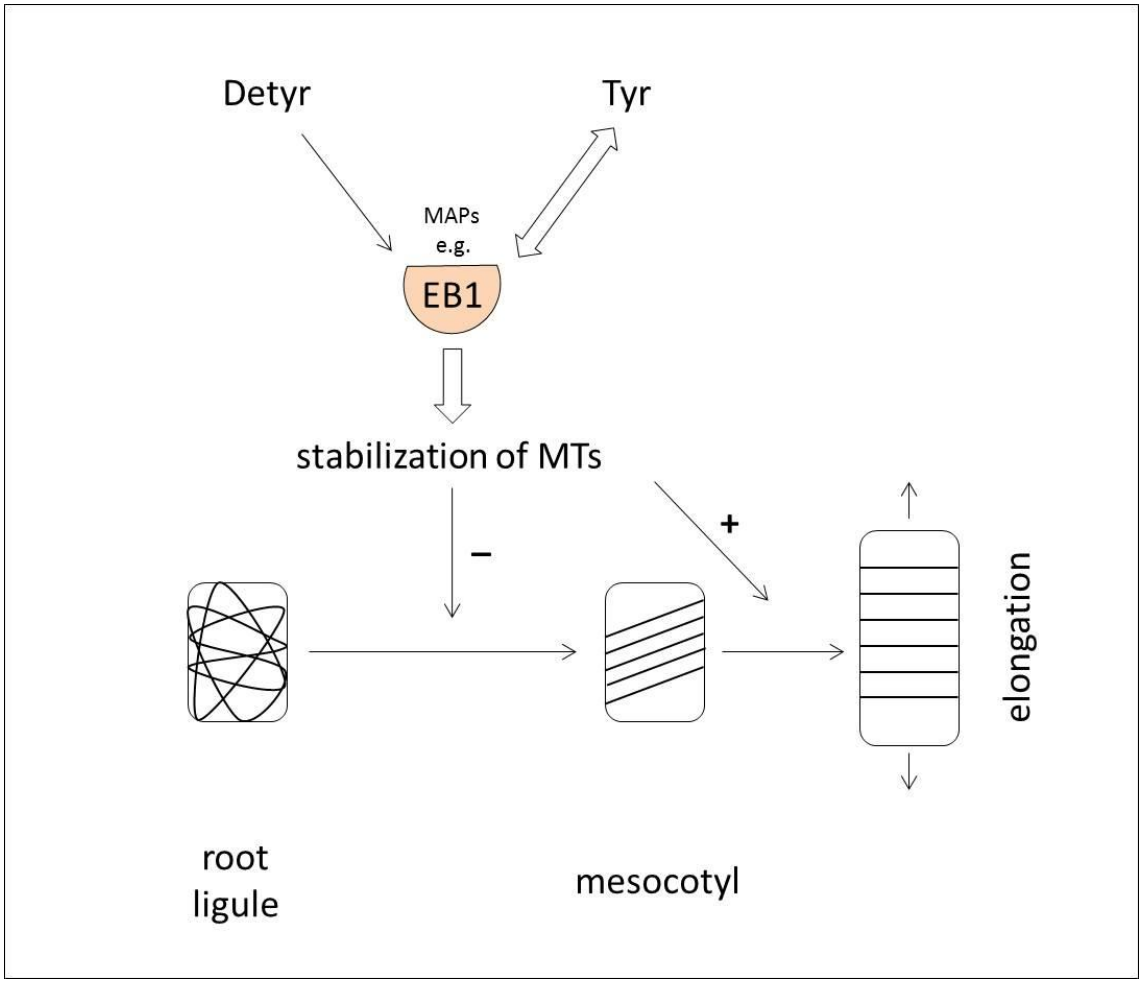


Figure B: Interaction of microtubule redistribution, MAPs and tyrosinated microtubules. In order to simplify the scheme EB1 is shown as an example of MAPs. In the cell different MAPs are interacting together. Microtubules are illustrated by black lines. The minus symbolizes the inhibition of redistribution, whereas the plus is indicating an amplifying.

In the beginning of cell elongation a redistribution of formerly randomized microtubules is performed. Therefore, microtubules undergo depolymerization and are rebuilt. Dynamic microtubules depolymerize faster due to the velocity of assembly and disassembly. But, microtubule dynamics is also influenced by MAPs. EB1 preferentially binding to tyrosinated microtubules, for example is stabilizing microtubule plus ends. Furthermore it is among

others located at the cortex (Akhmanova & Steinmetz, 2010). In Figure B the interaction of microtubule redistribution, MAPs and tyrosinated microtubules is shown. When more tyrosinated microtubules are present due to TTL overexpression, EB1 amongst other associated proteins is binding more microtubules, thus, stabilization of MT plus ends at the cortex is increased. Redistribution of microtubules is blocked. This situation can be seen in root and ligule development, where the microtubules are randomized in the beginning. Therefore, cell elongation is inhibited in TTL overexpressing rice seedlings, leading to a decrease of length. In mesocotyl cells microtubules are already distributed in a transverse manner. Stabilization of MTs is leading to an increase in elongation.

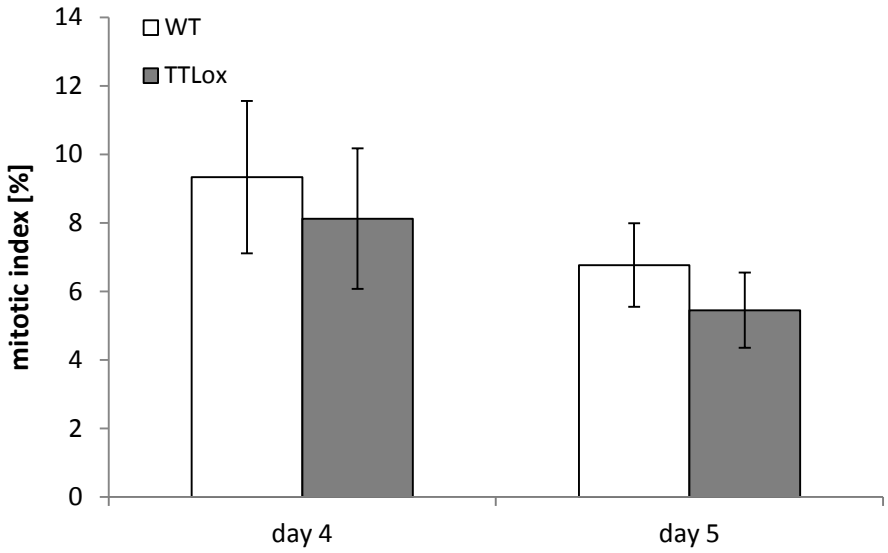
Shifting tubulin contents towards tyrosinated or detyrosinated microtubules *in vitro* led to a significant increase in microtubule length compared to control polymerizations. The effects of tyrosinated and detyrosinated tubulin on microtubule polymerization *in vitro* were confirming the findings given in the physiological approach in plants. It could be shown that detyrosination state influences the binding of EB1 and therefore also the length of microtubules. The fact that detyrosinated and tyrosinated tubulin is influencing microtubule length without further interaction with microtubule associated proteins gave a further insight into the correlation between microtubule detyrosination and stability. Detyrosinated microtubules seem to bundle giving an explanation for their influence on stability.

4.5. Outlook

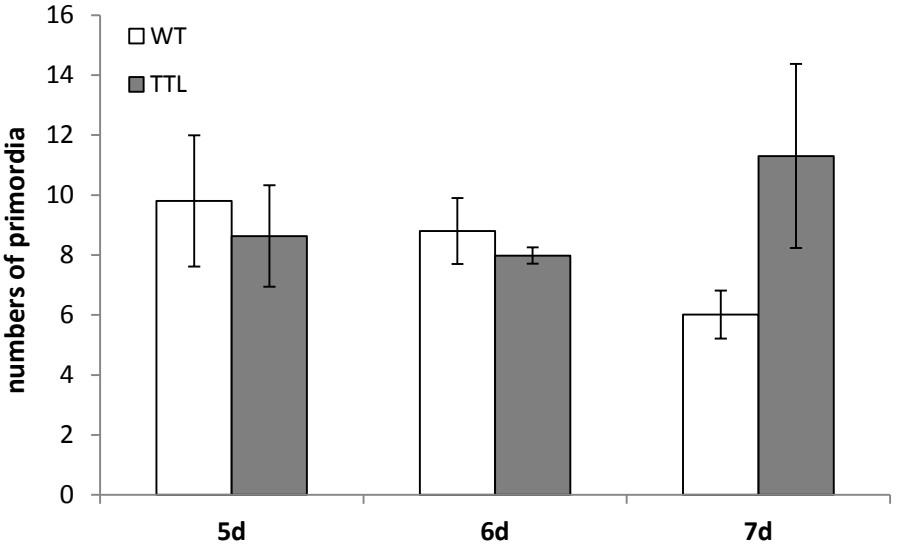
The next step will be to analyze microtubule detyrosination in a biochemical approach. Therefore, expression of TTL and also possible TTC candidates, since the TTC has remained elusive (Ersfeld *et al.*, 1993) so far, will be necessary. Next to a possible characterization of TTC, it would also be interesting to know, what happens to microtubules *in vitro*, when enzymes like TTL and TTC are added.

A further aspect referred to plant growth would be to examine whether phytohormone treatment results in diverse effects in wild type and TTL overexpressing rice plants since phytohormones are an important key in plant growth regulation. Can the effect of TTL overexpression even be repealed by phytohormones?

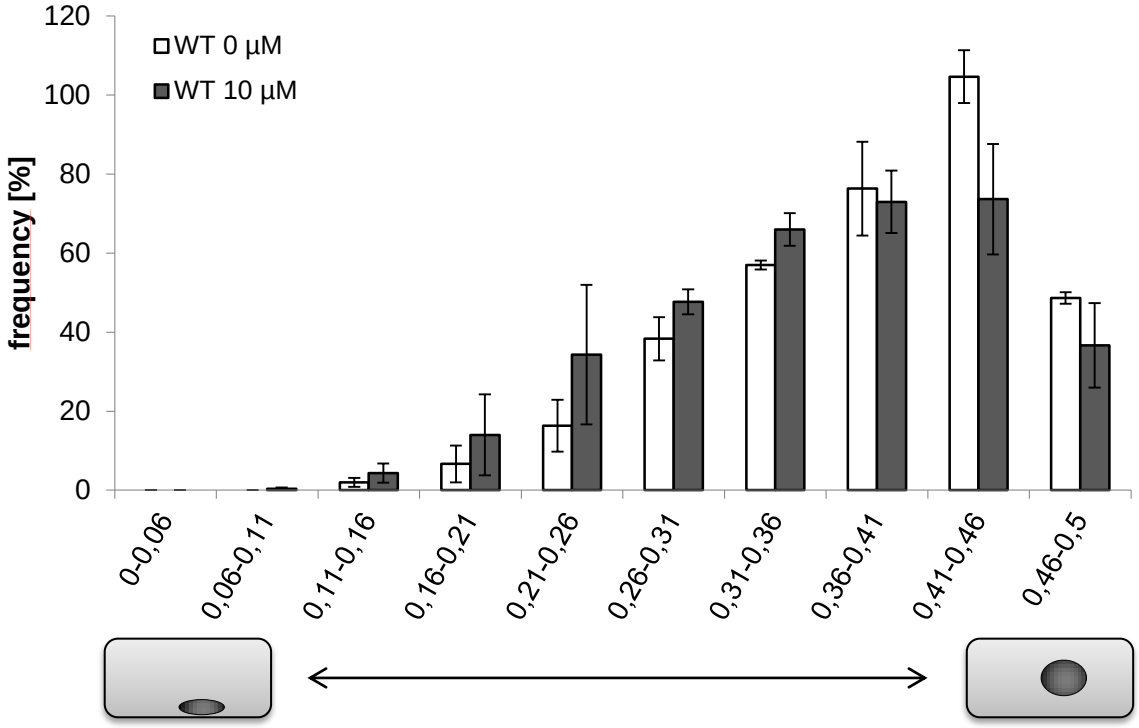
5. Supplemental Data



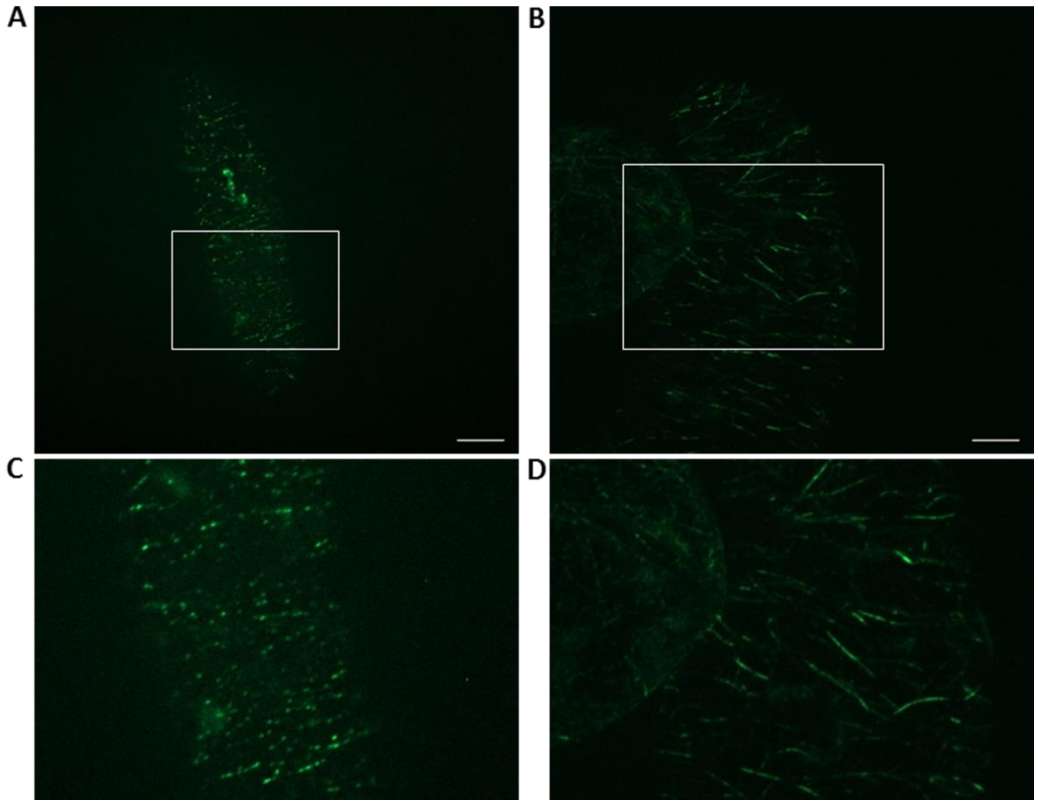
Supplemental figure 1: Measurement of mitotic index on 4 and 5 days old wild type (white) and TTLox (grey) rice seedlings in meristematic zone. Data show mean and standard error from three independent experimental series (n=600).



Supplemental figure 2: Numbers of primordia in 5, 6 and 7 days old wild type (white) and TTLox (grey) rice seedlings. Data show mean and standard error from three independent experimental series (n=10).



Supplemental figure 3: Frequency distribution over nuclear positioning at day 1 after subcultivation and treatment with 0 µM parthenolide (white) or 10 µM parthenolide (grey) recording premitotic nuclear positioning. A value of 0.5 represents completion of the process, when the nucleus has reached the middle of a cell. Before mitosis the nucleus has to be moved towards the middle of the cell. Therefore, delay of mitosis can be seen in a shift of nuclear positioning with values <0,5. Nuclear positioning was measured at day 1 after subcultivation according to Frey *et al.* (2010). Data show mean and standard errors from 350 individual cells collected in 3 independent experimental series.



Supplemental figure 4: Parthenolide recruits the plant-specific kinesin KCH to cortical microtubules. Representative images show the localization of a KCH-GFP fusion in an untreated control cell (A), and a cell treated with 10 µM parthenolide for three days (B). (C-D) Enlargement of the rectangle, which can be seen in A and B.

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Publikationen

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