Front cover: 3D projection of VII-stage lateral root primodium.
(Left) Green: pMAP65::MAP65-2:YFP, Red: Propidium Iodide. (Right) A schematic drawing of MT plus-end shows tyrosinated tubulin.
Picture taken by Dr. Robert Kumpf.

In the name of Allah
Most Gracious, Most Merciful

Ghent University
Faculty of Sciences
Department of Plant Biotechnology and Bioinformatics

The role of the cytoskeleton in hormone-regulated root development

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الى روح والدتي الطاهرة.....

رحمها الله و أدخلها فسيح جناته.....

To my adored mother

God rest her soul in peace

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LIST OF ABBREVIATIONS

+TIP	+END TRACKING PROTEIN
ABA	ABSCISIC ACID
ABP1	AUXIN BINDING PROTEIN1
ABPs	ACTIN-BINDING PROTEINS
ACT	ACTIN
ADF	ACTIN-DEPOLYMERIZING FACTOR
AFB	AUXIN-RELATED F-BOX PROTEIN
AFs	ACTIN FILAMENTS
AHK	ARABIDOPSIS HISTIDINE PROTEIN KINASE
AHP	ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN
ARF	AUXIN RESPONSE FACTOR
ARP	ACTIN-RELATED PROTEIN
ARR	ARABIDOPSIS RESPONSE REGULATOR
ATP	ADENOSINE-5'-TRIPHOSPHATE
AUX/IAA	AUXIN/INDOLE-3-ACETIC ACID
AUX/RE	AUXIN RESPONSIVE ELEMENT
AUX1	AUXIN RESISTANT1
AXR	AUXIN RESISTANT
BA	6-BENZYLAMINOPURINE
BASL	BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE
BDL	BODENLOS
BFA	BREFELDIN A
BR	BRASSINOSTEROID
BRK1	BRICK1
BRX	BREVIX RADIX
CA	CONSTITUTIVELY ACTIVE
CAP	CYCLASE-ASSOCIATED PROTEINS
CES	CELLULOSE SYNTHASE
CK	CYTOKININ
CKX	OXIDASES/DEHYDROGENASE
CLASP	CLIP-ASSOCIATED PROTEIN
CLSM	CONFOCAL LASER SCANNING MICROSCOPY
cMTs	CORTICAL MICROTUBULES
DN	DOMINANT NEGATIVE
EB1	END BINDING 1
ER	ENDOPLASMIC RETICULUM
FC	FOUNDER CELL
GAs	GIBBERELLIC ACID
GEF	GUANINE NUCLEOTIDE EXCHANGE FACTOR
GFP	GREEN FLUORESCENT PROTEIN
GTP	*GUANOSINE 5'* TRIPHOSPHATE
GUS	β-GLUCURONIDASE
IAA	INDOLE-3-ACETIC ACID
IPT	ISOPENTENYLTRANSFERASE
LatB	LATRUNCULIN B
LAX	LIKE-AUX
Ler	Landsberg *erecta*
LOG	LONELY GUY
LR	LATERAL ROOT
LRD	LATERAL ROOT DENSITY
LRI	LATERAL ROOT INITIATION

LRP	LATERAL ROOT PRIMORDIUM
MAPs	MICROTUBULE-ASSOCIATED PROTEINS
MDP40	MICROTUBULE-DESTABILIZING PROTEIN 40
MDR	MULTIDRUG RESISTANT
MP	MONOPTEROS
MS	MURASHIGE AND SKOOG
MTs	MICROTUBULES
NAA	NAPHTHALENE-1-ACETIC ACID
NLS	NUCLEAR LOCALIZATION SIGNAL
NPA	N-1-NAPHTHYLPHTHALAMIC ACID
ORYZ	ORYZALIN
PAC	PRIMORDIA ON AUXIN AND CYTOKININ
PAT	POLAR AUXIN TRANSPORT
PI	PROPIDIUM IODIDE
PIN	PIN-FORMED
PPB	PREPROPHASE BAND
PTMs	POST-TRANSLATIONAL MODIFICATIONS
QC	QUIESCENT CENTRE
RFP	RED FLUORESCENT PROTEIN
RIC	ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEIN
ROP	RHO OF PLANTS
SA	SALICYLIC ACID
SCAR	SUPPRESSOR OF cAMP RECEPTOR
SEM	STANDARD ERROR OF THE MEAN
SLR	SOLITARY ROOT
SNPs	SINGLE NUCLEOTIDE POLYMORPHISMS
SPR1	SPIRAL 1
TIR1	TRANSPORT INHIBITOR RESPONSE1
TTCP/TCP/ TTC	TUBULIN TYROSINE CARBOXYPEPTIDASE
TTL	TUBULIN TYROSINE LIGASE
TUA	α-TUBULIN
TUB	β-TUBULIN
TYR-TUB	TYROSINATED-TUBULIN
***t*-Z**	*trans*-ZEATIN
WASP	WISKOTT-ALDRICH SYNDROME PROTEINS
WAVE	(WASP) FAMILY VERPROLIN HOMOGOUS
WT	WILD TYPE

SCOPE OF THE RESEARCH

Plant growth and development are controlled by exogenous factors, such as light, water and nutrient availability. These signals are perceived and translated into endogenous signals, such as plant hormones. Over the past decades, studies that focused on the mechanisms of phytohormonal regulation of plant development clearly demonstrated that the individual hormone action is largely determined by complex interactions with other hormonal signaling pathways. Thus, a tightly interconnected hormonal network is generated and it governs and coordinates proper plant development. It has been shown that the plant hormones auxin and cytokinin are key regulators of lateral root (LR) organogenesis and they exhibit an antagonistic mode of action. Since the initial discoveries in 1950s, indicating that auxin and cytokinin are required to induce cell divisions and growth in plant tissue culture, both hormones have been shown to interact in several physiological and developmental processes. However, a molecular basis for their cross-talk remains largely unknown.

In plants, a unique mechanism of directional auxin transport is crucial for proper lateral root formation and plant development. The PIN-FORMED (PIN)-mediated polar auxin transport is involved in the establishment of an auxin maximum in the center of the primordium, because the expression of *PIN1*, *PIN2, PIN3, PIN4, PIN6* and *PIN7* is activated during primordium formation. In contrast, increased cytokinin biosynthesis in pericycle cells disrupts LR initiation and patterning. Cytokinins have been shown to control LR formation through their crosstalk with auxin because cytokinin influences the formation of auxin gradients during lateral root development. Moreover, it has been shown that the auxin gradient is dependent on cytoskeleton which is necessary to position the PINs precisely in the morphological context. Although the role of cytoskeleton on trafficking and cell expansion has been described, how hormonal and cytoskeleton activities are coordinated is largely unknown.

The main objective of the work presented in this thesis was to investigate the role of the cytoskeleton in auxin – cytokinin regulated LR organogenesis.

In the first part we focused on cytokinin – auxin interaction with microtubule cytoskeleton. Using confocal-based imaging of MT orientation and dynamics we show that cytokinin antagonizes auxin effect on MT cytoskeleton orientation to regulate LR initiation. Furthermore, we demonstrate how hormone-dependent posttranslational modifications correlate with changes in the cytoskeleton dynamics (**Chapter 2**).

In the second part of the thesis, we addressed the link between cytokinin and the actin cytoskeleton in modulating the auxin efflux carrier PIN1 vacuolar

trafficking to control root growth and development. We show that the PIN1 trafficking mechanism to the vacuole relies on the intact actin cytoskeleton. Genetic interference with several components controlling actin activity modulates PIN trafficking and root system sensitivity to cytokinin (**Chapter 3**).

Finally, a forward genetic screen was designed to identify molecular components of the auxin-cytokinin crosstalk. Among twenty-two *pac* (*primordia on auxin and cytokinin*) mutant plants that produce LRs when auxin is applied simultaneously with cytokinin at inhibiting concentrations *pac7* was selected for detailed studies. Using combination of the positional cloning and SHOREmap strategies The GARP-G2 type transcription factor was found to encode for *PAC7* gene. This TF has a MYB-like motif domain and encodes a putative novel molecular regulator that is predicted to control transcription of cytokinin-related genes. Hence, the GARP-G2 type transcription factor might represent novel component of auxin – cytokinin crosstalk controlling lateral root organogenesis (**Chapter 4**).

1

Introduction

Plant hormones shaping the plant's body

Plant hormones shape the plant by modulating growth in response to internal and environmental signals. They are involved in most of the aspects of plant growth and development, such as embryogenesis, seed growth and flowering, senescence of leaves and fruits, gravitropism, phototropism, leaf formation and stem growth, fruit development and ripening and many other processes. Recently, significant progress has been made in our understanding of hormone synthesis, metabolism, transport and signaling (Santner et al., 2009; Wolters and Jürgens, 2009). Hormones are molecules that are present at very low concentrations and that are released in one part of the body where they can act either locally, closely or at the synthesis or are transported and affect cells in other parts of the organism (Chow and McCourt, 2006; Santner et al., 2009). The phytohormones include abscisic acid (ABA), indole-3-acetic acid (IAA or auxin), brassinosteroids (BRs), cytokinin (CK), gibberellin, ethylene, jasmonic acid, salicylic acid and strigolactone.

The major developmental growth regulators are auxin, brassinosteroids, CK, strigolactone and gibberellin, whereas ABA, ethylene and jasmonic acid are often implicated in stress responses (Santner et al., 2009; Depuydt and Hardtke, 2011). CKs regulate cell proliferation, whereas gibberellins and auxins are involved in cell proliferation and elongation (Depuydt and Hardtke, 2011). Moreover, brassinosteroids are essential for cell elongation, but might also play a role in cell proliferation (Santner et al., 2009; Depuydt and Hardtke, 2011). Gibberellins function in germination, dormancy, flowering and leaf and fruit senescence (Yamaguchi, 2008). Furthermore, auxin mediates a surprising variety of processes, such as embryo development, organ initiation and growth, tropic responses as well as apical dominance (Petrášek and Friml, 2009; Vanneste and Friml, 2009). Strigolactones are a novel class of plant hormones that are involved in shoot branching and root system architecture (Xie et al., 2010). ABA plays an important role during many phases of the plant life cycle, including seed development and dormancy, and in plant responses to various environmental stresses, such as heat stress, water stress and salt stress (Seo and Koshiba, 2002). Similarly to ABA, ethylene is involved in several responses to environmental cues. Moreover, this plant hormone regulates seed germination, root initiation, flower development, fruit ripening and senescence (Lin et al., 2009). Phytohormones interact with specific target tissues to trigger physiological responses, although each response is often the result of the combined action of two or more hormones. This phenomenon of hormonal crosstalk has been extensively studied in recent years and the elucidation of the molecular mechanisms by which hormones interact to control growth and development has contributed to a better understanding of basic plant physiology. This thesis will focus on the growth hormones auxin and CK and on their crosstalk.

Auxin controls plant growth and development

The plant hormone auxin has been studied since a very long time. The first effects related to auxin were described by Darwin as early as the 19th century in his book "*The power of movements in plants*" (1881). Together with his son Francis, Charles Darwin performed experiments on coleoptiles of *Phalaris* sp. and *Avena* sp. and they found that the top coleoptile part was responsible for the seedling bending towards a light source. This observation suggested that the coleoptile tips produced some light-promoted substance that moved to the growing region of the coleoptiles. Went (1974) demonstrated the presence of a chemical product in the tip of *Avena* coleoptiles that was designated auxin from the Greek 'αυξάνω' (*afksano*), meaning 'to grow' in English, of which the chemical name is indole-3-acetic acid (IAA).

The word auxin highlights already its importance: indeed, it is represented by its prevalent form IAA, which is the key endogenous plant hormone that regulates many aspects of plant growth and development, namely it controls and moderates division, cell expansion and changes in developmental programs depending on the cellular position within the plant body (Teale et al., 2006; Benjamins and Sheres, 2008). Auxin is essential throughout the plant life cycle, because it is implicated in the axis formation regulation during embryogenesis (De Smet and Jürgens, 2007; Friml et al., 2003) and maintenance of the apical dominance in shoots and roots, but also during root (Friml et al., 2002; Blilou et al., 2005; Peret et al., 2009; Heisler et al., 2005) and shoot (Leyser, 2003) branching. Furthermore, it is involved in vascular tissue differentiation and regeneration (Sauer et al., 2006; Scarpella et al., 2006). Within the meristems, it governs the growing organs in respect to phyllotaxis (Reinhardt et al., 2003; Kierzkowski et al., 2013) and, likewise, during gravitropic and phototropic responses (Abas et al., 2006; Ding et al., 2011; Friml et al., 2002; Kleine-Vehn et al., 2010).

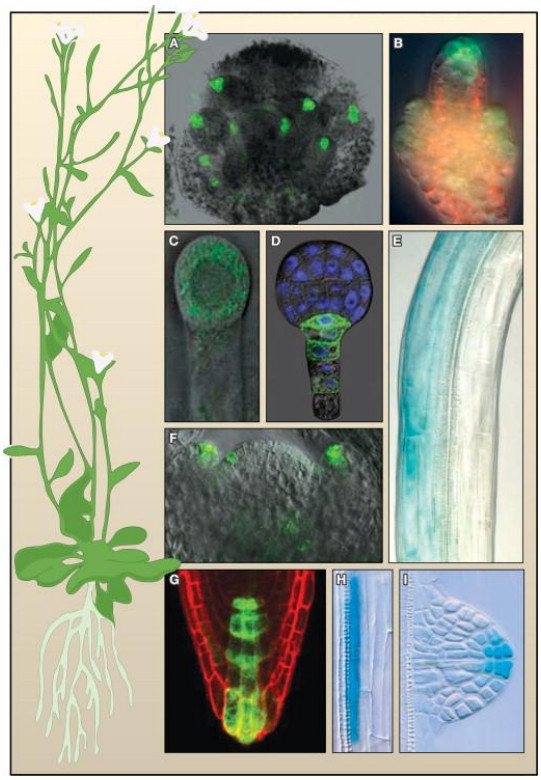

Figure 1: Auxin distribution during *Arabidopsis* development (Vanneste and Friml, 2009).

Locations of auxin activity. (A) Tips of floral organ primordia within developing flowers. (B) Tip of the ovule primordium. (C) Apical cell of a divided zygote. (D) Root pole of the globular-stage embryo. (E) Shade side of photostimulated hypocotyls. (F) Position of incipient organ initiation and tips of flower primordia at the shoot meristem. (G) Root apical meristem. (H) Lateral root initiation sites. (I) Tip of an emerging lateral root primordium. Auxin activity distribution as visualized by the activity of auxin response reporters expressing the green fluorescent protein (DR5rev::GFP) (A, B, C, D, F, and G) and DR5::GUS (E, H, and I).

Auxin Biosynthesis

In plants, high IAA levels are detected in meristematic tissues of shoot and roots, in cotyledons, and in young leaves, of which the latter have the highest biosynthetic capacity (Ljung et al., 2001; Ljung et al., 2005). The identification of the molecular components of the IAA biosynthesis revealed the existence of at least two separate major pathways, one that depends on the precursor tryptophan and one that does not (Woodward and Bartel, 2005). Auxin can also be conjugated (reversibly deactivated) and degraded (Ljung et al., 2002). In two independent genetic screens, the importance of indole-3-pyruvic acid (IPA) has been demonstrated in auxin biosynthesis. Both screens identified mutants in a tryptophan aminotransferase, designated TAA1 that converts indole-3-pyruvic acid (IPA) into indole-3-acetaldehyde (Stepanova et al., 2008; Tao et al., 2008). The IPA pathway is very short, because IPA is converted directly into IAA by the flavin monooxygenases from the YUCCA family (Mashiguchi et al., 2011). Plants overexpressing *YUCCA* genes contain high levels of free auxin and display auxin overproduction phenotypes that depend on the TAA1 activity (Stepanova et al., 2011; Won et al., 2011; Zhao et al., 2001). The quadruple mutant *yuc1yuc4yuc10yuc11* lacks a hypocotyl, a root meristem and floral organs, a phenotype that is very similar to that of some signaling or transport mutants (Cheng et al., 2007), implying a loss in auxin signaling. Further characterization of the *Arabidopsis thaliana* mutant of the *WEAK ETHYLENE INSENSITIVE8* (*WEI8*) gene revealed that it encodes the long-anticipated TAA1, involved in the IPA branch of the auxin biosynthetic pathway (Stepanova et

al., 2011). The *YUCCA* genes code for enzymes that limit the auxin biosynthesis rate and their expression is highly regulated by both environmental and developmental pathways (Zhao et al., 2001).

Auxin Transport and Regulation

Establishment of effective auxin levels in particular cells or group of cells always involves auxin biosynthesis, conjugation, degradation and utilization by the gene expression machinery and transport. Auxin is synthesized in young tissues, especially in emerging leaf primordia and, to a lesser extent, in the main root and lateral root meristems (Ljung et al., 2001, 2005) and is transported from the source to the sink tissues via two different routes. Nonpolar auxin transport takes place through the phloem and, in this manner, auxin is quickly and passively transported following a concentration gradient. Nonpolar auxin transport probably accounts for most of the long-distance transport (Robert & Friml, 2009).

For the other route, the chemiosmotic hypothesis had been formulated (Rubery and Sheldrake, 1974; Raven, 1975) that described the polar trafficking of the main naturally occurring auxin across the plasma membrane. As auxin is weakly acidic (pKa = 4.75), a portion (~15%) of the IAA molecules remains undissociated (protonated IAAH) in a relatively acidic environment of the apoplast (pH= 5.5), thereby being capable to penetrate passively through the cell membrane. Once in the neutral cytoplasm (pH=7), IAA dissociates and becomes deprotonated (IAA^-), so that it is no longer able to cross through the plasma membrane and gets "trapped" inside the cell. To exit the cell, auxin must be transported actively through the plasma membrane by specific efflux carriers (Löfke et al., 2013) (Figure 2). This second pathway is the the polar auxin transport pathway (PAT). For this short-distance transport, plants developed unique, coordinated cell-to-cell transport machinery (Robert and Friml, 2009; Petrášek and Friml, 2009). This type of transport is polar because of the uneven distribution of the auxin transport proteins within the plasma membrane (Friml, 2010). Direction and intensity of the auxin flow are determined by the localization of the auxin efflux carriers. Overall, PAT is crucial for conveying a certain amount of auxin to the perception sites of auxin signals in particular tissues or cell types (Friml and Palme, 2002).

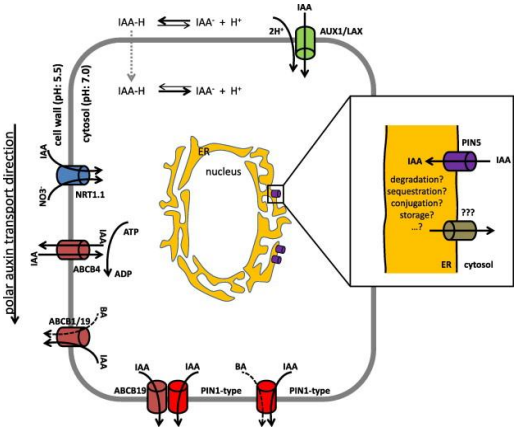

Figure 2: Schematic model of the chemiosmotic hypothesis for the Polar Auxin Transport (Löfke et al., 2013).

The low pH in the apoplast (cell wall) is maintained through the activity of the plasma membrane H^+-ATPases. In the relatively acidic environment, a fraction of the weakly acidic IAA, the major form of auxin, becomes protonated. The protonated (IAAH) form is more lipophilic and can diffuse freely through the plasma membrane into the cell. Besides passive diffusion, auxin is also actively taken up from the apoplast by H^+/IAA^- symport mediated by AUXIN-RESISTANT1(AUX1)/LIKE-AUX1 (LAX) influx carriers. Once inside the neutral cytosol, auxin is deprotonated and becomes trapped inside the cell. Auxin can leave the cell by auxin efflux carriers, such as PIN-FORMED (PIN) proteins. Nonpolarly distributed ATP-binding cassette (ABC) transporters facilitate the nondirectional auxin efflux. ABC, subfamily B4 (ABCB4) can switch the transport direction upon changes in auxin levels. The interaction of ABCB19 and PIN1 at the plasma membrane increases the substrate specificity to auxin (reduced affinity to BA). The nitrate transporter NRT1.1 mediates the auxin influx at low NO_3^- concenttrations. PIN5 auxin carriers, mediates the import of auxin into the lumen of the endoplasmic reticulum (ER), where auxin might undergo degradation, conjugation and/or storage. Auxin exporters in the ER are still unknown.

Auxin transport at the cellular level

Mutants in auxin transport display pleiotropic phenotypes, including impaired tropic growth, lack of apical dominance, disturbed morphology and embryo lethality (Paciorek and Friml, 2006; Tanaka et al., 2006; Vanneste and Friml, 2009). The agravitropic mutant *auxin-resistant 1 (aux1)* was characterized as the first member of the *AUXIN-RESISTANT1/LIKE-AUX1 (AUX1/LAX1)* gene family and has been identified in *Arabidopsis*, and encodes an amino acid permease-like protein (Bennett et al., 1996), that acts as a H+/ IAA− symporter (Bennett et al., 1996). The AUX1 protein facilitates loading of auxin into the leaf phloem (Marchant et al., 2002) and unloading in the roots (Swarup et al., 2001). Transport disruption in *aux1* mutants leads to defects in lateral root formation (Marchant et al., 2002). Moreover, the LAX3 protein is important for correct development of lateral roots, by establishing increased auxin levels in cells of the future emerging LR primordia (LRP) (Swarup et al., 2008; Kumpf et al., 2013).

Several proteins have been shown to be involved in the auxin efflux. The first characterized auxin efflux carrier PIN1 was a member of the PIN-FORMED (PIN) family from Arabidopsis (Galweiler et al., 1998). The *pin1* mutant shows a disturbed development of floral buds, resulting in bare, needle-like inflorescence stems (Okada

et al., 1991). The function of the PIN1 protein in the auxin transport machinery was suggested by treatments with the auxin transport inhibitor N-(1-naphthyl)-phtalamic acid (NPA) that mimics the pin1 mutant phenotype (Okada et al., 1991). Eight members of this family have been described (PIN1 to PIN8) (Zažímalová et al., 2007; Kreček et al., 2009) and recently, the PIN-LIKES (PILS) proteins have been detected (Barbez et al., 2012). All the PIN auxin efflux carriers have 6-10 transmembrane domains separated by a hydrophilic loop (Müller et al., 1998). This protein family can be divided into two subclades: PIN1, PIN2, PIN3, PIN4 and PIN7 that are localized at the plasma membrane of various cell types (Petrášek et al., 2006) and PIN5, PIN8 and partially PIN6 that differ from these canonical PIN proteins and are located in the endoplasmic reticulum (ER) (Mravec et al., 2009; Ding et al., 2012). The PILS proteins are also localized in the ER and involved in the regulation of the intracellular auxin homeostasis (Barbez et al., 2012). PIN proteins are targeted to either side of the plasma membrane according to their phosphorylation status, which is mediated by PINOID (PID), a serine-threonine protein kinase and the protein phosphatase 2A (PP2A) (Friml et al., 2004; Michniewicz et al., 2007). Moreover, at the shoot apex, PID might assign the apical or basal localization of the PIN proteins (Friml et al., 2004). However, PID-independent PIN phosphorylation at one single site is adequate to change the PIN polarity and thus, direct the auxin transport (Zhang et al., 2010). Other proteins implicated in auxin transport are the ATP-binding cassette B-type (ABCB) transporters, also known as multidrug-resistant/phosphor (P)-glycoproteins (MDR-PGPs) (Noh et al., 2001; Geisler and Murphy, 2006; Titapiwatanakun and Murphy, 2009). The ABCB1/PGP1 and ABCB19/MDR1/PGP19 proteins have been shown to be capable of exporting auxin or fluoroindole from cells in heterologous yeast and mammalian systems (Geisler el al., 2005; Santelia et al., 2005; Bouchard et al., 2006), whereas ABCB4/PGP4 operates as an auxin influx carrier in plant cells (Terasaka et al., 2005). Even though some ABCB transporters can function as auxin efflux carriers, the corresponding plant *abcb/pgp* mutants cannot be mimicked by treatment with auxin efflux inhibitors, such as NPA. Furthermore, the double *abcb/pgp pin* mutant exhibits additive phenotypes, hinting at the existence of an independent auxin transport mechanism (Blakeslee et al., 2007). Moreover, ABCB1 and ABCB19 have been proposed to play a supportive role in the regulation of the auxin availability for PIN proteins (Mravec et al., 2008).

Vesicle trafficking and targeting of auxin carriers

The regulation of the amount and localization of the PIN proteins within the plasma membrane is not static, but it is achieved by a constitutive cycling between the plasma membrane and the endosomal compartments. Proteins that are secreted to the plasma membrane might undergo repeated endocytosis and recycling via recycling endosomes or mature into prevacuolar compartments (PVCs)/multivesicular bodies before being targeted to the vacuole for degradation.

This protein trafficking plays a crucial role in the rapid nongenomic regulation of auxin signaling. The auxin transport polarity and the auxin gradient formation are directed by the coordinated localization of the auxin efflux (PIN) proteins to the plasma membrane (Geldner et al., 2001; Kleine-Vehn et al., 2006). Whereas the trafficking pathway in plants is still poorly understood, PIN endocytosis is known to depend on clathrin (Dhonukshe et al., 2007; Kitakura et al., 2011) and it is recycling back to the plasma membrane to rely on ADP-ribosylation factor-GTP-exchange factors (ARF-GEFs), such as GNOM, (a membrane-associated guanine nucleotide exchange factor for ADP-ribosylation factor) (Geldner et al., 2001; Kleine-Vehn et al., 2008a). This process can be inhibited with the fungal toxin Brefeldin A (BFA) that interferes with the ARF-GEF GNOM activity. The PIN1 localization dynamics can be affected by BFA in wild-type, but not in *gnom* mutant embryos. GNOM has been shown to regulate the targeting of PIN1 proteins (Steinmann et al., 1999; Geldner et al., 2001, 2003). After BFA treatment, PIN proteins accumulate in the endosomal compartment, thereby inhibiting auxin transport (Geldner et al., 2001). Moreover, auxin itself inhibits endocytosis of the auxin efflux carrier PIN1 and, as a consequence, promotes its own cell-to-cell transport (Paciorek et al., 2005). The recycling of PIN proteins is also dependent on the actin cytoskeleton and on microtubules (Geldner et al., 2001; Kleine-Vehn et al., 2008a). The vesicular cycling of PIN1 from endosomal compartments to the plasma membrane depends on actin and is sensitive to BFA, whereas the retrograde pathway is resistant to BFA. Another efflux carrier from the PIN family is mediated by other ARF-GEF, but nonetheless, the cycling of PIN2 is similar: its apical localization in root epidermal cells is essential for the gravitropic response (Kleine-Vehn et al., 2008). ARF-GEF-dependent endosomal sorting is also involved in PIN protein trafficking to the lytic vacuolar pathway through the PVC, from where the PIN proteins might be retrieved again into the trans-Golgi network (TGN) through the assistance of the subunits of the retromer complex, SORTING NEXIN1 (SNX1) and VACUOLAR PROTEIN SORTING29 (VPS29) (Jaillais et al., 2006, 2007; Kleine-Vehn et al., 2008).

Auxin Signaling

Auxin signaling involves auxin metabolism, transport, perception and response. Auxin regulates its own amount by rapidly modulating levels of auxin/indole-3-acetic acid (Aux/IAA) transcription factors (repressors) through various stages of plant development. Members of the auxin response factor (ARF) family are transcription factors that bind to auxin-responsive elements (AuxREs) in the promoters of primary auxin-responsive genes, mediating their transcription (Teale et al., 2006). ARF proteins act as activators or repressors (Paciorek and Friml, 2006). IAA binds to its binding protein TRANSPORT INHIBITOR RESPONSE1 (TIR1), the F-box subunit of the ubiquitin ligase complex Skp-Cdc53-F-Box Transport Inhibitor Response 1/Auxin receptor F-Box (AFB) ($SCF^{TIR1/AFB}$) and thus stabilizes the interaction between TIR1 and Aux/IAA substrates (Dharmasiri et al., 2005;

Kepinski and Leyser, 2005). In this process, auxin does not induce conformational changes in the crystal structure of TIR1, but rather functions as 'molecular glue' between Aux/IAA and TIR1 (Tan et al., 2007). Another auxin receptor is the AUXIN-BINDING PROTEIN1 (ABP1) that binds auxin with high affinity and specificity (Hertel et al., 1972). ABP1 is located at the plasma membrane and in the lumen of the ER, but this glycoprotein lacks a transmembrane domain, (Henderson et al., 1997). ABP1 is secreted in the lumen of the ER and found in the apoplast. It is not clear how auxin binding to ABP1 is transduced, but ABP1 signaling has been shown to act independently of the TIR1/AFB system at a posttranscriptional level (Robert et al., 2010; Xu et al., 2010). Plants overexpressing *ABP1* exhibit an auxin-dependent increase in the size of the differentiated cells that are normally unresponsive (Jones et al., 1998), whereas loss of ABP1 function causes embryonic arrest and results in defects in cell division and cell elongation (Chen et al., 2001). Moreover, inducible inactivation of ABP1 affects plant growth by interfering with the cell cycle during postembryonic shoot (Braun et al., 2008; David et al., 2007) and root development (Tromas et al., 2009).

Auxin regulates postembryonic development

Lateral root priming, founder cell specification and primordium initiation

After germination during the vegetative growth, LRs arise from pericycle cells adjacent to the xylem pole. Because LRs are initiated constantly while the primary root grows, they are an excellent model to study organogenesis in plants. LR formation follows a periodic pattern and several studies have associated the presence of a LR with a bend formation in the primary root (De Smet et al., 2007; Laskowski et al., 2008; Ditengou et al., 2008; Moreno-Risueno et al., 2010). The last years, substantial progress has been made in our understanding on formation process of LRs. Although LRs are only visible in the maturation zone, the first events leading to LR formation take place in a region close to the root apical meristem, termed the basal meristem (De Smet et al., 2007). By intermittent activation of the synthetic AuxRE, known as the *DR5* promoter in protoxylem cells of that region, the *DR5pro::GUS* oscillatory signal correlates with the LR sites that are initiated later, when pericycle cells enter the maturation zone. Therefore, pericycle cells that will develop into LRs are first primed, shortly after leaving the root meristem. This event probably involves auxin signaling. Indeed, DR5 activation in the basal meristem also corresponds to an oscillatory gene transcription mechanism (Moreno-Risueno et al., 2010) that specifies the so-called branching points at early stages of LR formation. Accordingly, two groups of genes have been identified, of which the transcription oscillates periodically in phase or antiphase with the observed *DR5pro::luciferase* fluctuations. Auxin has been shown to regulate spacing of pericycle founder cells by generating auxin accumulation sites in the protoxylem that prime the adjacent

pericycle cells to become founder cells (De Smet et al., 2007). Local auxin accumulation causes the degradation of IAA14, thereby releasing the repression on ARF7 and ARF19, and allowing them to directly activate the expression of LBD/ASL18 and LBD/ASL16 transcription factors (Okushima et al., 2007).

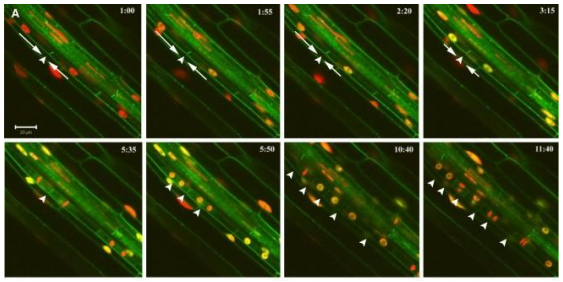

Figure 3: Confocal live imaging of the nuclear migration event in the xylem pericycle pole of a wild-type root in a p35S::FH6-GFP×p35S::H2B-RFP×pDR5::NLSGFP triple marker line. As nuclei of the founder cells move toward the common wall, they become yellow, indicative of *DR5* activation. Arrows and arrowheads mark movement of the nuclei and common cell walls, respectively **(De Rybel et al., 2010).**

Primed pericycle cells in the xylem pole that give rise to LRs must be specified as founder cells before the LR initiation can occur. The transcription factor GATA23 acts in the region above the basal meristem and is active in branching sites (De Rybel et al., 2010). Founder cell specification also involves auxin accumulation within pericycle cells prior to primordium initiation (Benková et al., 2003; Dubrovsky et al., 2011). Soon after specification, two adjacent founder cells start to divide in a well-defined pattern to initiate the primordium (Casimiro et al., 2001; De Smet et al., 2008; Dubrovsky et al., 2011). The LR initiation process includes a few rounds of anticlinal cell divisions that yield a first primordium layer (Malamy and Benfey, 1997). These initial anticlinal divisions are asymmetric and generate daughter cells of different size and fate (Casimiro et al., 2001; De Smet et al., 2008; De Rybel et al., 2010). Asymmetric divisions during LR initiation are crucial for correct primordium organogenesis (De Smet & Beeckman, 2011; Van Damme et al., 2011). Afterwards, the central daughter cells change the division plane by 90 degrees and start dividing periclinally (parallel to the main root axis). At later stages, the combination of both anticlinal and periclinal divisions results in the formation of the primordium (Figures 3 and 4).

Auxin signaling during lateral root initiation

As in many other developmental processes, auxin has a prominent role during LR initiation. Auxin accumulates in the founder cells before the first anticlinal division (Benková et al., 2003; De Rybel et al., 2010). The auxin gradient probably acts as a morphogen during primordium development (Dubrovsky et al., 2008). Auxin accumulation within the primordium induces the transcription of genes involved in patterning through the degradation of Aux/IAA proteins, allowing ARF-

dependent transcriptional activation. Accordingly, some genes encoding Aux/IAA and ARF proteins have been shown to play a role in LR development. For example, in the *solitary root1* (*slr1)* mutant, a dominant mutation resulting in the IAA14/SLR protein stabilization prevents LR formation (Fukaki et al., 2002). The IAA14/SLR protein interacts with ARF7 and ARF19 (Fukaki et al., 2005) and this IAA14/SLR-ARF7-ARF19 module regulates the expression of downstream transcription factors, including members of the LATERAL ORGAN BOUNDARIES-DOMAIN/ASYMMETRIC LEAVES-LIKE (LBD/ASL) family (Okushima et al., 2007). An additional IAA-ARF module, including BODENLOS (BDL)/IAA12 and MONOPTEROS (MP)/ARF5, has been reported to control gene transcription during LR initiation and to act downstream of the IAA14/SLR-ARF7-ARF19 module (De Smet et al., 2010). Mutations in the *BDL/IAA12* and *MP/ARF5* gene provoke irregularly positioned LRs. One prominent Aux/IAA-ARF pair is Aux/IAA28 that regulates the expression of GATA23 (De Rybel et al., 2010).

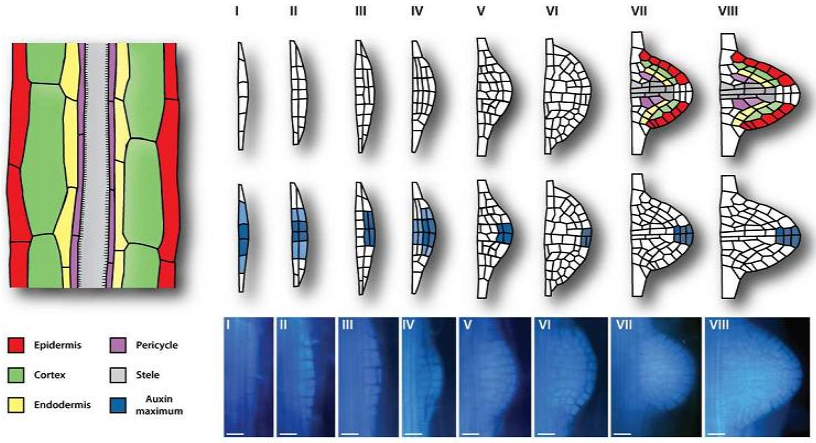

Figure 4: Morphological changes during lateral root development (Péret et al., 2009).

LRs originate within the primary root from the pericycle cells (left). Right top, the eight stages of primordium development (Malamy and Benfey, 1997); right middle, establishment of the auxin signaling maximum as demonstrated with the *DR5:GUS* reporter (blue gradient) (Benková et al., 2003); right bottom, the cartoons were drawn from aniline-blue-stained roots for each stage of lateral root development.

PAT during lateral root initiation

PAT is required for the establishment of auxin gradients within the primordium. Plants grown on high concentrations of the PAT inhibitor NPA do not form LRs. PIN-mediated PAT is involved in the start of an auxin maximum in the center of the primordium, because the expression of *PIN1*, *PIN2, PIN3, PIN4, PIN6* and *PIN7* is activated during primordium formation (Benková et al., 2003). In addition, the *pin1*, *pin3*, *pin4* and *pin7* mutants and their higher order combinations are defective in LR formation. Although no exhaustive studies have been reported to

define the localization of all these PIN proteins during primordium formation, the analysis of the dynamics in the PIN1 protein distribution revealed that PIN1 is polarized within the auxin-directing cells toward the primordium tip (Benková et al., 2003). Mutations in genes, such as *GNOM*, that affect the PIN protein intracellular trafficking also impact on the proper LR development (Geldner et al., 2004) and furthermore, mutations in genes coding for the influx carrier AUX1 and for PGP4 also resulted in defective LR numbers (Marchant et al., 2002; Mravec et al., 2008). Last but not least, the importance of PIN3 has recently been discovered during LR initiation (Marhavý et al., 2013; Vermeer et al., 2014).

To date, an extensive crosstalk between auxin and CK has been reported in many aspects of plant development at multiple levels, including metabolism, signaling, transport and transcriptional, posttranscriptional and posttranslational control. Therefore, to unravel the complexity of this crosstalk, CK metabolism, transport and signaling will be discussed in the following sections.

Cytokinins

Cytokinin (CK) is a crucial phytohormone that modulates many important developmental processes and responses to the environment, including embryogenesis, seed development, organogenesis, vascular patterning, and stress tolerance (Aloni et al., 2006; Riefler et al., 2006; To and Kieber, 2008). The interplay of CK metabolism, transport and signaling, as well as interactions of CKs with other plant hormones give these hormones the capacity to coordinate their multiple functions throughout the plant life. CK regulates the auxin pathway mainly by affecting the expression of its signaling components (Vanstraelen and Benková, 2012).

Biosynthesis and metabolism

CK biosynthesis requires two reaction types: modifications of an adenine moiety and of a side chain. Important metabolic steps can be shared with the purine metabolic pathway (Mok and Mok, 2001; Sakakibara, 2006). The rate-limiting and first step of CK biosynthesis in *Arabidopsis* is catalyzed by the ATP/ADP isopentenyltransfereses IPT1 and IPT4 and their homologs IPT3, IPT5, IPT6, IPT7 and IPT8 (Kakimoto, 2001, 2003). The isoprenoid side chain of CK can be synthesized in two pathways, the IPT-dependent methylerythiritol phosphate (MEP) and the IPT-independent mevalonate (MVA) pathways (Miyawaki et al., 2006; Sakakibara, 2006). Promoter-reporter analysis revealed a specific expression pattern for each gene and supports the hypothesis that CKs are produced in a wide range of organs and cell types (Miyawaki et al., 2004).

The distribution of bioactive CKs is finely regulated by metabolic enzymes, among which LONELY GUY (LOG) has been identified in rice (*Oryza sativa*) as an enzyme controlling CK biosynthesis and shoot meristem activity (Kurakawa et al., 2007). LOG is involved in CK activation and hydrolyzes CK nucleobase and ribose 5/monophosphate (Kuroha et al., 2009). In *Arabidopsis*, nine *LOG* genes are present that are differentially expressed in various tissues during plant development. Analyses of the triple loss-of-function mutant *log3log4log7* suggested a crucial role for *LOG* genes in the control of the CK activity during normal development in *Arabidopsis* (Tokunaga et al., 2012; Kuroha et al., 2009). CK oxidases/dehydrogenases (CKX) catalyze the irreversible degradation of isopentenyladenine, zeatin, and their ribosides by oxidative side chain cleavage. *CKX* genes are differentially expressed during plant development and their activity is confined to active growth zones (Werner et al., 2001, 2003).

Transport of cytokinins

The presence of CKs in xylem and phloem sap indicates that CKs can be transported over long distances, both acropetally and basipetally (Gillissen et al., 2000; Burkle et al., 2003; Bishopp et al., 2011b; Zhang et al., 2014). The root apical meristem is believed to be the main synthesis site of free CKs. Therefore, CKs are translocated via basipetal transport from the tip to the base of roots and acropetally from the root base to the aerial parts of plant (Gillissen et al., 2000; Burkle et al., 2003). The basipetal transport of CKs occurs through symplastic connections in the phloem and stabilizes the root vasculature pattern (Bishopp et al., 2011b). Recently, in *Arabidopsis*, ABCG14, has been found essential for the acropetal (root to shoot) translocation of root-synthesized CKs (Zhang et al., 2014). However, distinct and tissue-specific expression patterns of *IPT* genes imply the existence of different CK synthesis sources (Miyawaki et al., 2004). Hence, CKs must be moved to target cells by diffusion and/or by a selective transport system. In contrast to the very well-characterized PAT, very little is known about the cell-to-cell transport of CKs (Sakakibara, 2006). Based on the high permeability of cell membranes to free CK bases and ribosides, a diffusion mechanism for CK uptake has been proposed (Laloue et al., 1981). Thus, plasma membrane transporters do not seem to be crucial as is the case for auxins. Nevertheless, two purine permeases, PUP1 and PUP2, involved in CK transport have been described in *Arabidopsis* (Gillissen et al., 2000; Burkle et al., 2003). Transport studies in cell cultures and yeast indicated that adenine and CKs are transported by a common system. Direct measurements demonstrated that PUP1 can mediate the uptake of radiolabeled *trans*-zeatin (tZ), whereas PUP2 is able to transport a number of different CKs (Burkle et al., 2003). Thus far, an equilibrative nucleoside transporter (*ENT)* gene family has been suggested as candidate CK transporters (Li et al., 2003; Sun et al., 2005).

Cytokinin perception and signaling

Cytokinin perception in plants is similar to the two-component signaling system through which bacteria sense and respond to environmental stimuli. The signaling cascade is a multistep phospho-relay, consisting of histidine protein kinase (HK), histidine phosphotransfer proteins (HPs) and response regulators (RRs). In higher plants, two–component systems occur in CK signaling, ethylene signaling and osmoregulation (Stock et al., 2000). In a screen for *Arabidopsis* mutants in which hypocotyl explants failed to form shoots under shoot formation-inducing conditions in control explants (medium with high CK/auxin content, the *CYTOKININ RESISTANT1* (*CRE1*) gene has been identified (Inoue et al., 2001). Loss-of-function *cre1* mutants were insensitive to CK in root elongation assays. Moreover, expression of *CRE1* in a heterologous yeast system could complement a HK mutant in a CK-dependent manner (Inoue et al., 2001). Almost simultaneously, mutants in the same gene were characterized, *WOODEN LEG* (*WOL*) (Mähönen et al., 2000) and *ARABIDOPSIS HISTIDINE KINASE4* (*AHK4*) (Ueguchi et al., 2001). Subsequently, the active CKs were confirmed to bind to the extracellular domain of CRE1 and two additional genes were identified, *AHK2* and *AHK3*, which encode CK receptors in *Arabidopsis* (Higuchi et al., 2004; Nishimura et al., 2004). In *Arabidopsis*, CK is perceived by three receptors of which the large majority is localized to the ER, hinting at a central role for this compartment in CK signaling (Caesar et al., 2011; Wulfetange et al., 2011). Nevertheless, the possibility should be considered that a small part of the CK receptors signal from the plasma membrane as well (Wulfetange et al., 2011). Negative regulation of the CK signaling pathway occurs through type-A *Arabidopsis* RRs (ARRs) (To et al., 2007) of which the genes are direct targets of the type-B ARRs (Hwang and Sheen, 2001; Sakai et al., 2001) and are strongly and rapidly induced in response to CK (To et al., 2007). Genetic analysis revealed that type-A ARRs are partially redundant negative regulators of CK signaling (To et al., 2007) and that their transcription rapidly increases by exogenous CK (D'Agostino et al., 2000). Type-B ARRs act as positive regulators in the CK signaling pathway and genetic analysis revealed that ARR1, ARR2, ARR10-12 and ARR18 have overlapping functions (Sakai et al., 2001; Mason et al., 2005; Yokoyama et al., 2007). Moreover, plants lacking ARR1, ARR10 and ARR12 exhibit an almost complete insensitivity to CK, indicating that these type-B ARRs are essential for the primary CK transcriptional response (Mason et al., 2005).

Cytokinin as inhibitor of lateral root organogenesis

Besides auxin, several other plant hormones have been found to regulate LR organogenesis (Ruegger et al., 1997; Sponsel et al., 1997; Bao et al., 2004; De Smet et al., 2006b; Mouchel et al., 2006) and CK seems to be one of the major ones. In addition to auxin, CKs also influence the patterning of new LRP. The exogenous application of CK or the increase of endogenous CK levels via enhanced *IPT*

expression significantly reduced LR initiation and decreased root growth (Laplaze et al., 2007; Růžička et al., 2009). Exogenous CK leads to abnormal tangential and oblique divisions in stage-II LRP, followed by ectopic divisions in the inner layer and rounds of periclinal divisions in the central cells of the outer layer, giving rise to flattened LRP (Laplaze et al., 2007). Interestingly, a similar deviation from the otherwise tightly controlled cellular divisions during LRP organogenesis has been observed in a mutant defective for the AP2/EREBP transcription factor PUCHI (Hirota et al., 2007).

Cytokinin signal transduction during lateral root formation

CKs have been shown to inhibit LR initiation by blocking the pericycle founder cells in the G2-to-M transition phase progression (Li et al., 2006). This inhibition process seems to be mediated by the CK receptor CRE1/AHK4 (Li et al., 2006) and requires AHPs (Hutchison et al., 2006). Furthermore, exogenous auxin can only rescue cell divisions but not LR initiation and development (Li et al., 2006; Laplaze et al., 2007), indicating that CK accumulation in pericycle cells does not prevent the auxin-mediated activation of cell divisions, but rather blocks the developmental program of LR initiation (Laplaze et al., 2007). In contrast, overexpression of *CKX* enhanced LR formation (Werner et al., 2001, 2003). Regarding the different elements of the CK signaling pathway, mutations in CK receptors and in the CK signaling components, type-B ARRs increase the number of LRs, whereas mutations in type-A ARRs decrease them, in agreement with the inhibitory role of CK on LR development (Mason et al., 2005, To et al., 2007).

Auxin-cytokinin: An antagonistic effect

A fundamental question in developmental biology is how multicellular organisms coordinate cell division and differentiation to determine organ size. Since the initial studies on auxin in the 19th century, many *in vivo*, *in vitro* and *in silico* experiments have provided a broad knowledge on plant hormones, namely on their role during the plant life cycle, their biosynthesis, metabolism, transport, perception and their signaling pathways. In the meantime, hormones have been proposed to act through a network of interacting responses rather than through isolated linear pathways. This suggestion raises the question of how hormone signaling pathways trigger specific outcomes while operating in a communication network with other pathways (Coenen and Lomax, 1997; Chandler, 2009; Kuppusamy et al., 2009).

Auxin-cytokinin crosstalk and the regulation of plant development

The interaction of auxin and CK signaling pathways is crucial in the control of the shoot apical meristem activity and the specification of the embryonic root. In

Arabidopsis, CK and auxin are known to act antagonistically to control plant growth and development. CK promotes cell differentiation by repressing both auxin signaling and transport, whereas auxin sustains root meristem activity by promoting cell division. Their coordinated action is essential for the maintenance of the root meristem size and for ensuring root growth (Dello Ioio et al., 2007; Růžička et al., 2009; Moubayidin et al., 2009). Accordingly, CK influences the formation of auxin gradients, both at the transcriptional and posttranscriptional levels. In root apical meristems, the interaction between the auxin and CK signaling pathways is mediated through the primary CK response transcription factor, *ARR1* and *SHORT HYPOCOTYL2 (SHY2)/IAA3*, an auxin signaling repressor (Dello Ioio et al., 2008). CK activates the *SHY2/IAA3* promoter specifically at the tissue transition zone (Dello Ioio et al., 2008). Via an AHK3 receptor and an ARR1 response regulator, the *SHY2/IAA3* activation results in auxin signaling repression and consequently, in downregulation of the expression of the *PIN* auxin efflux carriers (Dello Ioio et al., 2008; Růžička et al., 2009). Exogenous CK treatments interfere with the PIN protein transcription (Laplaze et al., 2007). Besides repression of *PIN1* transcription, the expression of other PIN family members is also affected by CK. For example, *PIN4* and *PIN3* are down-regulated, whereas the *PIN2* expression is CK insensitive and *PIN7* is up-regulated after CK treatments (Růžička et al., 2009). Besides transcriptional regulation, CK also controls the PIN proteins at the posttranscriptional level (Marhavý et al., 2011; Yoshida et al., 2011; Zhang, W. et al., 2011). Indeed, during the early stages of LRP formation, CK-treated PIN1 is rapidly degraded in lytic vacuoles and prevents the creation of an auxin gradient, which is required for normal LRP patterning (Laplaze et al., 2007; Marhavý et al., 2011). The CK signal is transduced through the CRE1/AHK4 receptor that modulates the PIN1 endocytic trafficking and redirects this membrane protein for lytic degradation into the vacuoles. Stimulation of the lytic PIN1 degradation is not a default effect of general down regulation of proteins from plasma membranes, but a specific mechanism to rapidly modulate the auxin distribution in CK-mediated developmental processes (Marhavý et al., 2011) (Figure 5). However, the mechanism underlying the interference with the PIN1 endocytic trafficking remains poorly understood (Marhavý et al., 2011).

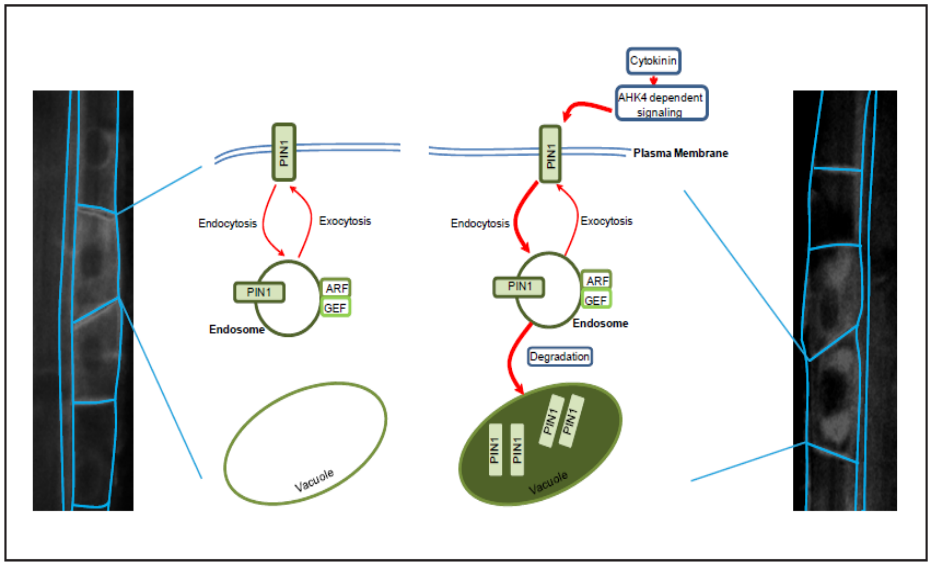

Figure 5: Cytokinin-mediated posttranscriptional regulation during plant organogenesis (Marhavý et al., 2011).

Cytokinin regulates PIN1 trafficking to the vacuole, through the endocytosis machinery. Functional CK perception is required for the CK-induced PIN1 lytic degradation.

The negative regulators of the CK signaling pathway, ARR7 and ARR15, integrate CK and auxin signals in the embryonic root as well as in shoot stem cell niche (Müller and Sheen, 2008; Zhao et al., 2010). Auxin-controlled activity of *ARR7* and *ARR15* is necessary for proper embryo development and embryos in which both genes are not functional show strong patterning defects (Müller and Sheen, 2008). In the shoot meristem, the expression of *ARR7* and *ARR15* is induced by CK, whereas auxin has a negative effect, at least in part, by the ARR5/MONOPTEROS (MP) transcription factor (Zhao et al., 2010). These regulatory mechanisms confirm the antagonism between auxin and CK in the root meristem, but they hint at a synergy between the two hormones in the shoot apical meristem, as supported by classic shoot regeneration experiments. In conclusion, the auxin-CK crosstalk is spatially and temporally regulated. To fully understand the developmental processes and the responses to the environment, interactions with other hormones must be considered too. Further studies by means of genome-wide profiling for epigenetics, transcriptomics, and proteomics will provide new information about the interconnected web of hormonal actions.

Cytoskeleton: The spider's web

The cytoskeleton is a scaffold made of proteinaceous fibers that is required for the majority of essential cellular functions, such as cell morphogenesis, organogenesis and development. Cell division, cell expansion, cell differentiation and cell-to-cell communication are fundamentally important in the development of

multicellular organisms (Kueh et al., 2009). The cytoskeleton, as a dynamic network of continuously changing polymers, quickly reacts by its reorganization to various internal and external stimuli. Its features, resulting from the actin and tubulin polymer structures, designate its function as polar transport vector for molecules and organelles within cells. Proteins interacting with the cytoskeletal polymers modulate the cytoskeleton structure and function. Plants contain two groups of cytoskeletal filaments: microtubules (MTs) and actin filaments.

Microtubule organization, structure and dynamics

In both plant and mammalian cells, MTs are essential for fundamental cellular functions, including determination of cell shape and polarity, intracellular organization, organelle transport and cell division (Sedbrook, 2004). MTs are hollow cylinders with a diameter of around 25 nm. They consist of laterally grouped protofilament fibers that are composed of α- and β-tubulin heterodimer subunits (Desai and Mitchison, 1997). Both the α- and β-subunits are bound to a molecule of GTP (Guanosine-5'-triphosphate). The GTP bound to α-tubulin is stable, but the GTP bound to β-tubulin may be hydrolized to GDP shortly after assembly. Both ends of the microtubule cylinders are distinctly different: the plus end is terminated by β-tubulin, whereas the minus end has α-tubulin at its end. The difference between minus- and plus-ends can be specifically recognized by various microtubule-binding proteins and the ends show different assembly/disassembly rates of subunits. Taken together, the MTs have a polar head-to-tail fashion, which is essential for the functioning of the MTs as tracks. MTs can show treadmilling, which occurs when the filament adds subunits at the plus end, but simultaneously losses subunits from the minus end. As a result, the MT seems to translocate. However, marks created by photobleaching on these migrating MTs remained stationary as the MT moved, demonstrating that MTs do not physically move, but rather reposition through the addition and loss of tubulin dimers. MTs are very dynamic structures that undergo periods of growth (polymerization), pausing, and shortening (depolymerization) and the transitions are called catastrophe (from assembly to disassembly) and rescue (from disassembly to assembly). This behavior of MTs is known as dynamic instability (Figure 6). Assembly-polymerization and disassembly-depolymerization of MTs are driven by GTP hydrolysis. Tubulin subunits that are added to the MT are in the GTP conformation. The GTP from β-tubulin is hydrolysed to GDP after incorporation, while the GTP from α-tubulin is stable. It has been proposed that this GTP hydrolysis changes the conformation of a protofilament from a slightly curved tubulin GTP to a more profoundly curved tubulin-GDP structure. This model therefore predicts that growing MTs contain a “GTP cap” which forces the rest of the more curved tubulin-GDP subunits to remain straight in the MT lattice. When the amount of incorporated tubulin-GDP subunits increases, the GTP cap is lost and the contacts between the protofilaments are destabilized, resulting in depolymerization

of the MTs. Additionally, the dynamic instability of microtubules is regulated by a range of MAPs (microtubule associated proteins).

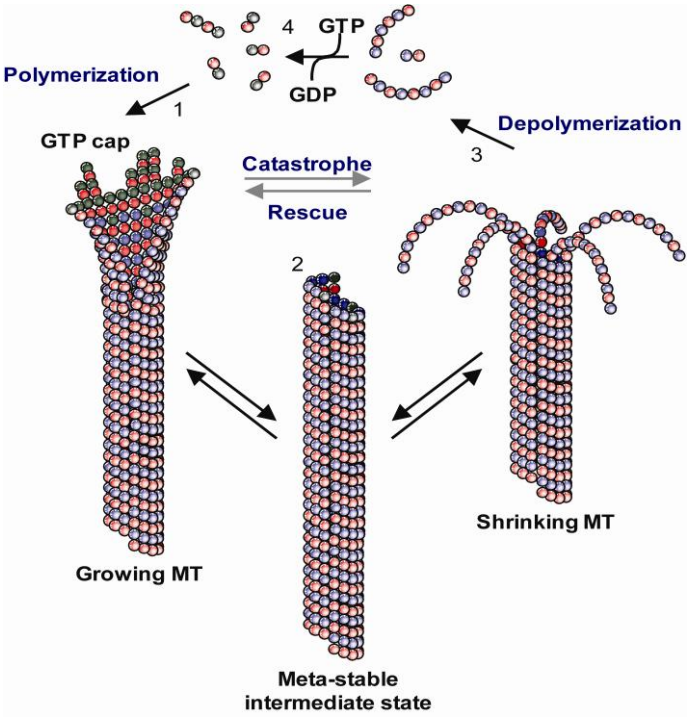

Figure 6: MT dynamic instability (Akhmanova and Steinmetz, 2008).

Schematic drawing of dynamic instability. Tubulin GTP assembles at the end of a MT, forming a stabilizing structure, whereas the GTP cap prevents MTs from shrinking (1). The closure of the tubulin open sheet at the end of a growing MT generates a metastable, blunt-ended MT intermediate (2). When the GTP cap is lost due to GTP hydrolysis, the MT switches to a shrinking state and rapidly disassembles by protofilament peeling. This conformational change, presumably dependent on GDP tubulin conformation, may destabilize the lateral contacts between adjacent protofilaments (3). The cycle is completed by exchange, upon depolymerization, of the GDP of β-tubulin dimers to GTP (4).

Tubulins

Tubulins are globular proteins consisting of two closely related 55-kDa polypeptides, the α- and β-subunits (Williams et al., 1999). The α-tubulin subunit binds GTP irreversibly and does not hydrolyze it, whereas GTP bound to the β-tubulin subunit is hydrolyzed to GDP during or shortly after MT assembly. The GTP cycle is essential for the dynamic instability of MT. GTP hydrolysis reduces the binding affinity of tubulin to adjacent molecules, thereby favoring MT depolymerization (Shivanna et al., 1993; Davis et al., 1994; Jayaram and Haley 1994). In *Arabidopsis*, tubulin genes form a large group including at least six α-tubulin (*TUA*) (Kopczak et al., 1992), nine β-tubulin (*TUB*) (Snustad et al., 1992), and two γ-tubulin (*TUG*) genes (Liu et al., 1994). Dominant-negative point mutations in either the *TUA6* or *TUA4* genes (*lefty1* or *lefty2*, respectively) cause a right-handed

cortical microtubule (cMT) array in root epidermal cells that twist roots in a direction roughly perpendicular to the array orientation. The *lefty1*/*lefty2* double mutants show helical growth in hypocotyl and radial cell expansion in the root elongation zone. CMTs in the double mutant are more fragmented and oriented randomly. Analyses of *lefty* mutants show that altered microtubular conformations induce twisted phenotype indicating an important role of MTs in directional plant growth (Thitamadee et al., 2002). Similar mutant phenotypes, helical MT arrays and left- or right-handed organ twisting were observed in plants with point mutations in either *TUA* or *TUB* genes (Ishida et al., 2007). The direction of the helical organ growth depends on the mutation site and the exchanged amino acid in the tubulin. It was shown that the direction of helical organ growth depends on mutation site and the exchanged amino acid in tubulin (Ishida et al., 2007). Additionally, the reduced *TUA6* gene expression in antisense *Arabidopsis* plants leads to a dwarf phenotype and abnormal gravitropic response (Bao et al., 2001). Taken together, normal MT functions are critical for transverse MT organization and maintaining directional anisotropic growth. Moreover, plant tubulin undergoes various reversible posttranslational modifications, including tyrosination, acetylation, detyrosination and polyglutamylation (Smertenko et al., 1997) that influence organization and function of MTs (Wang et al., 2004).

Tubulin posttranslational modification

Tubulin is a target of multiple posttranslational modifications (PTMs). These modifications, conserved throughout evolution, are thought to act individually or in combination to control specific MT based functions. Some of these PTMs are ubiquitous protein modifications, such as acetylation, phosphorylation or palmitoylation, while others are less common, and some appear to be unique to tubulin. Among these rare modifications are the removal of gene-encoded amino acids by detyrosination and the follow-up deglutamylation of α-tubulin, or the addition of amino acids by tyrosination, polyglutamylation, or polyglycylation (Figure 7). Many of these modifications were known for decades, yet their functional roles in different MT structures are only now starting to emerge. Most post-translational modifications occur on MTs rather than tubulin dimers and are more abundant in stable MTs rather dynamic ones (Smertenko et al., 1997).

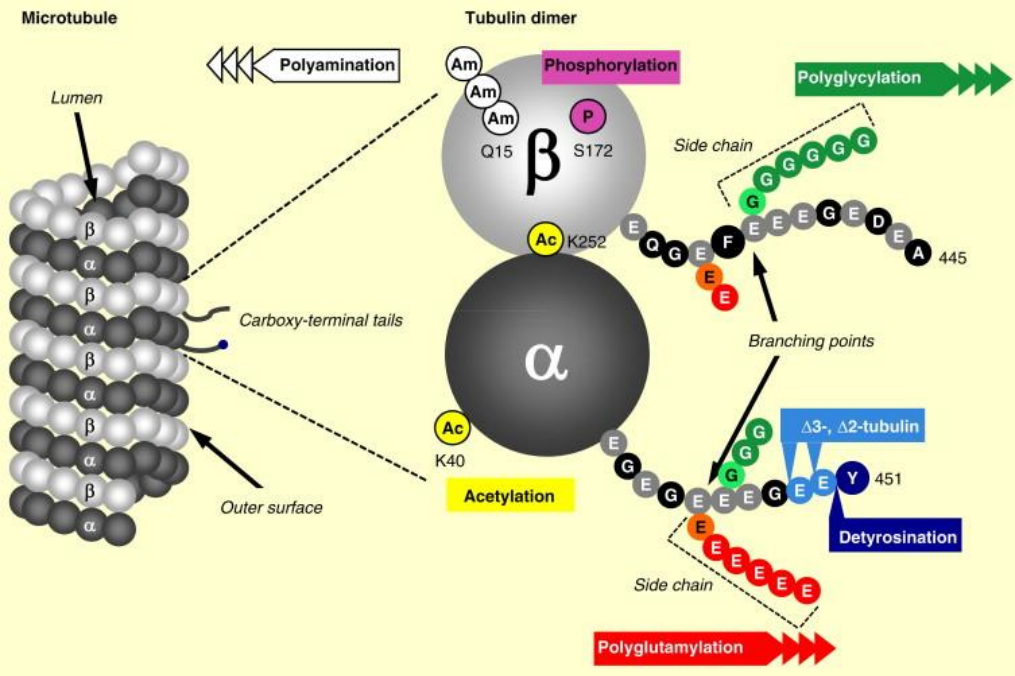

Figure 7: Molecular localization of tubulin posttranslational modifications (Magiera and Janke, 2014).

Microtubules are assembled from α-tubulin–β-tubulin dimers, which form hollow tubes composed of 13 protofilaments. The globular parts of the tubulins form the microtubule walls and the luminal surface, while the carboxy-terminal tails decorate the outer surface of microtubules, where many MAPs and motors bind. PTMs are found on various regions of the tubulin dimer: the α-tubulin K40 acetylation site is present at the luminal surface of microtubules, while the K252 acetylation site of β-tubulin is present on the boundary between α- and β-tubulins. Polyamination at Q15 and phosphorylation at S172 are found in the globular, folded part of β-tubulin and might influence the assembly rates and stability of microtubules. Detyrosination/tyrosination, Δ2- and Δ3-tubulin modulate the carboxy-terminal tails of α-tubulins, and polyglutamylation and polyglycylation are found within the carboxy-terminal tails of both α- and β-tubulin. All modifications of the carboxy-terminal tails are likely to regulate interactions between microtubules and associated proteins.

Tubulin tyrosination/detyrosination

Detyrosination of tubulin is a fairly common PTM. In fact, tubulins are normally synthesized with tyrosine as the last C-terminal amino acid. The enzymatic removal of this amino acid generates detyrosinated tubulin (also called Glu-tubulin); MTs containing significant amounts of Glu-tubulin may exhibit different properties. The opposite process of detyrosination, tyrosination, requires the enzymatic addition of tyrosine by the tubulin tyrosine ligase (TTL) enzyme which was the first tubulin-modifying enzyme to be identified. Biochemical and structural work has demonstrated that TTL exclusively modifies unpolymerized tubulin, which implies that the detyrosination/retyrosination cycle of tubulin depends critically upon the dynamic instability of MTs. This mechanism, together with the preference of the tubulin-detyrosinating enzyme for polymerized MTs, results in an accumulation of

detyrosinated tubulin in stable MTs. In plant cells, different MT subsets show dissimilar levels of tyrosinated/detyrosinated tubulin, but these ratios do not always correspond exactly with the supposed MT stability. The majority of the plant cell MTs seems to be tyrosinated and only a small subgroup is detyrosinated (Smertenko et al., 1997).

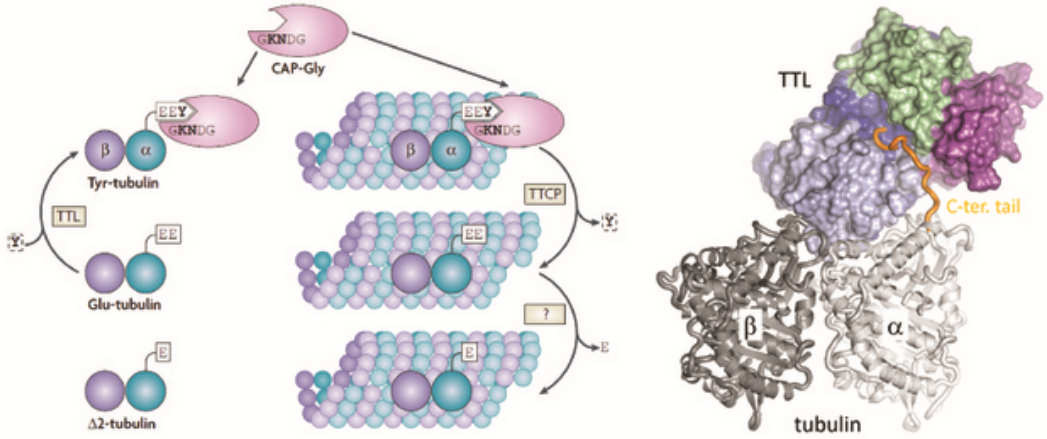

Figure 8: Tubulin tyrosination/detyrosination cycle (Akhmanova and Steinmetz, 2008).

In most eukaryotic cells, the C-terminal EEY/F tail of α-tubulin is subject to a detyrosination/tyrosination cycle, in which the C-terminal tyrosine is repeatedly cleaved and re-added by a tubulin tyrosine carboxypeptidase (TTCP; acting on microtubules) and tubulin tyrosin ligase (TTL; acting on tubulin), respectively (left image). αTubulin tyrosination has been linked to the ability of the MTs end to recruit CAP-Gly proteins such as the +TIPs CLIP170, and CLIP115. Crystal structure of TTL in complex with tubulin (right image).

In maize (*Zea mays*), for example, the tyrosinated α1–α4(α5) isoforms are common to all tissues tested, thus confirming that tyrosinated α-tubulin is universally the predominant isoform in plant cells (MacRae, 1997) and that the relative tyrosinated/detyrosinated tubulin ratio is unrelated to the MT stability. Recently, inhibition of the enzymatic removal of tyrosine by incorporation of a chemically modified terminal tyrosine has revealed a number of effects, especially in dividing cells, with the concomitant stimulation of cell length and changes in cell wall organization (Jovanović et al., 2010). These findings suggest that the detyrosination/tyrosination cycle of tubulin might regulate the transition from the elongation to the division stage. As a consequence of its limited distribution, detyrosinated α-tubulin does not occur commonly in plant tissues, but accumulates specifically in particular tissues (Wang et al., 2004). The cellular processes inherent to the tyrosination/detyrosination of tubulin are probably linked to the impact of this C-terminal amino acid on the binding of specific tubulin-associated proteins. It has been demonstrated that CAP-Gly proteins, a subgroup of plus-end tracking proteins (+TIPs; Akhmanova and Steinmetz, 2008) (Figure 8), are regulated by the tyrosination status of alpha-tubulin (Peris et al., 2006). Therefore, the binding

between modified tubulin and associated proteins with a depolymerizing activity might represent the link between MT stability and relative content of tyrosinated/detyrosinated tubulin (Paroota et al., 2013).

MAPs: Cellular markers for microtubule orientations

The dynamic instability of MTs and their organization can be regulated by their interactions with other proteins, the so-called microtubule-associated proteins (MAPs). MAPs were defined as proteins that copurify with MTs *in vitro* after several polymerization and depolymerization cycles (Lloyd and Hussey, 2001). Currently, MAPs are considered to be proteins that associate with MTs by direct or indirect interactions, co-localize with MTs *in vivo*, or are homologous to a known MAP. A large variety of MAPs have been identified with a wide range of functions, such as MT destabilization either by activity severing or by promoting depolymerization of tubulins from the MT ends, binding of other MAPs to MTs, stability increase, or interfilament interaction promotion (Sedbrook, 2004). MAP4 has no homologues in plants, but phosphorylation and dephosphorylation also influence the organization and stability of the cytoskeleton. When root cells were treated with both phosphatase and kinase inhibitors, the cortical MTs became considerably disorganized, demonstrating that protein phosphorylation and dephosphorylation are important for the control of cortical MT arrays (Baskin and Wilson, 1997).

MAPs can be divided into two groups. One group includes structural MAPs that mediate interactions with MTs and other cell structures that stabilize MTs and govern their polymerization (Mandelkow and Mandelkow, 1995). Structural MAPs reduce the critical threshold concentration of tubulins required for MT polymerization and, thereby, increasing its speed (Nick, 1998). Many linker proteins are present between cortical MTs and the plasma membrane, of which phospholipase D has been identified as one of them (Gardiner et al., 2003). Severing of the MTs at the cortical cytoplasm of the isotropically growing root hair is ensured by plant homologs of the mammalian protein katanin (Bartolini and Gundersen, 2006). Also homologs of the mammalian plus-end-tracking proteins have been detected in plants, namely SPIRAL1 (SPR1), CLIP-associated protein (CLASP) and END BINDING1 (EB1) (Hamada, 2007). These plus-end-residing proteins have been described as regulatory proteins of the MT instability and as sensors of environmental determinants in the cell (Sedbrook and Kaloriti, 2008).

In *Arabidopsis*, 3 homologs of the highly conserved +TIP EB1 are found, namely AtEB1a, AtEB1b and AtEB1c. Of these at least both AtEB1a and AtEB1b were shown to accumulate at the MTs plus ends in the typical comet-like fashion (Mathur et al., 2003; Chan et al., 2003; Van Damme et al., 2004a). All three EB1 proteins contain a conserved N-terminal calponin homology domain (CH) required for MT binding and a conserved coiled-coil domain, predicted to be involved in protein-

protein interaction (Bu and Su, 2003). This domain is necessary and sufficient for binding to MTs and recognizing growing MT ends (Hayashi and Ikura, 2003; Komarova et al., 2009). In budding yeast and *Drosophila* cells, EB1 recruits several other proteins to growing MT ends and participates in control of MT dynamics, EB1 has been shown to stimulate both catastrophes and rescues, making MTs more dynamic, while it decreased the time MTs spend on pausing (Tirnauer et al., 1999; Rogers et al., 2002).

In addition, MTs are organized in specific structures during the cell cycle. During the preprophase, they form the preprophase band (PPB) of MTs marking the site of the future localization of the newly formed cell plate. The importance of MTs is obvious during mitosis and cytokinesis, where they form the mitotic spindle and phragmoplast, respectively. PPB disappears in the prometaphase, after the mitotic spindle is formed, to segregate the two daughter chromosomes. The PPB position is memorized throughout mitosis for the eventual positioning of the cell plate insertion site during cytokinesis (Van Damme, 2009; Rasmussen et al., 2011) (Figure 9). Recently, the *Arabidopsis SABRE* (*SAB*) gene was identified, mechanistically linking its action to CLASP-mediated microtubule organization. It was shown that SABRE stabilizes the orientation of CLASP-labelled preprophase band microtubules predicting the cell division plane, and of cortical microtubules driving cell elongation (Pietra et al., 2013). In the interphase cells, MTs are localized in the cortical cytoplasm where they form the cortical MT network that underlines the cell wall. Cortical MTs are generally parallel to each other and, in most cases, perpendicular to the preferred cell growth direction (Nick, 1998). The orientation of cortical MTs plays an important role in the cell polarity maintenance by positioning newly synthesized cellulose microfibrils on the inner side of the cell wall (Giddings and Staehelin, 1991).

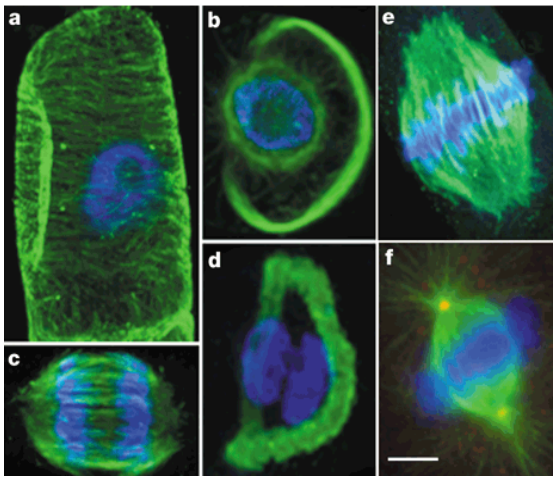

Figure 9: The Plant Microtubule cycle (Lloyd and Hussey, 2001).

(a) Interphase cortical MTs. (b) Preprophase band. (c) Plant spindle during anaphase. (d) Phragmoplast. (e) Plant metaphase spindle. (f) Mammalian metaphase spindle. Green and blue, MTs and DNA, respectively. Scale bar = 7.5 μm.

In most cells F-actin filaments are not co-aligned with MT during interphase. However, in root hairs the orientation of F-actin filaments depends on MTs. So, in

the next section we highlight the role of actin filament on root growth and development.

Actin Filaments (AFs)

AFs consist of two linear polymers of globular actin monomers (G-actin) covered with a spectrum of associated proteins. In the vegetative cells of *Arabidopsis*, three actin isoforms are prevalently present, encoded by the *ACTIN2, ACTIN7,* and *ACTIN8* genes (Kandasamy et al., 2009). The dynamic behavior of AFs highly depends on the presence of actin-binding proteins (ABPs). ABPs can be roughly divided into the following groups: nucleating factors, capping proteins, severing proteins and side-binding proteins (Staiger et al., 2010). These proteins control the G-actin to F-actin ratio, participate in the nucleation of AFs, control the polymerization speed and are involved in the organization of filaments into higher structures.

The monomer-binding proteins are profilins, ACTIN-DEPOLYMERIZING FACTOR (ADF) proteins and CYCLASE ASSOCIATED PROTEINs (CAPs) that control the AF turnover (Staiger et al., 2010). Profilin makes a complex with G-actin and this binding prevents spontaneous nucleation at the pointed end of AFs. In case of uncapped, barbed-end AFs, profilin contributes to the filament elongation by delivering monomeric actin (Staiger et al., 1997). Formins, plasma membrane-associated actin nucleation proteins interact with profilin and are major AF nucleators in plants (Cvrckova et al., 2004b). ADF proteins regulate the actin dynamics by keeping the monomeric actin pool, whereas CAPs bind ATP-G-actin as well as ADP-G-actin, facilitating the nucleotide exchange on actin. The monomer-binding proteins work probably in synergy. CAPs that can bind monomers and exchange nucleotides might function in-between the ADF-mediated depolymerization and profilin-based shuttling of ATP-G-actin onto the barbed-end AFs (Staiger et al., 2010). The proposed actin dynamics model in plant cells assumes that the major monomeric actin pool is bound by profilin, preventing its spontaneous nucleation and pointed-end growth. New filaments with available noncapped, barbed ends are created by nucleating factors (formins or the Arp2/3 complex), either *de novo* or along the mother filament sides or grow from the fragment ends or from recently severed filaments with free barbed ends by the cooperation of actin with ABPs (Staiger et al., 2010).

In the interphase cell, AFs are localized in the cortical cytoplasm and, although they do not coalign with cortical MTs, they seem to stabilize them at the plasma membrane (Shibaoka, 1994). AFs were found along the MTs in the PPB and the phragmoplast (Kakimoto and Shibaoka, 1987). Despite some of the actin bundles are often aligned with the long cell axes, actin is present around the interphase nucleus and in the cytoplasmic strands, where, together with associated proteins,

they ensure cytoplasmic streaming, organelle movements and vesicle trafficking. In mammalian and plant cells, the actin and microtubule cytoskeleton cooperatively interact to regulate cell polarity and vesicle trafficking (Goode et al., 2000; Petrašék and Schwarzerova, 2009).

AFs and cell polarity

AFs play a crucial role during diffuse cell growth as well as in the polarized apical growth of root hairs, trichomes and pollen tubes (Hussey et al. 2006; Szymanski, 2009). In tip growth, actin serves as a scaffold for transport and docking of secretory vesicles at the plasma membrane (Staiger et al., 2010). Actin polymerization is necessary for the membrane vesicle accumulation at the pollen tube tips, whereas actin depolymerization accompanies vesicular docking and fusion at the plasma membrane (Lee et al., 2008). Small GTPases have an important function in these processes. In growing pollen tubes, small GTPases Rop (Rho of plants) were found to have similarities with the Rho GTPases of mammals and yeast (Lin et al., 1996). These signaling molecules are involved in the AF organization by modeling of the Ca^{+2} flow (Li et al., 1999). Tip-localized Rop GTPases act by antagonistically working with the ROP-interactive CRIB motif-containing proteins RIC3 and RIC4 (Hwang et al., 2005).

The actin-related protein (Arp2/3) complex is one of the possible nucleators of actin in plants (Staiger and Blanchoin, 2006). This complex is activated by the Wiskott-Aldrich Syndrome Protein (WASP/WAVE) complex. Some of the subunits of the WAVE complex appear to be ROP effectors that translate ROP GTPase signals into nucleation responses of AFs. The WAVE-Arp2/3 activation might generate new actin nucleation domains at the cortical cytoplasm and, thus, contribute into a coordinated cell expansion (Szymansky, 2009). ROP proteins appear to interact directly with the upstream regulators of the Arp2/3 complex that plays a role in the modulation of the spatial distribution of cortical F-actin and in the activation of the polymerization of some types of AFs in cells with complex shapes (Li et al., 2003). The ROP function depends on the class 1 ADP-ribosylation (ARFs), which are core components of the vesicle transport machinery that is involved in the polar localization of PIN auxin efflux carriers (Xu and Scheres, 2005a; Boutte et al., 2007; Geldner, 2009).

Similarly, leaf epidermal pavement cells of *Arabidopsis* and their jigsaw morphology make them a favorite model to study the establishment of cell polarity during cell shaping. The fine actin network with MTs (Fu et al., 2005) and its ROP GTPase-dependent reorganization have been shown to play an important role in pavement cell development (Settleman, 2005). The coordinated communication between adjacent cells permits a local growth inhibition concomitant to the outgrowth activation of the neighboring cells by the regulation of cytoskeletal

components. Two mutually exclusive ROP pathways modulate this process (Fu et al., 2005, 2009). Whereas cortical MTs inhibit the growth at the indentation, the F-actin array at the lobe promotes local elongation. Active ROP2 locally stimulates RIC4 at the lobe tips to allow the formation of fine cortical AFs and lobe development, but ROP6 activates RIC1, leading to organized MT formation at the indentation, thus constraining growth. The RIC1-dependent MT organization not only inhibits the local outgrowth, but, also suppresses the ROP2 activity, whereas the lobe-activated ROP2 inhibits RIC1 activity and MT organization. Thus, both pathways antagonize each other for the cell shape formation by using different cytoskeletal components. Interestingly, PIN proteins, auxin and their interactions with ROP GTPases have been hypothesized to play a role in lobe development (Fu et al., 2009; Xu et al., 2010; Lin et al., 2013).

Small GTPases seem to be important in trafficking events as well as in the control of cytoskeletal changes and signaling events in the cells. In yeast and mammalian cells, clathrin-mediated endocytosis and Arp2/3-mediated actin assembly have been linked (Galleta and Cooper, 2009). Although many ABPs have been identified in *Arabidopsis*, the direct proof of a connection between actin and the clathrin-mediated endocytic machinery is still missing. As mentioned above, the intracellular polarization of auxin transporters is associated with the vesicle trafficking processes (Steinmann et al., 1999; Geldner et al., 2003; Kleine-Vehn et al., 2006). AFs are considered guides for vesicle trafficking and polar growth in plants (Voigt et al., 2005) and have been shown to play important roles in auxin transport, PIN recycling (Dhonukshe et al., 2008b; Geldner et al., 2001) and polarity (Baluška et al., 2001; Kleine-Vehn et al., 2008a). MTs have been proposed previously to partake in the PIN protein positioning by means of anti-tubulin drugs (Boutté et al., 2006), but the information is limited regarding the mechanism of the cytoskeleton participation in the auxin efflux carrier trafficking and targeting. The basally localized PIN1 in the stele and PIN2 in young cortex cells are sensitive to MT disruption by oryzalin, whereas apical PIN2 in the epidermis is largely insensitive to the MT array disruptions (Kleine-Vehn et al., 2008a). The PIN1 enrichment at the expanding lobe tip is necessary for the generation of a local auxin gradient and for auxin-promoted pavement cells (PC) interdigitation. At the lobe tip, ROP2 attenuates the auxin-inhibited, clathrin-mediated PIN1 endocytosis (Nagawa et al., 2012). ROP2-activated, RIC4-dependent cortical F-actin formation at the lobe inhibits PIN1 endocytosis in a manner similar to that of the chemical AFs stabilization in root tips (Dhonukshe et al., 2008b). This mechanism hints at a conserved function for the actin cytoskeleton in the PIN internalization regulation, as further supported by the required ROP/RIC signaling for clathrin-mediated PIN internalization in roots (Chen et al., 2012; Lin et al., 2012). These data suggest that delivery and maintenance of polar cargos to apical or basal cell domains depend on different arrays of cytoskeletal components.

Hormonal regulation of MT dynamics and organization

Phytohormones are the principal regulatory molecules in plant cells in which they control essentially all physiological processes. The cytoskeleton is the basis for the cell architecture, intracellular and cellular motility, and cellular proliferation capacity; moreover, it is a scaffold for most biochemical processes and the target for external and internal regulatory signals (Klyachko, 2003) that have been demonstrated for anisotropic cell expansion, stomata opening, gravitropic bending, and some other processes (Klyachko, 2003). There is a clear relationship between the components of the cytoskeleton and phytohormones because MT orientation/organization, their dynamic properties, expression of genes encoding various tubulin and actin isotypes, and also their posttranslational modifications are determined by the action of various growth regulators on the plant cell.

Actin microfilaments, arranged in parallel with MT, are evidently tightly connected with them and affect the rate of MT reorientation (Blancaflor, 2000). Several factors influence MT reorientation: change during cell growth and development under the influence of various abiotic (photoperiod, mechanical stimuli, temperature changes, etc.) and biotic (infection with viral, bacterial, or fungal pathogens) factors. Phytohormones are one of the most effective factors, and their effects on MT reorientation were considered in a number of comprehensive reviews (Shibaoka, 1994; Nick, 1998). Auxins, gibberellins, ethylene, abscisic acid, and brassinosteroids exert such an action. Auxins facilitate transverse MT orientation in stems and coleoptiles. GA, which stimulates elongation of stems and roots, usually induces transverse MT orientation. Ethylene and ABA increase the proportion of longitudinal MT in the systems where these hormones retard growth. The most studied effects of phytohormones are those on the morphology and spatial localization of MTs and actin that are exerted by auxin, gibberellins, CKs, ethylene, ABA, and brassinosteroids. In fact, phytohormone effects on MT orientation are much more complex than it is briefly considered above. They can differ in different tissue types, in different growth zones, and even near different parts of the cell wall in one and the same cell (Blume et al., 2013). Although some hormones, such as auxin, gibberellins, and ethylene, have been reported to reorient cortical MTs in plant cells (Shibaoka, 1994; Polko et al., 2012). Auxins facilitate the transverse MT orientation in stems and coleoptiles, where they induce growth acceleration and promote the longitudinal orientation of MTs in roots, of which growth is affected by auxins (Blancaflor, 2000). Both auxin and gibberellic acid affect hypocotyl cell elongation, and both hormones alter MT array organization when exogenously applied (Vineyard et al., 2013). In the next section, we will summarize some of the phytohormonal effect on MTs orientation and organization.

Auxin and microtubule

Proper development of plant organs, flowers and leaves in particular, is determined by the localized growth controlled by auxins and directed growth dependent on MTs (Heisler et al., 2010). In spite of the abundance of data concerning polar auxin transport and its physiological importance for plants, auxin action on the cytoskeleton dynamics and expression of its constituent proteins is relatively poorly studied. It was shown that auxin induced root organogenesis is preceded by MT rearrangement required for polar cell growth (Baluška et al., 1996; Verbelen et al., 2008). It was established that auxin treatment increased the number of cells with transverse MT orientation in maize coleoptiles (Baluška et al., 1996). It is known that transverse MT orientation prevails under conditions favorable for cell growth, in particular, in the presence of auxins or gibberellins, whereas longitudinal MT orientation prevails under conditions unfavorable for cell growth. Moreover, it is proposed that a transition of cortical MT orientation from longitudinal to transverse under the control of endogenous auxins is a physiological process necessary for root growth and differentiation (Baluška et al., 1996).

MT reorientation by auxins may be mediated by the increase in the concentration of cytoplasmic Ca^{+2} and by the rearrangement of MT organizing centers (MTOCs), due to direct auxin action on phosphorylation of cytoskeletal proteins (Baluška et al., 1996) or due to changes in the interaction between MTs and MAPs. Transverse or longitudinal orientation of MTs may be also dependent on posttranslational modifications of α- or β-tubulins. In particular, auxin induced α-tubulin tyrosination is correlated with the enhanced sensitivity of plant growth to ethyl-N-phenylcarbamate, a compound with anti-microtubule activity, which is explained by suppression of MT dynamics (Wiesler et al., 2002). Dynamic MTs accumulated under the influence of auxins comprise mainly tyrosinated α-tubulin, whereas more stable longitudinal MTs, predominated under auxin deficiency, comprise detyrosinated α-tubulin. It seems likely that auxins suppresses α-tubulin detyrosination via their effect on the activity of tyrosine carboxypeptidases (Wiesler et al., 2002).

Gibberellin and microtubule

In addition, gibberellins (GAs) are phytohormones that promote plant growth by stimulating axial cell expansion. The growth-stimulatory effect of GAs is correlated with an increased transverse orientation of the cortical MT array (Sambade, et al., 2012; Sauret-Gueto et al., 2012). Whereas the GA signaling pathway has been studied extensively (Daviere and Achard, 2013; Hauvermale et al., 2012), the regulation of cortical MTs by these hormones remains a mystery. Recently, an important role for GAs has been demonstrated (Locascio et al., 2013) in the control of the MT orientation through a physical interaction between the nuclear-localized

DELLA proteins and the prefoldin complex, a cochaperone required for tubulin folding. This finding provides a mechanistic link between the growth-promoting phytohormone GA and cortical MT organization.

ABA and microtubule

Abscisic acid (ABA) is also involved in plant development and responses to environmental stresses, including formation of longitudinal MT arrays in elongating cells, by a still uncharacterized mechanism. ABA was found to interfere with the dynamics and orientations of cortical MTs in a dose-dependent manner in *Arabidopsis* root epidermal cells (Seung et al., 2013). ABA changed MT orientation to longitudinal one in the cells of dwarf pea epicotyls and maize hypocotyls with the subsequent retardation of their growth (Shibaoka, 1994). Longitudinal deposition of cellulose microfibrils within the cell wall occurring after cortical MTs become oriented longitudinally reduced cell wall extensibility of ABA treated maize coleoptiles (Shibaoka, 1994). ABA decreased the abundance of MTs, suppressed the growth of coffee embryo cells, caused MT randomization, reduced the activity of DNA replication in the embryo axis, prevented the accumulation of β-tubulin transcripts, and, correspondingly, seed maturation (da Silva et al., 2008). Under the influence of various abiotic stressors, the intracellular ABA content increases, and this affects MT organization. The opposite situation is also possible: MT organization/dynamics determines ABA accumulation. For example, osmotic stress increased the proportion of obliquely and/or longitudinally oriented MTs in cucumber hypocotyls, which provided for their growth inhibition (Shibaoka, 1994).

Ethylene and microtubule

Ethylene is known to induce MT reorientation (Shibaoka, 1994). It was demonstrated for the first time by indirect immunofluorescence in the cells of the epidermis and outer cortex of pea epicotyls and mungbean hypocotyls, that MTs mostly were oriented transversely with respect to the cell axis, some MTs- obliquely and helically, and rare MTs – longitudinally. However, after treatment with ethylene, most MTs acquired longitudinal or oblique orientation (Roberts et al., 1985; Verbelen et al., 2008). In addition, a parallel orientation of cortical MTs and cellulose microfibrils was also disturbed, and this was accompanied by the inhibition of longitudinal elongation of pea epicotyls and mungbean hypocotyls with simultaneous activation of their lateral expansion (Roberts et al., 1985).

Brassinosteroid and microtubule

Furthermore, the brassinosteroids (BRs) play crucial roles in regulating plant cell growth and morphogenesis, particularly hypocotyl cell elongation, in which the participation of the MT cytoskeleton is also known. BRs have been shown to mediate

hypocotyl cell elongation by influencing orientation and stability of cortical MTs (Wang et al., 2012). This regulation occurs through the MICROTUBULE DESTABILIZING PROTEIN40 (MDP40), which is the key regulator of the cortical MT reorientation by BRs and mediator of hypocotyl growth (Wang et al., 2012). The identification of microtubule regulatory proteins specifically involved in BR-mediated hypocotyl cell elongation will facilitate the understanding of underlying mechanisms of BR-regulated cell growth.

Moreover, CKs and ethylene increase the proportion of longitudinal MTs in the systems where these hormones retard growth. However, as little is known on the mechanisms of CKs effect on MT orientation and dynamics, which will be discussed in this thesis (Chapter 2).

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Cytokinin modulates microtubule cytoskeleton dynamics in Arabidopsis roots

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Author contribution:

A.A and E.B initiated the project and designed most of the experiments, A.A carried out most of the experiments. X.C analyzed the EB1 trajectories movies related to figures 2E-2H and S2B-S2E. E.D analyzed statistics of the MT dynamics related to figures 4B, 4D and S4C, S4G. J.P, K.S and M.V. helped in the discussion of the data. A.A and E.B wrote the manuscript. All authors saw and commented on the manuscript.

Cytokinin Modulates Microtubule Cytoskeleton Dynamics in *Arabidopsis* Roots

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The microtubule cytoskeleton is a remarkable structure that can adopt a distinct architecture uniquely suited for the individual needs of a particular cell type or process. Whereas well-ordered transverse microtubules promote cell elongation and restrict radial cell expansion, extensive microtubule cytoskeleton rearrangements occur to form the preprophase band, spindle and the phragmoplast in dividing cells. Hence, microtubule dynamics are of fundamental importance to the intracellular functions of the microtubule cytoskeleton and developmental responses to different endogenous signals. As well, hormones have been shown to depend on the dynamic nature of microtubules. The plant hormones auxin and cytokinin play a key role in the regulation of plant development. In roots, auxin and cytokinin are known to act antagonistically in the control of root meristem activity and root branching. Here, we demonstrate that both auxin and cytokinin in root cells regulate microtubule dynamics in an opposite manner. By means of several microtubule markers, we show that the frequency of catastrophe events is increased by auxin, but significantly reduced by cytokinin treatments. We propose that this opposite regulation of the microtubule dynamics might be part of the regulatory mechanism underlying the auxin–cytokinin antagonism in the control of root development and lateral root initiation.

Introduction

Cytokinin plays an important role in the root system architecture establishment. It inhibits the rapid elongation of root cells and promotes differentiation of cells that leave the meristematic zone, with meristem shortening as a consequence. Whereas the root meristem size is under cytokinin-specific control, inhibition of the elongation growth is mediated through ethylene, which is overproduced in response to cytokinin and can be reverted by inhibition of either ethylene biosynthesis or signaling (Růžička et al., 2009). In the pericycle, cytokinin disrupts lateral root initiation and organogenesis and leads to primordia with an abnormal division pattern and morphological defects (Rani Debi et al., 2005; Li et al., 2006; Laplaze et al., 2007; Růžička et al., 2009). Developmental responses to different endogenous signals, such as the phytohormones auxin (Blancaflor and Hasenstein, 1995), gibberellins (Sauret-Gueto et al., 2012) or brassinosteroids (Catterou et al., 2001) have been shown to depend on the dynamic nature of the microtubules (Ehrhardt, 2008; Lucas and Shaw, 2008; Nick et al., 1990; Nick et al., 1992; Shaw et al., 2003).

In plants, microtubules (MTs) and F-actin microfilaments serve as spatial organizers that determine cell growth and shape, cell division orientation and layering and composition of cell walls and stress responses (Wasteneys and Ambrose 2009). Given their cellular functions, MTs and F-actin are essential for plant growth and development and abiotic and biotic stress responses. Accumulating evidence indicates that cortical MTs (cMTs) serve as tracks for cellulose synthase (CESA) complexes, thereby regulating the positioning of cellulose microfibrils and, consequently, the cell growth orientation and shape (Paredez et al., 2006; Lloyd, 2011). MT breakdown and reformation and actin reorganization have been found to be important for salt stress tolerance (Henty et al., 2011; Wang et al., 2011a/b) and reorganization of both MTs and F-actin is important for stomatal closure (Gao et al., 2009; Eisinger et al., 2012). Following a fungal pathogen attack, MTs and F-actin form a basket-like structure underneath the appressorium. This reorganization is thought to facilitate vesicle transport to the infection site, enhancing cell wall formation and delivery of defense compounds and proteins (Hardham et al., 2007, 2008; Day et al., 2011; Huesmann et al., 2012). MTs are highly dynamic and have several distinctive structures in the course of the cell cycle. During interphase, MTs are organized in cortical arrays (cMTs) that underlie the plasma membrane. In contrast to mammalian and yeast cells, the plant interphase MTs assemble without an organizing center with their plus and minus ends distributed throughout the cell cortex (Ehrhardt, 2008; Wasteneys and Ambrose, 2009). Collectively, studies on MT organization, cell growth, cell wall formation and hormone-dependent patterning highlight the intricate links between MTs and plant growth and development.

Microtubules (MTs) are highly dynamic polymers that control many aspects of cellular architecture. The fast-growing end of a microtubule, also called the 'plus end', shows alternating periods of growth (polymer assembly) and rapid shrinkage (polymer disassembly). This stochastic switching between growing and shrinking states is known as dynamic instability of microtubules (Dhonukshe and Gadella, 2003; Dixit and Cyr, 2004). The dynamic nature of the cMTs and their ability to respond to diverse stimuli is governed by proteins that regulate their nucleation, stability, cross-linking, severing, membrane interaction and orientation. The link between MTs and cell structure is emphasized in mutants or overexpression of MT-regulating proteins, such as MICROTUBULE ORGANIZATION1 (MOR1), ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEIN1 (RIC1), SPIRAL1 (SPR1), CLIP-ASSOCIATED PROTEIN (CLASP) and KATANIN, in which altered MT stability and organization are associated with compromised cell growth (Wasteneys and Ambrose, 2009). The dynamic instability of MTs is controlled by several proteins that preferentially bind to the plus ends, also called +TIPs or plus-end tracking proteins, such as the highly conserved END BINDING1 (EB1) family members (Tirnauer and Bierer, 2000; Chan et al., 2003). The EB1 proteins are involved both in the regulation of MT dynamics and in the recruitment of other MICROTUBULE-ASSOCIATED PROTEINS (MAPs) to a "plus-end complex". During growth, cMTs lie in a parallel pattern perpendicular to the cell elongation axis and are called transverse MTs, whereas a switch to the longitudinal alignment accompanies growth inhibition (Sedbrook and Kaloriti, 2008). The regulatory cytoskeleton-associated proteins themselves also need to be controlled by a mechanism that might possibly be phosphorylation. In mammalian cells, the microtubule-associated protein4 (MAP4) is phosphorylated during mitosis by the checkpoint cdc2 kinase complex that lowers the MT-stabilizing activity of MAP4 during mitosis (Ookata et al., 1995, 1997).

One hypothesis that has recently gained experimental support is that posttranslational modifications (PTMs) of the tubulin-building block generate functional MT diversity. These modifications may act individually and/or in a combinatorial fashion to recruit specific protein complexes and, thus, regulate the organelle-specific properties of MTs (Hammond et al., 2008). Although, in mammals, many tubulin PTMs have been known for decades – detyrosination and the related Δ2 modification, glutamylation, glycylation, acetylation, phosphorylation, and palmitoylation (Janke and Bulinski., 2011), their functional roles in different MT structures are just beginning to be discovered. Most PTMs occur on MTs rather than on unpolymerized tubulin and it has long been known that stable MTs are more prone to modifications than dynamic MTs. The PTMs are postulated to play a role in specific functions of stable MTs because the tubulin PTM patterns differ in stable MTs (Hammond et al., 2008). The majority of plant cell MTs seem to be tyrosinated and only a small subgroup exists in a detyrosinated state (Smertenko et al., 1997). Tubulin tyrosine ligase (TTL) binds nitrated Tyr to α-tubulin both *in vitro* and *in vivo* (Westermann and Weber, 2003) and tubulin tyrosine carboxypeptidase (TTCP)

removes it (Bisig et al., 2002). However, the reversibility of α-tubulin tyrosine nitration is still an open question, because no enzyme (TTCP) has been identified in plant cells. Recently, under physiological conditions, 3-NO_2-Tyr has been found to affect the MT organization in *Arabidopsis thaliana* root cells and cytoskeleton-related processes, such as root growth and differentiation (Blume et al., 2013).

In this chapter, we demonstrate that modulation of cytokinin levels in plants impact on cytoskeleton dynamics and antagonize auxin effects. We show that cytokinin interferes with the principal processes that determine MT dynamics, including MT growth rate, shrinking rate, frequency of growth-to-shrinkage transition (catastrophe frequency), and frequency of shrinkage-to-growth transition (rescue frequency).

Results

Microtubule cytoskeleton is involved in cytokinin-regulated root development

To gain more insight into the role of the MT cytoskeleton in the cytokinin-regulated root growth and development, we analyzed in detail mutants in genes encoding components of the MT cytoskeleton. In the cell, tubulin exists in two interchangeable pools: soluble tubulin dimers made of α- and β-tubulin molecules and a MT polymer pool that is assembled from tubulin heterodimers. Several mutants in genes coding for tubulin subunits (*TUA1, TUA5, TUA6, TUB1, TUB2, TUB3, TUB6, TUB7,* and *TUB8*) were chosen to assess the role of the MT cytoskeleton in the development of the root system and its sensitivity to cytokinin. We found that lack of either TUA or TUB subunits dramatically reduced the lateral root density (LRP/cm) (Figure 1A). In contrast, most of these mutants, with the exception of *tua6*, *tub1* and *tub6*, did not severely change the root elongation (Figure S1A). Closer examination of the root cytokinin sensitivity revealed that the lateral root density of all tested mutants was reduced significantly, except in *tub7* and *tub8* (Figure 1A), but not the root elongation (Figure S1A).

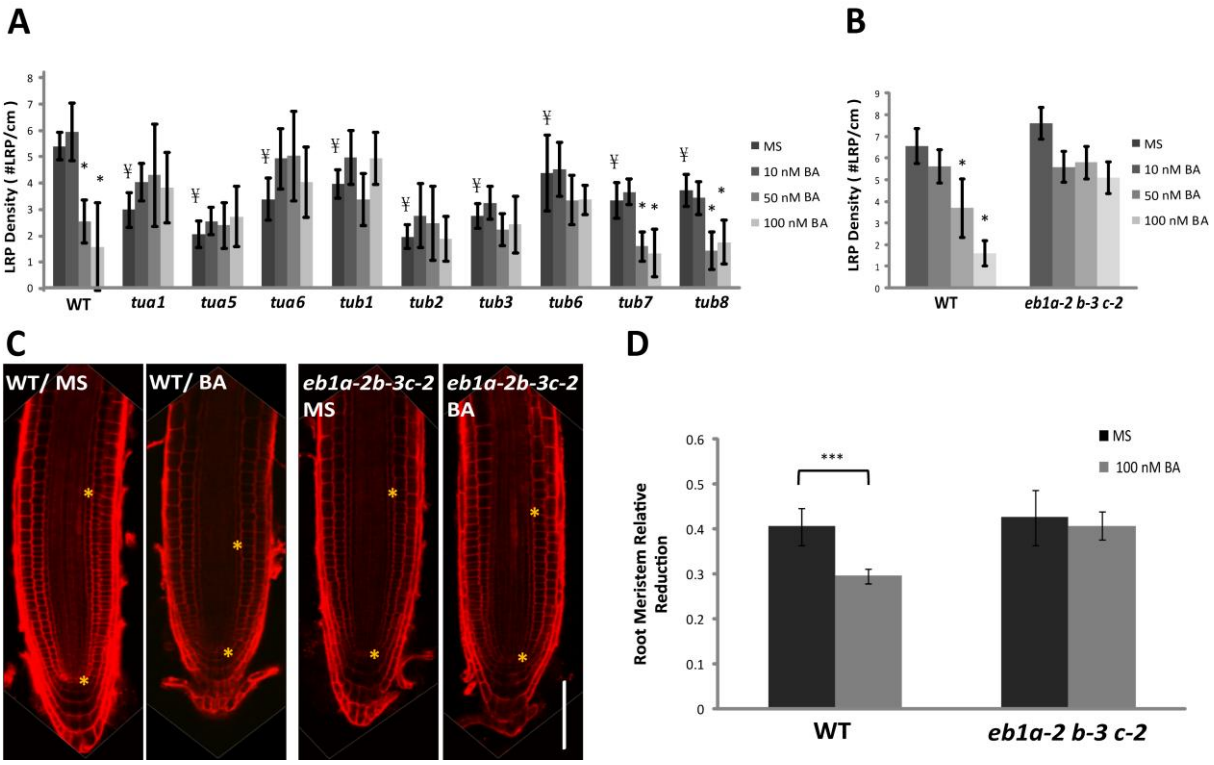

Figure 1: Cytokinin regulation of root development requires intact MT cytoskeleton.

(A and B) Mutants defective in MT components exhibit cytokinin-insensitive root growth. Mutants of tubulin α and β subunits display reduced lateral root density (LRP/cm), reduced cytokinin sensitivity, except for *tub7* and *tub8* mutants (A). The lateral root density phenotype of the EB1 triple mutant has a cytokinin-insensitive phenotype for lateral root density (B). Seedlings grown on 10, 50, and 100 nM BA-containing media for 7 days (¥p<0.05, *p<0.05, n= 15 roots) ¥ corresponding to lateral root initiation significance between the wild type and tubulin mutants on MS; * corresponds to the cytokinin sensitivity significance between MS and each treatment of each genotype. Error bars mark ± Standard Deviation (±SD). (C-D) Root meristem size of the triple mutant *eb1a-2b-3c-2* is cytokinin insensitive when grown on MS media and media supplemented with 0.1 μM BA for 7-days and then stained with propidium iodide (scale bars= 50 μm). (***p<0.001, n=15 roots). Yellow asterisks mark the distance between the quiescent center (QC) and the cortex transition zone. Error bars mark ± Standard Deviation (±SD). Significant values were evaluated using Student's *t*-test. MS, Murashige and Skoog; BA, 6-Benzylaminopurine.

In budding yeast and *Drosophila* cells, EB1 recruits several proteins to the growing MT ends and participates in the MT dynamics control. EB1 has been shown to stimulate both catastrophes and rescues, making MTs more dynamic and reducing the MT pausing time (Tirnauer et al., 1999; Rogers et al., 2002). In *Arabidopsis*, three homologs of the highly conserved +TIP EB1 have been found, namely AtEB1a, AtEB1b and AtEB1c, of which at least both AtEB1a and AtEB1b accumulate at the MT plus ends in the typical comet-like fashion (Mathur et al., 2003; Chan, 2003; Van Damme et al., 2004a). Interestingly, a triple mutant, lacking

all three *EB1* gene homologs, *eb1a-2b-3c-2* had a decreased sensitivity to cytokinin at the level of lateral root density and root meristem size, but the root elongation sensitivity to the cytokinin inhibitory effect was normal (Figure 1B, 1C-1D, Figure S1B).

The spatial regulation of MTs depends on the function of the RHO family small G proteins, called Rho of Plants (ROPs). RIC1 was shown to be a MT-associated protein (MAP), providing the first direct link between MT and ROPs (Fu et al., 2005). To corroborate the role of the MT cytoskeleton in the cytokinin-regulated root growth, we examined transgenic plants with a defective ROP6 signaling pathway (Fu et al., 2009; Lin et al., 2013). Modulation of either the ROP6 or RIC1 activity significantly affected the overall root growth, because *rop6-1* and *ric1-1* mutants had slightly shorter roots, whereas OX-ROP6 slightly longer roots, but without impact on lateral root initiation (Figure S1C and S1D). When exposed to cytokinin, lateral root initiation in both *rop6-1* loss-of-function and RIC1 overexpression plants was attenuated (Figure S1D), but the root meristem size of *rop6-1* and *ric1-1* loss-of-function mutants and overexpressor of RIC1 was cytokinin insensitive (Figure S1E and S1F). Altogether, analyses of mutants in genes related to the MT cytoskeleton and its dynamics control hint at an involvement of the MTs in the regulation of root development, in particular, of lateral root organogenesis and its sensitivity to cytokinin.

Cytokinin interferes with MT dynamics

To examine the interaction between cytokinin and the MT cytoskeleton, we monitored the effect of cytokinin on the MT cytoskeleton organization. MT-associated proteins, such as the MAP4-GFP reporter (Marc et al., 1998), decorate MT filaments and enable their visualization in cells. Initially, we attempted to observe MTs in the pericycle cells, where the lateral root initiation takes place. However, due to technical difficulties to image MTs in narrow, elongated pericycle cells attached to the vascular cylinder, we looked for another cytokinin-sensitive cell type to carry on these experiments. Epidermal cells of the root elongation zone with MTs mostly aligned perpendicularly to the growth axis appeared to be well suited for our analysis (Figure S2A). No dramatic changes in the MT orientation, which were visualized by 35S::MAP4-GFP, could be noticed in roots treated with cytokinin. Within 50 min of treatment, MT filaments maintained the perpendicular orientation as in untreated root epidermal cells (Figure 2B and 2D). In contrast, treatment of roots with auxin induced a rapid reorientation of MTs and realigned them longitudinally (Chen et al., unpublished, Figure 2A and 2D). After 10 min of exposure to auxin, the regular transversal orientation of MTs became randomized and within 50 min gradually became longitudinal (Figure 2A and 2D). Pretreatment of roots with cytokinin for 30 min interfered with the auxin-induced reorientation of MTs

and after 50 min of treatment no longitudinal MT orientation could be observed (Figure 2C and 2D).

Whereas MAP4-GFP visualizes mainly polymerized MTs, it gives little information about the MT growth trajectories. To examine the auxin and cytokinin effects on the trajectories of growing MTs, we followed the EB1b protein fused to GFP that preferentially accumulates at the rapidly growing plus ends of MTs (Vitre et al., 2008).

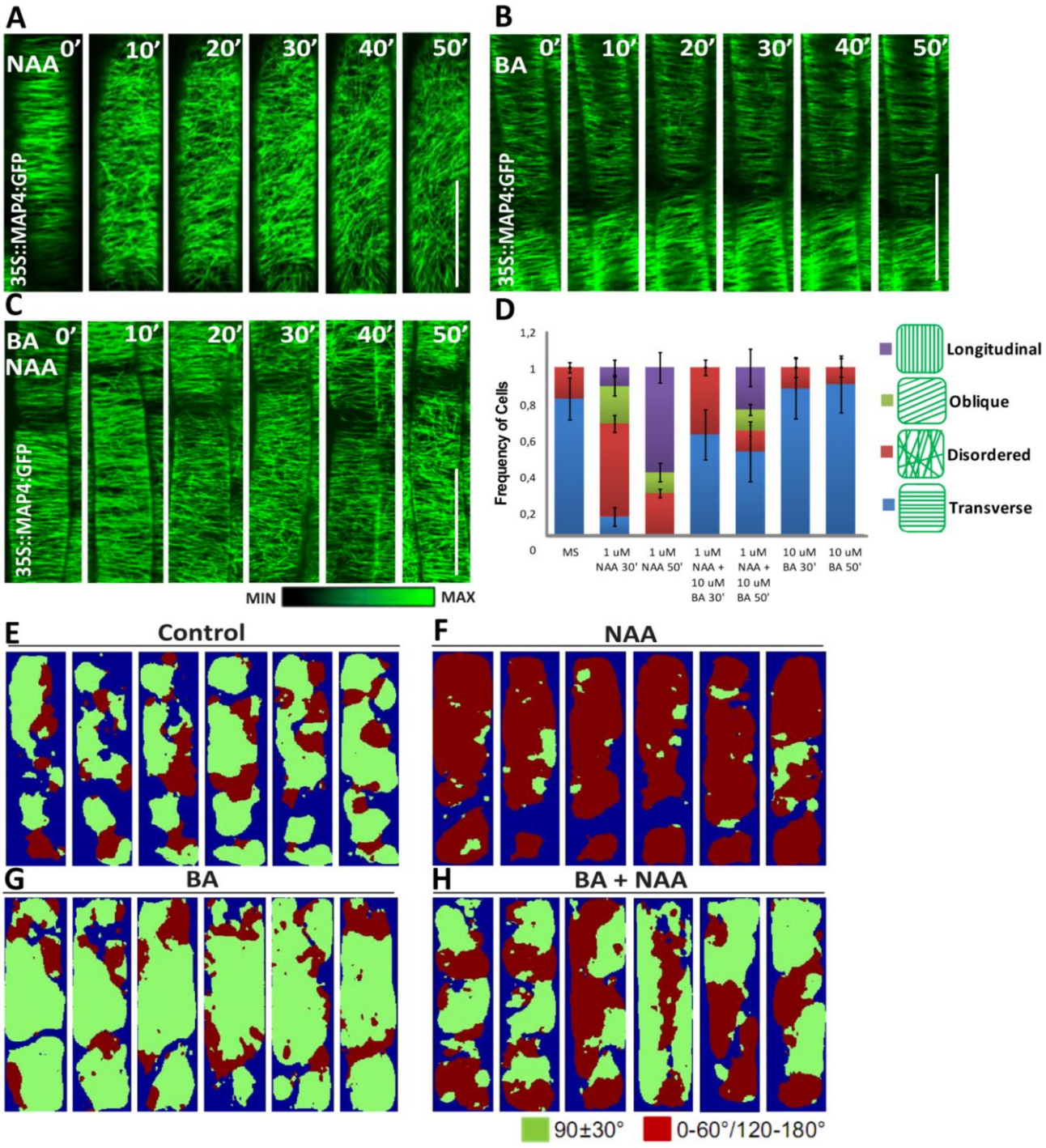

Figure 2: Cytokinin antagonizes the auxin effect on MTs.

(A-D) Auxin and cytokinin impact on the MT orientation of root epidermal cells (4-5 cells above the transition zone). Time lapse analysis of 0.1 µM auxin (NAA) (A), 10 µM cytokinin (BA) (B), and effect of 0.1 µM NAA + 10 µM BA double treatment (C) on MTs using 35S::MAP4-GFP transgenic line. Quantitative analysis of the normalized number of cells showing four different MT orientations, indicate that auxin influence longitudinal orientation while cytokinin influence transverse orientation when applied separately, cytokinin antagonize auxin effect when applied simultaneously (D). Error bars mark ± Standard Error of the Mean (±SE). (E-H) Quantification method and artificial cartoon of the EB1b trajectories. Control treated with MS (E), 0.1 µM auxin (NAA) (F), 10 µM cytokinin (BA) (G), and 0.1 µM NAA + 10 µM BA double treatment (H), incubated for 1 h in the dark. Color maps show the EB1b trajectory simulated with the Image J and MATLAB software, indicate that auxin influence longitudinal orientation while cytokinin influence transverse orientation when applied separately, cytokinin antagonize auxin effect when applied simultaneously. Green and red areas represent EB1b moving transversally (90±30°), and obliquely and longitudinally (0-60°/120-180°). (Scale bar = 10 µm). NAA, naphthalene acetic acid; BA, 6-Benzylaminopurine.

An unbiased, quantitative measurement of the MT dynamics orientation in a single cell over time by means of an image analysis tool automatically assigned the EB1b trajectories to transversal (depicted as green area) or longitudinal (red area) directions (Figure 2E-2H) (Chen et al., unpublished). Consistently with the MAP4::GFP analysis, the prevailing EB1b::GFP trajectories in untreated epidermal cells corresponded to the MT growth in a transversal direction. Treatment with cytokinin (either [benzylaminopurine BA] or *trans*-zeatin) did not interfere with the visualized EB1b-GFP trajectories and the MT growth maintained mainly a transversal direction (Figure 2G, Figure S2D). In contrast, auxin promoted oblique and longitudinal movements of EB1b, corresponding to the extension of the red area (Figure 2F and Figure S2C). When pretreated with cytokinin, MTs were unable to react to auxin and the trajectory of the EB1b reporter remained transversal as reflected by the prevalence of the green area in monitored cells (Figure 2H, Figure S2E). These results indicate that auxin and cytokinin regulate the MT dynamics and that their contributions might be opposite.

Mutants with modulated cytokinin levels exhibit abnormalities in the MT cytoskeleton structure

Next, we introduced the MAP4-GFP reporter into the cytokinin receptor mutants *arabidopis histidine kinase2* (*ahk2*), *ahk3* and *ahk4*/*cytokinin resistant1* (*cre1*) loss-of-function mutants. Whereas MTs maintained their regular transversal position in the *ahk2-2* mutant, they were more randomly arranged in the *ahk3* and *ahk4/cre1* mutants (Figure 3A-3D). To examine whether modulations of endogenous cytokinins affect the MT orientation and dynamics, we immunodetected α-tubulin in the root epidermal cells of mutants defective in either cytokinin biosynthesis or perception. The first rate-limiting step in cytokinin biosynthesis in *Arabidopsis* is

catalyzed by the ATP/ADP isopentenyltransferase (IPT) gene family (Kakimoto, 2001). Decrease in cytokinin levels in the loss-of-function mutants *ipt3*, *ipt5* and *ipt7* and higher order mutants *ipt3,5, ipt3,7, ipt5,7*, and *ipt3,5,7* resulted in a more randomized MT arrangement than the transversal organization of MTs observed in control roots (Figure 3E-3L and 3P). In roots overexpressing cytokinin oxidase/dehydrogenase3 (CKX3) and CKX2 that catalyze the irreversible cytokinin degradation (Werner et al., 2001; Werner et al., 2003), α-tubulin filaments appeared to be oriented randomly, to be thicker and more bundled than those of control roots (Figure 3N, 3O and 3P). In contrast, *IPT* overexpression that increases endogenous cytokinin levels did not dramatically affect the MT orientation (Figure 3M and 3P), in agreement with previous observations with exogenous cytokinin. Thus, these results support a role for cytokinin in the control of MT cytoskeleton arrangements and show that a decrease in either endogenous cytokinin levels or signaling interferes with the perpendicular orientation of MTs in root epidermal cells.

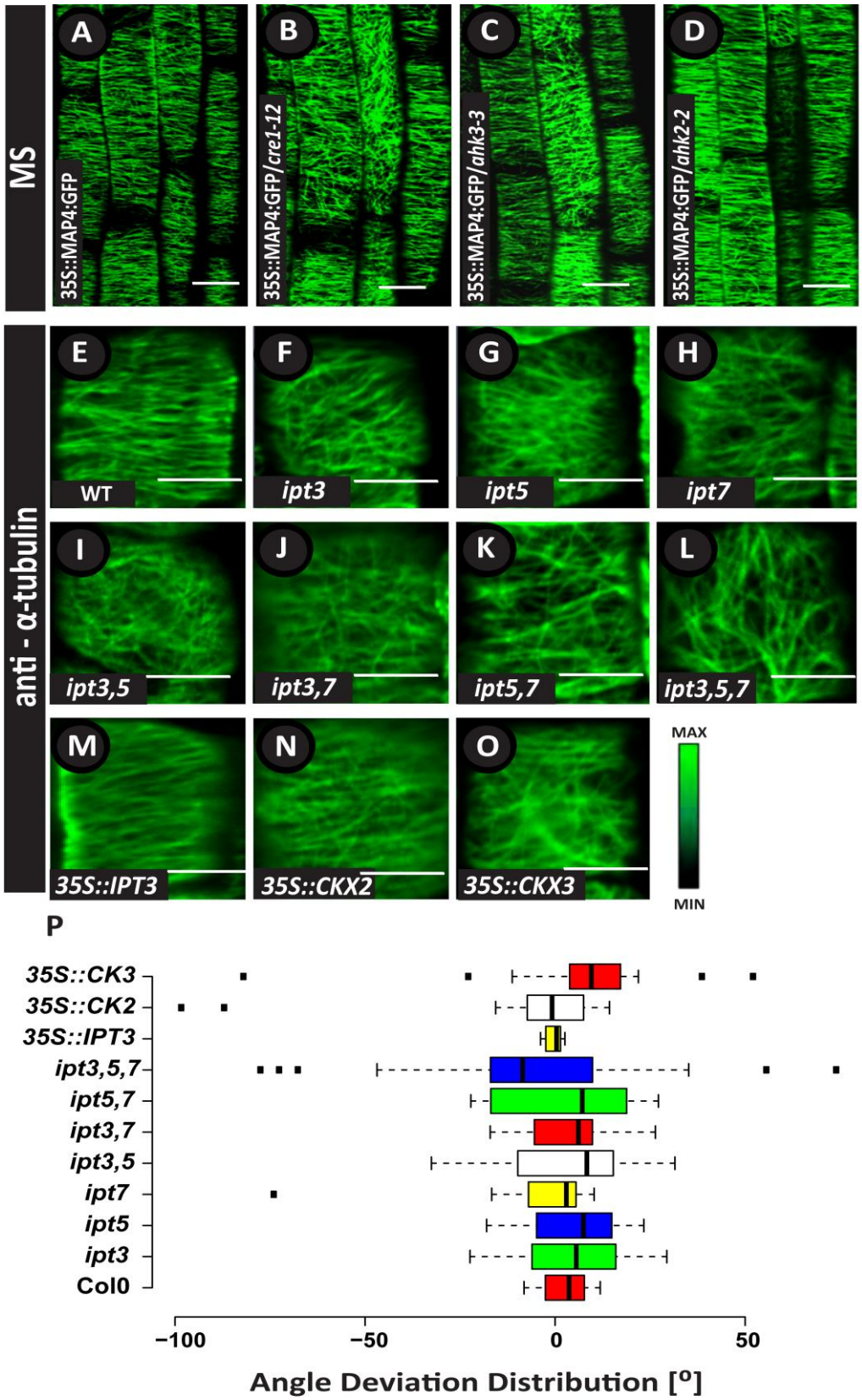

Figure 3: Mutants with modulated cytokinin levels exhibit defective MT cytoskeleton structures.

(A-D) MT structure of *MAP4-GFP* of root epidermal cells (4-5 cells above the transition zone) (A), *MAP4-GFP/cre1-12* (B), *MAP4-GFP/ahk3-3* (C), and *MAP4-GFP/ahk2-2* (D). (E-O) Anti-α-tubulin immunofluorescence of root epidermal cells (meristematic zone) in wild type (E), isopentenyltransferase mutants, *ipt3* (F), *ipt5* (G), *ipt7* (H), *ipt3,5* (I), *ipt3,7* (J), *ipt5,7* (K), *ipt3,5,7* (L) reveals more randomized MT structures in these mutants, but not in the *IPT3* overexpressor (M). Overexpressing cytokinin oxidases/dehydrogenase2 CKX2 (N) and CKX3 (O) exhibit randomized, thicker and bundled MTs. (Scale bars= 5 μm). Quantification of MT orientation and distribution (P). Isopentenyltransferase (*ipt*) mutants and IPT overexpressor, as well as cytokinin oxidases/dehydrogenases, indicate that the angle deviation from transverse orientation (0^0) is altered in all *ipt* mutants and lines overexpressing cytokinin oxidases/dehydrogenases, but reduced in the IPT3 overexpressor. Box plots indicate the 25th percentile (bottom boundary), median (middle line), 75th percentile (top boundary), nearest observations within 1.5 times the interquartile range and outliers. Quantification analysis was performed using Image J software package (FibrilTool), as described (Boudaoud et al., 2014) (n = 15, 5 cells each root).

Cytokinin affects the MT cytoskeleton dynamics

MT dynamics are characterized by four parameters: growth rate, shrinking rate, catastrophe frequency, and rescue frequency (Dhonukshe and Gadella, 2003; Dixit and Cyr, 2004). To explore the MT dynamics in the presence of plant hormones, we monitored MTs with either the α-tubulin6-RFP or β-tubulin6-GFP reporter in root epidermal cells and tobacco BY-2 cells, respectively by means of a spinning-disk microscope imaging system (see Materials and Methods for details). In both root epidermal cells and tobacco BY-2 cells, cytokinin significantly attenuated the MT shrinking rate and catastrophe frequency (Figure 4A, 4D and 4E, Figure S4E-S4G), but at the plus ends, it significantly enhanced the MT growth rate (Figure 4A and 4B, Figure S4A-S4C), but the frequency of these events per cell was severely diminished (Figure 4C, Figure S4D and S4H).

Unlike cytokinin, auxin had no significant effect on either MT growth or shrinking rates (Figure 4A, 4B and 4D), but significantly increased the catastrophe frequency (Figure 4E). When both hormones were applied simultaneously, cytokinin, regardless of the presence of auxin, significantly reduced the catastrophe frequency per cell as well as the shrinking rate (Figure 4D-4E). In contrast, auxin attenuated the cytokinin effect on the MT growth rate at the plus ends (Figure 4A and 4B).

Interestingly, pharmacological analyses revealed that MTs pretreated for 30 min with cytokinin were less sensitive to depolymerization by oryzalin, propyzamide, or tubulysine A (Figure S3A and S3B). Altogether these data indicate that both auxin and cytokinin modulate the MT dynamics and that their activities converge at the regulation of the transition from growth-to-shrinkage. The reduced catastrophe frequency in cytokinin-treated cell, hints at its role in MT stabilization,

but the molecular mechanisms underlying these cytokinin effects must be further explored.

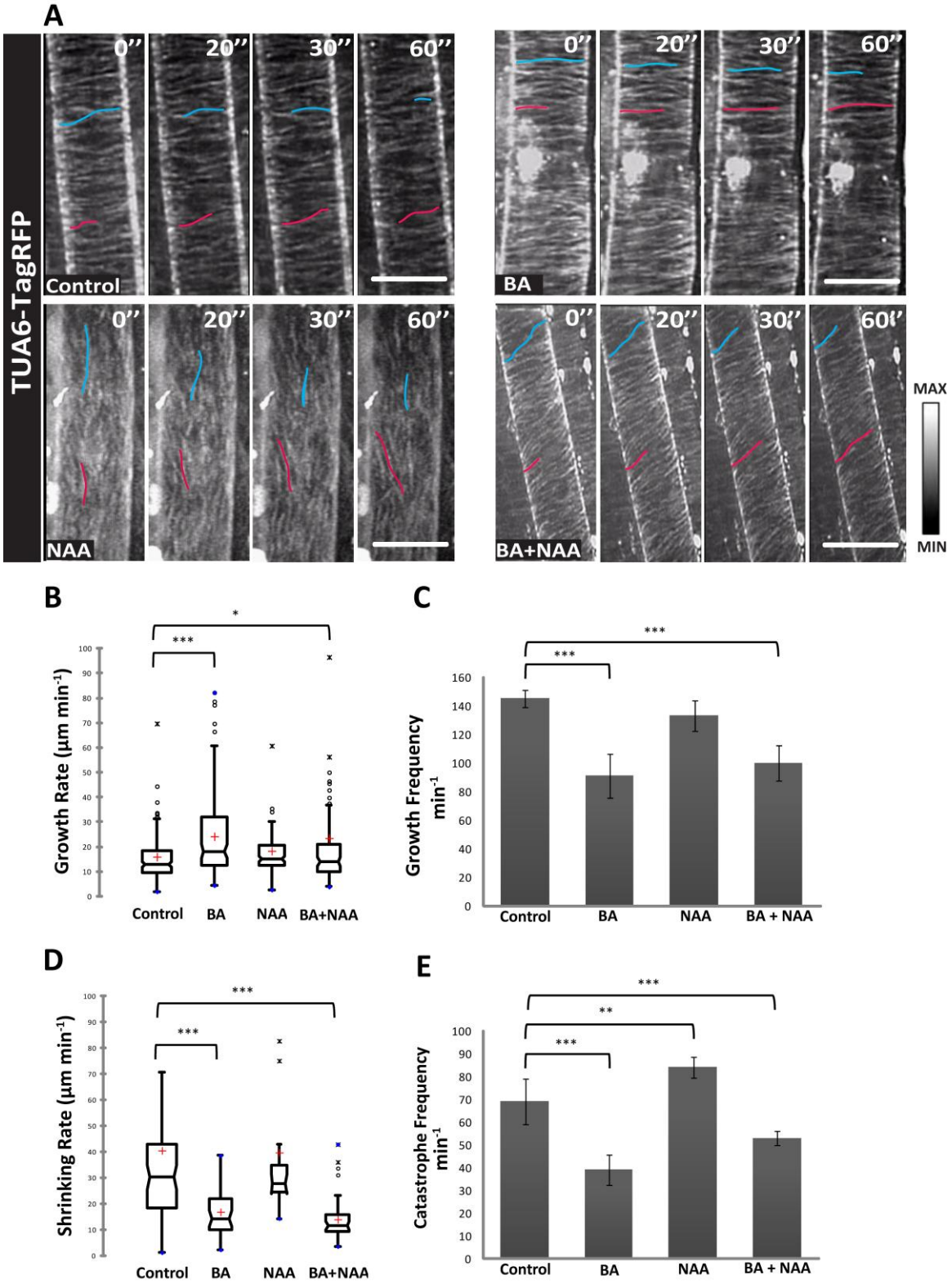

Figure 4: Cytokinin interferes with the MT dynamics.

(A) Time lapse images of *Arabidopsis thaliana* roots (4-5 cells above the transition zone) expressing TUA6-TagRFP marker, using the spinning disk microscope (100 frames x 500 msec), showing the MT growth rate (red lines) and the shrinking rate (blue lines) of control

seedlings and seedlings treated with (10 μM cytokinin (BA), 0.1 μM auxin (NAA), and 10 μM BA+ 0.1 μM NAA) for 1.5 h in the dark. Blue and red lines also present the orientation of MT of each treatment. (B) The growth rate of α-tubulin6 presented by a notched box plot. At the plus ends after 1.5 h of 10 μM cytokinin (BA) in the dark, the MT growth rate is enhanced, whereas 1.5 h of treatment with 0.1 μM auxin (NAA) in the dark had no significant effect, when both hormones applied, auxin attenuated the cytokinin effect on the MT growth rate (A). The growth frequency per cell was diminished after 1.5 h of treatment with 10 μM cytokinin (BA) in the dark, but no impact after 1.5 h of treatment with 0.1 μM auxin (NAA) (C). (D and E) Cytokinin (BA) reduced the shortening rate (C) and the catastrophe frequency (D) and interfered with the effect of auxin (NAA) on the catastrophe rates. Box plots indicate the 25th percentile (bottom boundary), mean (red plus), median (middle line), 75th percentile (top boundary), the nearest observations within 1.5 times the interquartile range and outliers. (Scale bars= 5 μm), (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Error bars for mark ± Standard Error of the mean (± SE) of the mean (B and D). Significant values were evaluated using Kruskal–Wallis test using MATLAB software.

Cytokinin enhances MT tyrosination

Mammalian tubulins undergo several types of PTMs, such as tyrosination, detyrosination, acetylation polyglutamylation and polyglycylation, leading to the appearance of various tubulin isoforms and of various MT classes (MacRae 1997). Tyrosination and detyrosination are reversible PTMs that occur mostly on α-tubulin monomers, but not on β-tubulin. Detyrosination is associated with MT stability because long-lived MTs in various cell types lack tyrosination. However, detyrosination itself does not confer stability to MTs (Khawaja et al., 1988). Taking in account that cytokinin and auxin affect MTs and their dynamics, we examined whether the tyrosination pattern of MTs is modified by these hormones. To examine the MT tyrosination levels, we performed co-localization experiments with antibodies specific to either α-tubulin or tyrosinated α-tubulin and we measured the Pearson's correlation coefficient to estimate the MT tyrosination levels (Figure S6, See Materials and Methods). We hypothesized that if both antibodies recognized specifically α-tubulin, an increase in the Pearson's correlation coefficient might indicate a higher tyrosination. Indeed, the Pearson's correlation coefficient increased and decreased in cells treated with cytokinin and auxin, respectively (Figure 5A-5C). Accordingly, decrease in the endogenous cytokinin levels due to loss of the IPT activity in the *ipt3, ipt7, ipt3,5, ipt3,7* and *ipt3,5,7* reduced the Pearson's correlation coefficient, whereas enhanced endogenous cytokinin as result of the *IPT* overexpression increased it (Figure 5D and 5E). Tyrosination analysis in the cytokinin receptor mutants revealed that a mutation in *AHK4/CRE1* receptor interfered with the tyrosination pattern when compared to control and the *ahk2* and *ahk3* mutants. In the *cre1* mutant, the Pearson's correlation coefficient increased, but without further increase in response to cytokinin (Figure S5A- S5H). These results further support our observation that cytokinin and cytokinin signaling modulate the MT dynamics, as also reflected in the tyrosination status modulation of the MT filaments.

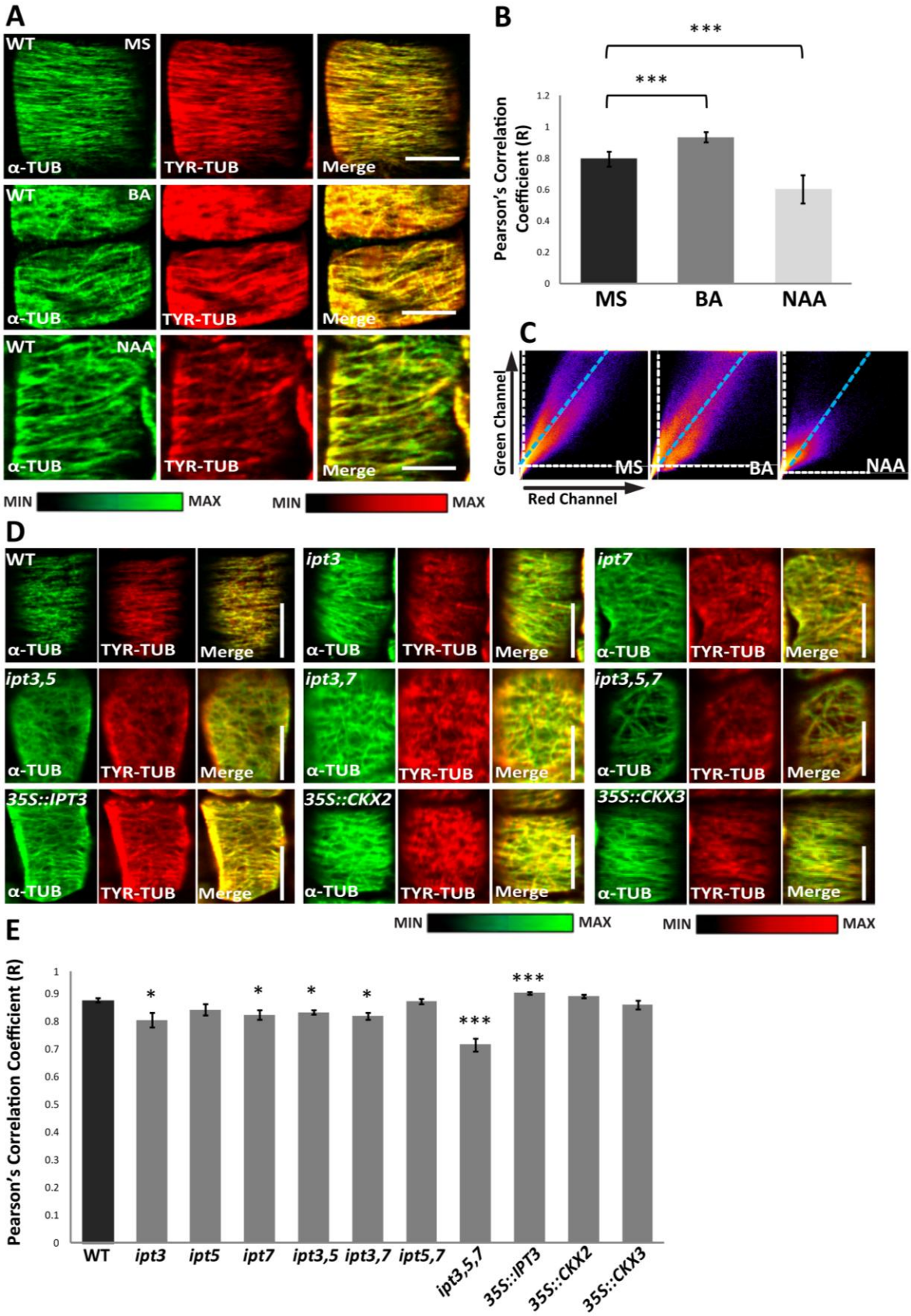

Figure 5: Cytokinin increases tyrosination of α-tubulin.

(A-C) Cytokinin and auxin have antagonistic effects on the tyrosination of α-tubulin. Immunodetection of α-tubulin (green channel) versus tyrosinated tubulin (red channel) of root epidermal cells (meristematic zone) on wild-type seedlings (Col0) in untreated (MS) and treated with 10 μM cytokinin (BA) and 0.1 μM auxin (NAA) for 1.5 h in the dark, shows enhanced co-localization in BA-treated samples while reduced co-localization in NAA-treated samples (A). Quantification analysis of the co-localization between α-tubulin and TYR-TUB with the Pearson's correlation coefficient (R) shows a significant increase and decrease in co-localization upon treatment with cytokinin and auxin, respectively (B).Scatter plots showing the co-localization between the two channels in untreated (MS) and treated samples with 10 μM cytokinin (BA) and 0.1 μM auxin (NAA). Dashed white lines represent the pixel intensity of the red channel (x-axis) and of the green channel (y-axis) (see Figure S6). Dashed blue line represents the line of the linear regression (C). (D and E) Mutants with modified cytokinin levels exhibit changes in tyrosination of α-tubulin. Immunodetection of α-tubulin (green channel) versus tyrosinated tubulin (red channel) of root epidermal cells (meristematic zone) on wild-type seedlings (Col0), cytokinin biosynthesis (*IPT*) and degradation (*CKX*) mutant genes, reveals an altered tubulin structure and co-localization with TYR-TUB (D). Quantification analysis of the co-localization between α-tubulin and TYR-TUB with the Pearson's correlation coefficient (R) for the wild type and cytokinin biosynthesis/degradation mutants (E). (Scale bars= 5 μm). (*$p<0.05$, ***$p<0.001$ n= 15 roots, 10 cells each root). Error bars mark ±Standard Deviation (±SD). Significant values were evaluated using *One-Way ANOVA*. NAA, naphthalene acetic acid; BA, 6-Benzylaminopurine.

Tubulin tyrosine ligase is involved in the cytokinin regulation of root growth

Tyrosination is a reversible PTM that is most probably unique for α-tubulin (MacRae, 1997). Most eukaryotic α-tubulin genes encode a C-terminal tyrosine that can be removed by a specific and, still unidentified, tubulin–tyrosine carboxypeptidase (TTC, also referred to as TCP or TTCP) with a preference for polymers rather than α/β-tubulin heterodimers (Ersfeld et al., 1993). Thus, TTC creates detyrosinated α-tubulin (detyr-tub) that forms a C-terminal glutamic acid (therefore often designated as Glu-tubulin). After depolymerization of MTs, the α-subunit of the soluble α/β-tubulin heterodimers can be (re-)tyrosinated by a TTL (Ersfeld et al., 1993), generating the initially tyrosinated α-tubulin (tyr-tub). TTL prefers the α-tubulin of soluble α/β-tubulin heterodimers as substrate and has a low affinity for assembled MTs (for a review, see Westermann and Weber, 2003). The first enzyme that had been identified as a tubulin-modifying enzyme is the one responsible for tyrosination of tubulin, namely TTL (Ersfeld et al., 1993). In mammalian cells, TTL tyrosinates α-tubulin, a component of the α/β heterodimers and re-tyrosinates the α-tubulin of depolymerized MTs. One TTL homolog was found in *Arabidopsis* (AtTTL;) (Gardiner and Marc, 2003) and a mutant line with a T-DNA insertion into the 10th exon of *AtTTL* (*salk_108909*) was obtained (Figure 6A). Reverse-transcription-polymerase chain reaction analysis confirmed that homozygous mutants did not express the full-length *AtTTL*, suggesting that this allele represented a full knockout (Figure 6B). Lack of *AtTTL* affected the sensitivity to cytokinin. In the *ttl* mutant, both lateral root density and root meristem size were insensitive to cytokinin (Figure 6C-6F), suggesting that cytokinin might tyrosinate α-

tubulin through TTL activation. In other words, cytokinin might interfere with the TTL activity to modulate the MT dynamics. However, further analysis is needed to prove the role of the TTL in tyrosination of plant MTs and the link with the cytokinin-regulated MT dynamics.

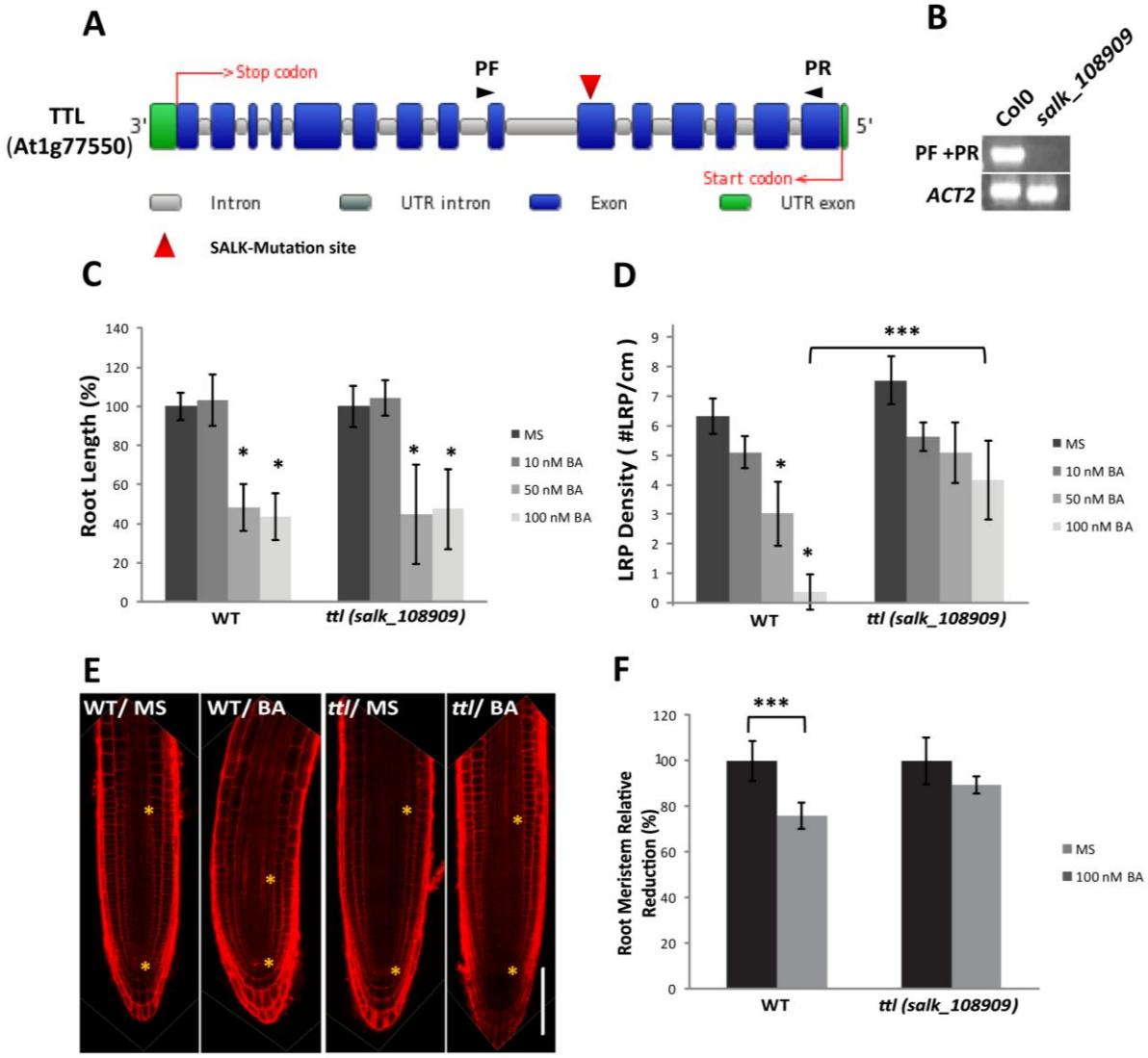

Figure 6: *ttl* mutant phenotype.

(A and B) Schematic representation of the intron (gray box)-exon (blue box) structure in the *TTL* gene. Red arrowhead indicates the T-DNA insertion site at the 10th exon (A). RT-PCR from RNA extracts of the *ttl* (SALK_108909) single mutant and wild-type Columbia-0 (Col-0) plantlets (B) The primer positions are shown in (A). (C and D) Root growth (C) and lateral root density (D) for the wild type and the *ttl* (SALK_108909) mutant, exhibit a cytokinin-insensitive phenotype for lateral root density but not for root length, when grown on MS media and media supplemented with 0.1 µM BA for 7-days (*p<0.05, ***p<0.001, n= 15 roots, Error bars mark ±Standard Deviation (±SD). Significant values were evaluated using Student's *t*-test. (E and F) Root meristem size stained with propidium iodide in the wild type (Col0) and *ttl* mutant on (MS) and MS supplemented with 0.1 µM BA grown for 7 days (C) (***p<0.001, n= 15 roots. Error bars mark ±Standard Deviation (±SD). Significant values were evaluated using Student's *t*-test. Yellow asterisks indicate the distance between the

quiescent centre (QC) and the cortex transition zone. (Scale bars= 50 μm). MS, Murashige and Skoog; BA, 6-Benzylaminopurine**.**

Discussion and Conclusions

The dynamic properties of the MTs are necessary for the rapid construction and function of each other succeeding different MT arrays during the cell cycle (Lloyd and Hussey, 2001). In this chapter, we have shown that MTs are essential for lateral root formation and that mutations in one of the MT components have an impact of the lateral root density (LRP/cm), but no effect on the overall root length. This suggest a specific and crucial role of MT for lateral root formation, this effect can be speculated by two scenarios, one pointing towards an abortion phenotype during very early divisions, as a consequence, is hard to detect many LRP. Secondly, we can point towards the LRP initiation where MTs also involved during cell division. Moreover, it was shown that *Arabidopsis* Aurora kinases also function during formative cell division, as double mutants of *aur1-2, aur2-2* showed less lateral root density (Van Damme et al., 2011), this also can verify the role of MT component and dynamics in regulating lateral root formation.

ROP-interactive CRIB motif-containing proteins (RICs) comprise a plant-specific family of ROP effectors (Wu et al., 2001). Several RIC family members mediate ROP signaling in the regulation of the polar cell growth in *Arabidopsis*. Hence, RIC1 seems to be a ROP effector involved in the control of both actin and MTs. It is still not well understood how MTs and actin are coordinated during cell growth. Our data show that the *ric1-1* primary roots are significantly shorter, whereas the roots produced by *RIC1-OX* plants are slightly longer than those of the wild type. As both cell division in the meristem and the cell elongation in the elongation zone contribute to root growth (Beemster and Baskin, 1998), we speculate that the root length phenotype is due to defective cell division or elongation that is inhibited in *ric1-1* mutants.

Many reviews have been devoted to the phytohormonal impact on the cytoskeleton arrangement (Shibaoka 1994; Grabski and Schindler, 1996; Klyachko, 2003; Foster et al., 2003). In plant cells, there is a strong connection between cytokinin signaling and other phytohormones, especially the auxin-cytokinin antagonism. Auxin has been reported to change the MT orientation from transverse to longitudinal in the elongation zone of *Arabidopsis* roots (Chen et al., unpublished). This can be an interesting difference concerning the reaction of MTs in the above ground tissues and in root tissues. There is very solid evidence on the reorientation of MTs in maize coleoptiles (Nick, 2000). Here, we provide evidence that cytokinin antagonizes the auxin effect and counteracts the orientation change in cortical MTs that is caused by auxin. Cytokinin reduces the frequency of catastrophe events at the plus end, whereas auxin increases it. Collectively, these results indicate that auxin

and cytokinin regulate the MT dynamics and that their contributions might be antagonistic. This cytokinin mode of action has been confirmed in cytokinin biosynthesis and perception mutants that are defective in MT structure and/or orientation, suggesting that cytokinin plays an important role in the modulation of the MT arrangements and in the maintenance of the perpendicular orientation. However, further analysis is needed to investigate the mechanism behind the cytokinin impact on the control of the MT orientation.

Detyrosination of the C-terminus of α-tubulin might play a role in cell division in plants (Jovanović et al., 2010). Furthermore, taking into account that cytokinin and auxin affect the MT orientation and dynamics and that cytokinin accumulation does not prevent the auxin-mediated activation of cell divisions but rather blocks the developmental program of lateral root initiation (Laplaze et al., 2007), we wanted to check whether the α-tubulin tyrosination might shed light on the MT stabilization by cytokinin. We hypothesized that to assess the tyrosination degree of MTs; the Pearson's correlation coefficient might be indicator of increased tyrosination in co-localization experiments with antibodies specific to either α-tubulin or tyrosinated α-tubulin. Indeed, exogenous cytokinin significantly increased the tyrosination level, whereas auxin decreased it. To further explore the cytokinin effect on the α-tubulin tyrosination level, we tested loss-of-function and gain-of-function mutants of the *IPT* genes. The tyrosination level of α-tubulin represented by the Pearson's correlation coefficient was lower in all *ipt* mutants and significantly higher in the overexpressor than that of the control, indicating a substantial impact of cytokinin on α-tubulin tyrosination.

Finally, tyrosination/detyrosination of α-tubulin is mediated by the TTL/TTCP activity, respectively (Westermann and Weber, 2003). In plants, a TTL homolog has been identified (Gardiner and Marc, 2003), but, until now, none for TTCP. Therefore, we investigated the cytokinin sensitivity of the *ttl* loss-of-function mutant. The mutant exhibited a strong cytokinin-resistant phenotype for lateral root density and root meristem size. Altogether, these data imply that cytokinin might interfere with the TTL activity to regulate lateral root initiation and root development. However, changes in the tyrosination of α-tubulin in the mutant with and without cytokinin should be further tested.

Collectively, the results of this chapter revealed a new cytokinin mode of action in the regulation of lateral root formation and development, by modulating the MT structure and dynamics, but the molecular mechanism behind this cytokinin effect needs to be further investigated.

Materials and Methods

Plant Material

The transgenic *Arabidopsis thaliana* (L.) Heynh. lines have been described elsewhere: *ahk4/cre1-12, ahk2-2, ahk3-3, ahk4/cre1-12ahk2-2, ahk4/cre1-12ahk3-3, ahk2-2ahk3-3* (Higuchi et al., 2004); the Columbia (Col-0) background-derived EB1 triple mutant (*eb1a-2b-3c-2*) (Komaki et al., 2010); the Col-0-derived *tua1* (N682691), *tua5* (N670645), *tua6* (SALK_060173), *tub1* (N653140), *tub2* (N573132), *tub3* (N639499), *tub6* (N554610), *tub7* (N656311) and *tub8* (N668903) mutants were ordered from NASC stock center; the isopentenyltransferase mutants *ipt3, ipt5, ipt7, ipt3,5, ipt3,7, ipt5,7,* and *ipt3,5,7 35S::IPT3* (Miyawaki et al., 2004); and the *35S::CKX2*; *35S::CKX3* lines (Werner et al., 2001, 2003). ROP gain-of-function and loss-of-function *Arabidopsis* lines include loss-of-function *rop6-1* and its RIC downstream effector mutant *ric1-1*, which was used in the *Wassilewskji* (WS) background; the other lines (overexpressors *OX-ROP6*, and *OX-RIC1*) were derived from the Col-0 background (Xu et al., 2010). T-DNA insertion lines of the *ttl* mutant (*SALK_ 108909*; N678944) were obtained from the Nottingham Arabidopsis Stock Centre. EB1b-GFP (Komaki et al., 2010) and 35S::MAP4:GFP (Marc et al., 1998) and α-tubulin6-RFP were used as MT markers.

Growth Conditions

Seeds of *Arabidopsis* (accession Columbia-0), RFP or GFP-fused lines, and mutants were plated on half-strength (0.5) Murashige and Skoog (MS) medium (Duchefa) with 1% sucrose and 1% agar (pH 5.7) and stratified for 2 days at 4°C. Seedlings were grown on vertically oriented plates in growth chambers under a 16-h light/8-h dark photoperiod at 18°C. The primers used for genotyping the *ttl* mutant were:
PF: 5'-CACAAATTCTGGGCGTGTCA-3' and PR: 5' AACCCTTGTGCAATGTCACC 3'. The primers used for RT-PCR for the *AtTTL* gene were PF: 5'-TGCACTGCTCCTCGTATTTG-3' and PR: 5'-TCGAAGGAGCTATCGAGCAT-3'.

Growth conditions and media preparation for BY-2 cells

Cells of tobacco line BY-2 FABD2 (fimbrin actin-binding domain 2):mCherry/(inducible) β-tubulin:GFP (Nicotiana tabacum L., cv. Bright-Yellow 2) (Nagata et al., 1992) were cultured in liquid medium 3% (w/v) sucrose, 4.3 g l^{-1} Murashige and Skoog (MS) salts (Sigma Chemical Company, St. Louis, MO, USA, cat. n. M5524), 100 mg l^{-1} inositol (Duchefa Biochemie B. V., The Netherlands, cat. n. I0609), 1 mg l^{-1} thiamine (Sigma Chemical Company, St. Louis, MO, USA, cat. n. T3902), 0.2 mg l^{-1} 2,4-D, and 200 mg l^{-1} KH_2PO_4 (Sigma Chemical Company, St. Louis, MO, USA, cat. n. D8407), pH 5.8] containing 100 μg.ml^{-1} kanamycin, 100 μg.ml^{-1} hygromycin and 100 μg.ml^{-1} cefotaxim in darkness at 27 °C on an orbital

incubator (Sanyo Gallenkamp, Schoeller Instruments Inc., Prague, Czech Republic; 150 rpm, 32 mm orbit) and subcultured weekly (1ml of 7-day-old BY-2 cells into 30 ml of MS cultivation medium). Stock tobacco BY-2 calli were maintained on the same media solidified with 0.6% (w/v) agar and subcultured monthly. Expression of β-tubulin:GFP was induced by the addition of estradiol (β-estradiol, 1 μM, 48 h) at the beginning of the subculture interval.

Pharmacological and Hormonal Treatments

Five- to 6-day-old seedlings were transferred onto solid MS media with or without the indicated chemicals and incubated for 1 to 1.5 h in the dark at 22°. Drugs and hormones used were as follows: 0.1 μM naphthalene acetic acid (NAA), 10, 50, 100 nM BA (for phenotypical analysis) and 10 μM BA (for short treatment), 5 μM *trans*-zeatin, 50 μM propyzamide, 10 μM oryzalin, and 0.5 μM tubulysine A. For double treatments, a 30-min pretreatment with 10 μM BA or 5 μM *trans*-zeatin was done prior to the application. Propyzamide, tubulysine A, oryzalin, and NAA were dissolved in dimethyl sulfoxide (DMSO). BA, 6-Benzylaminopurine was dissolved in water (H_2O).

Analysis of Primary Root and Lateral root primordia Organogenesis

For phenotypic analyses of root growth, lateral root initiation, at least 15 seedlings were processed. The lateral root primordia density was measured on 7-day-old seedlings as described (Malamy and Benfey 1997). By scoring the number of lateral roots (1^{st} stage-LR stage) and divide them by the root length of that specific root. Root growth parameters (root length and root meristem) were analyzed with the ImageJ software package (NIH;http://rsb.info.nih.gov/ij) as described (Růžička et al., 2009).

Immunodetection

Immunofluorescence in *Arabidopsis* roots was analyzed as previously described (Sauer et al. 2006). The anti-α-tubulin (1:500) YOL1/34, rat monoclonal IgG2a, (Santa Cruz Biotechnology, Inc.), Monoclonal anti-tyrosine-tubulin (1:800), mouse IgG3 (Sigma-Aldrich) were used as primary antibodies. AlexaFluor 488 anti-rat (green) for α-tubulin (1:600), and AlexaFluor 594 anti-mouse (red) for tyrosine-tubulin (1:600) were used as secondary antibodies. Fluorescence was observed with a Zeiss LSM710 confocal microscope with 488 nm excitation and 499-519 nm emission for AlexaFluor 488 and 594 nm excitation and 591-618 nm emission for AlexaFluor 594.

Confocal Microscopy

EGFP was observed at the excitation wavelength of 488 nm and 505-550 nm emission and RFP at 543 nm excitation and 512-570 nm emission. All images in a single experiment were captured with the same settings and repeated at least three times. For the visualization of all auxin or CK-treated seedlings, a 1-min manipulation time was needed for the application of auxin and/or BA before imaging.

Spinning-Disk Microscopy Analysis of MT Dynamics

Confocal imaging was done with a confocal Eclipse Ti inverted microscope (Nikon), equipped with a Yokogawa CSU-X1 spinning disk unit (NIS-Elements Advanced Research version 4.13.04), using a ×100 Plan Apo 1.4 N.A. oil immersion objective. For high-speed spinning-disk confocal experiments, images were acquired at 100–200 ms exposures. Images were analyzed with the ImageJ software (NIH;http://rsb.info.nih.gov/ij). For quantification of growth and catastrophe rates, the procedure for kymograph analysis was followed as described (Matove et al., 2010; Nakamora et al., 2010), (https://psbzim02.psb.ugent.be/service/home/~/?auth=co&loc=en_US&id=3861&part=2). For frequency of growth and catastrophe per minute, after applying the kymograph, these parameters were scored manually over time, taking into account the image scale for calculating the area of region of interest (ROI). Statistics analyzed by means of the MATLAB software package.

Angle Deviation and Distribution Measurements

For quantification of the angle deviation and distribution of the MT orientations, we used the plug-in FibrilTool from Image J software package as described in (Boudaoud et al., 2014). The steps we followed for this analysis are: we first set up the scale of the control orientation (transverse orientation= 0^0). We then select a cell in the image as one region of interest (ROI) and the whole process will be repeated until all ROIs in the image are analyzed. FibrilTool plug-in for each of the MTs in the ROI will recognize the signal intensity and draw a line. It then generates the orientation that corresponds to the average orientation of arrays in the image. The average degree and the distribution of the angle deviation will be calculated for the ROI as a last step.

Trajectory Analysis

The EB1b protein trajectory was simulated with the Image J and MATLAB softwares. Briefly, the programme was as described below: the lateral optical flow in subsequent movie frames was estimated by implementing the Horn-Schunck

method. The velocity fields were averaged over several frames. When the resulting average velocity at a location was predominately horizontal or vertical, the pixel was colored green or red. Blue indicates that the average velocity was below an arbitrary threshold. This was done by calculating the apparent motion of particles at each position (at each pixel) in the image employing a standard algorithm (Horn Schunk). Followed by picture a river: At each position in the river, we draw an arrow which indicates the direction and the speed (=length of the arrow) of the water flow, by looking at a movie of the river and on the river surface there are leaves, so we can clearly see the water motion. By comparing two constitutive frames of the movie, we can tell where the water is flowing at each location in the river. Then at each position we calculate the average of the water flow over time. And at the end we draw one image of the river. At each location we draw a green point if the water moves predominately from left to right or from right to left and so on.

Co-localization Analysis

For co-localization analysis we used Pearson's correlation coefficient (R). The purpose of a co-localization coefficient is to characterize the degree of overlap between two channels in a microscopy image, which in our case is α-tubulin versus tyrosinated tubulin. Co-localization analysis is based on the pixel intensity correlation over space and was performed using Image J software package (NIH;http://rsb.info.nih.gov/ij). After splitting the two channels, we first identified the region of interest (ROI). For our analysis we considered 1 cell as 1 ROI; in every image approximately 5 cells (5 ROIs) were considered and therefore the analysis was repeated for all of them. Then we ran the co-localization plug-in using an automatic threshold. This step generates a 2-D histogram, a scatter plot, a fluorogram and a table of results. This table contains among others Rcoloc value which is the Pearson's correlation coefficient for the co-localization. Rcoloc is defined in [-1,1], with 1 representing complete co-localization, 0 representing no co-localization and negative values representing objects that act repulsively or in other words objects that 'repel' each other (wherever there is a red signal the green one tends to be elsewhere).

Supplementary Data

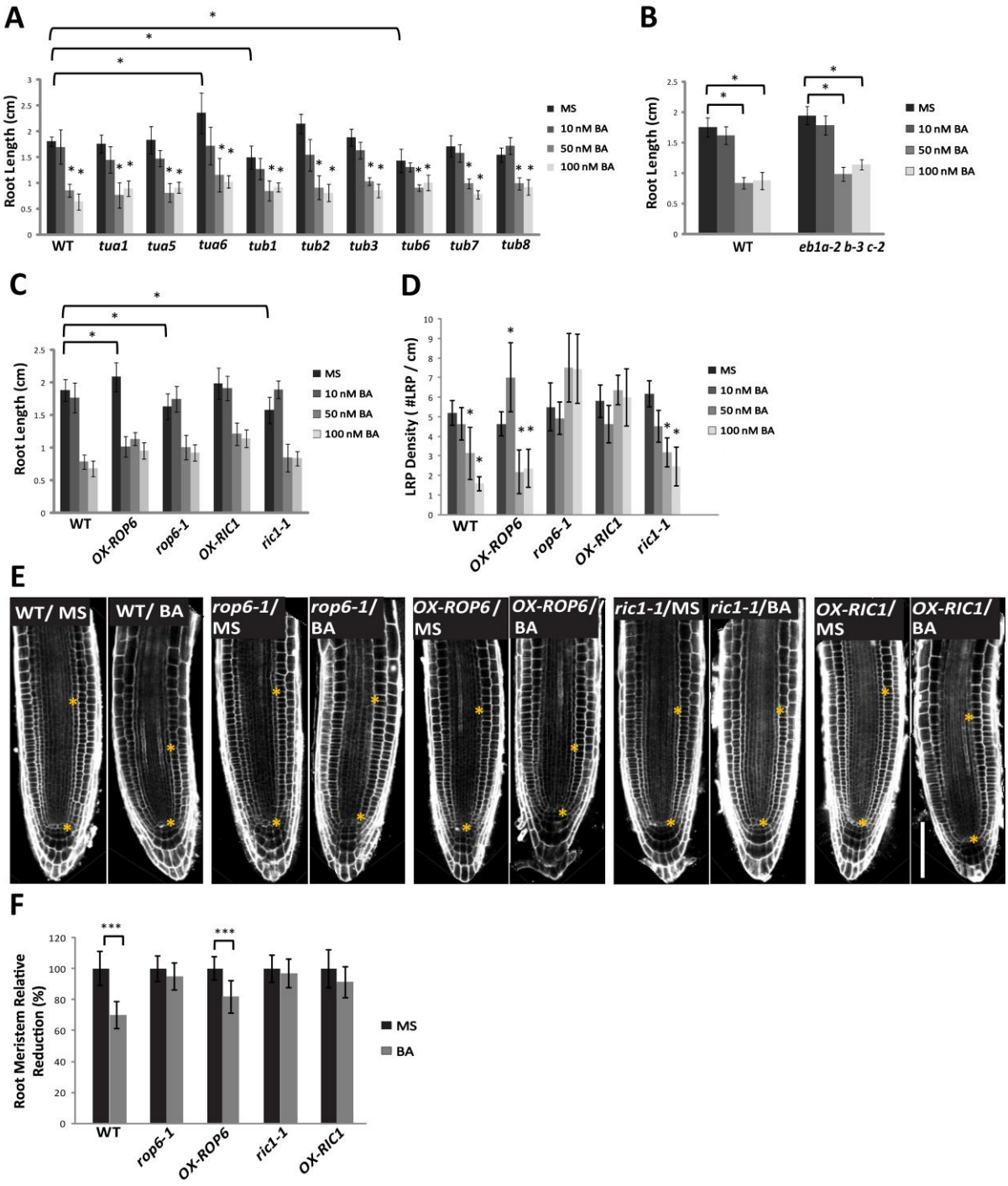

Figure S1: ROP6 and RIC1 are involved in the cytokinin regulation of root development (Related to Figure 1).

(A and B) Root length of tubulin mutants (A) and of the *eb1a-2b-3c-2* triple mutant (B) did not differ dramatically when compared to the wild type; except a slight difference for the *tua6*, *tub1*, and *tub6* mutants, no change were observed for the cytokinin (BA) sensitivity as well. (C and D) Mutants of *ROP6/RIC1* signaling are cytokinin sensitive for root length, whereas *ric1-1* and *rop6-1* mutants have reduced roots and the roots of *OX-ROP6* are

increased (C). *ROP6/RIC1* signaling mutants are CK-insensitive for LR initiation (D) for *rop6-1*, and *OX-RIC1,* in contrast to *OX-ROP6* and *ric1-1*, which are sensitive. Seedlings grown on 10, 50, and 100 nM BA-containing media for 7 days (*p<0.05, n= 15 roots). Error bars mark ±Standard Deviation (±SD). (E and F) Root meristem size of *rop6-1, ric1-1* and *OX-RIC1* mutants exhibit cytokinin insensitivity when grown on MS media and media supplemented with 0.1 µM BA for 7-days, then stained with propidium iodide, in contrast to *OX-ROP6,* which has a cytokinin-sensitive phenotype. (*p<0.05, ***p<0.001, n=15 roots). Yellow asterisks indicate the distance between the quiescent center (QC) and the cortex transition zone. (Scale bars= 50 µm). Error bars mark ±Standard Deviation (±SD). MS, Murashige and Skoog; BA, 6-benzylaminopurine).

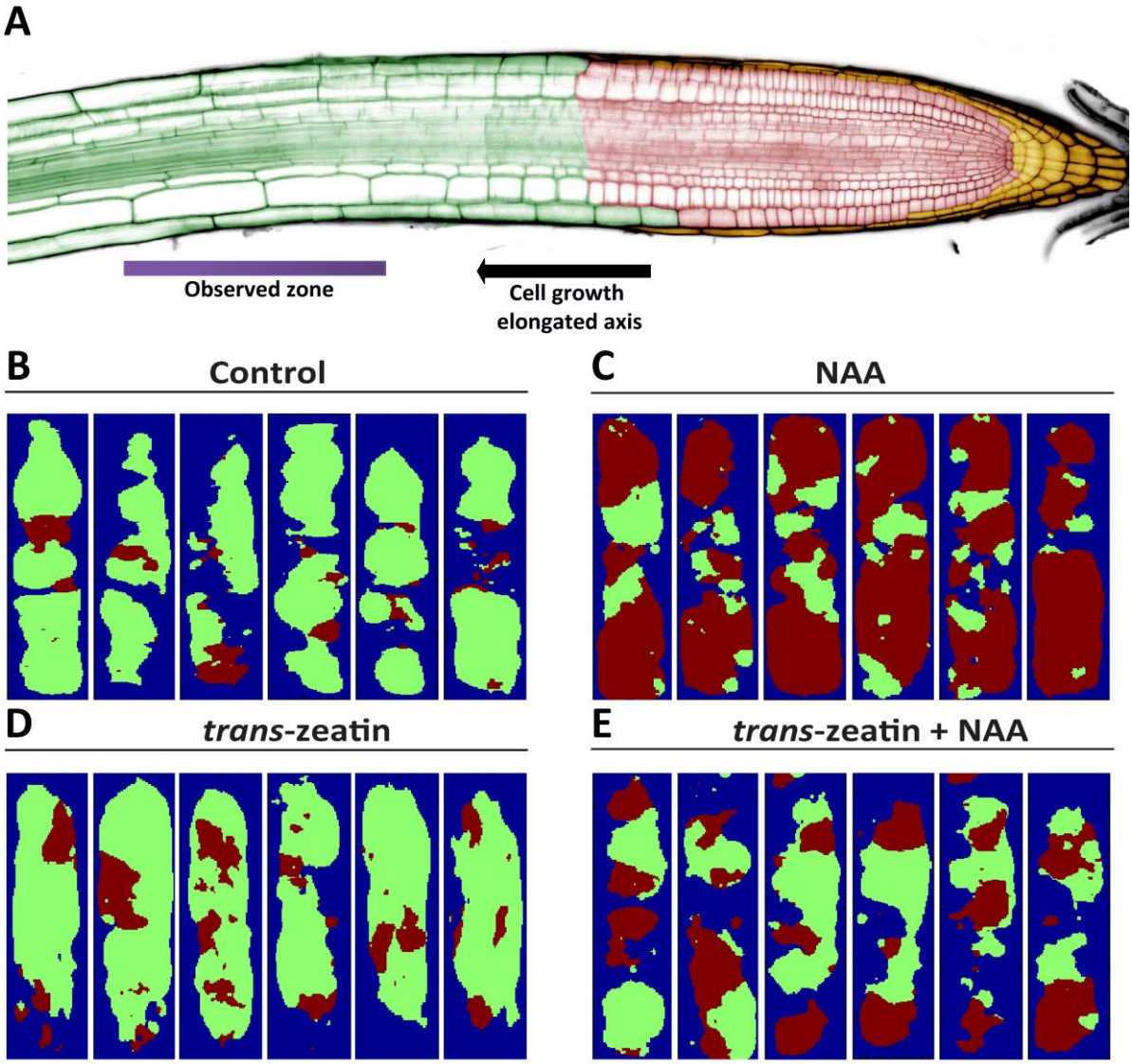

Figure S2: Cytokinin antagonizes the auxin effect on MTs (Related to Figure 2).

(A) Schematic representation of *Arabidopsis* main root showing the observed zone (4-5 cells) above the transition zone. In the transition and elongation zones of the root, cMTs arrange mostly perpendicularly to the elongation axis (A), designated transversal orientation. (B-E) The quantification method and artifical cartoon of the EB1b trajectories. Control is treated with MS (B), 0.1 µM auxin NAA (C), 5 µM cytokinin *trans*-zeatin (D), and 0.1 µM NAA+ 5 µM *trans*-zeatin double treatment (E), incubated for 1 h in the dark. Color maps show the EB1 trajectory simulated by the Image J and MATLAB softwares. Green and

red areas represent EB1b moves tranversally (90±30°) and obliquely and longitudinally (0-60°/120-180°), respectively.

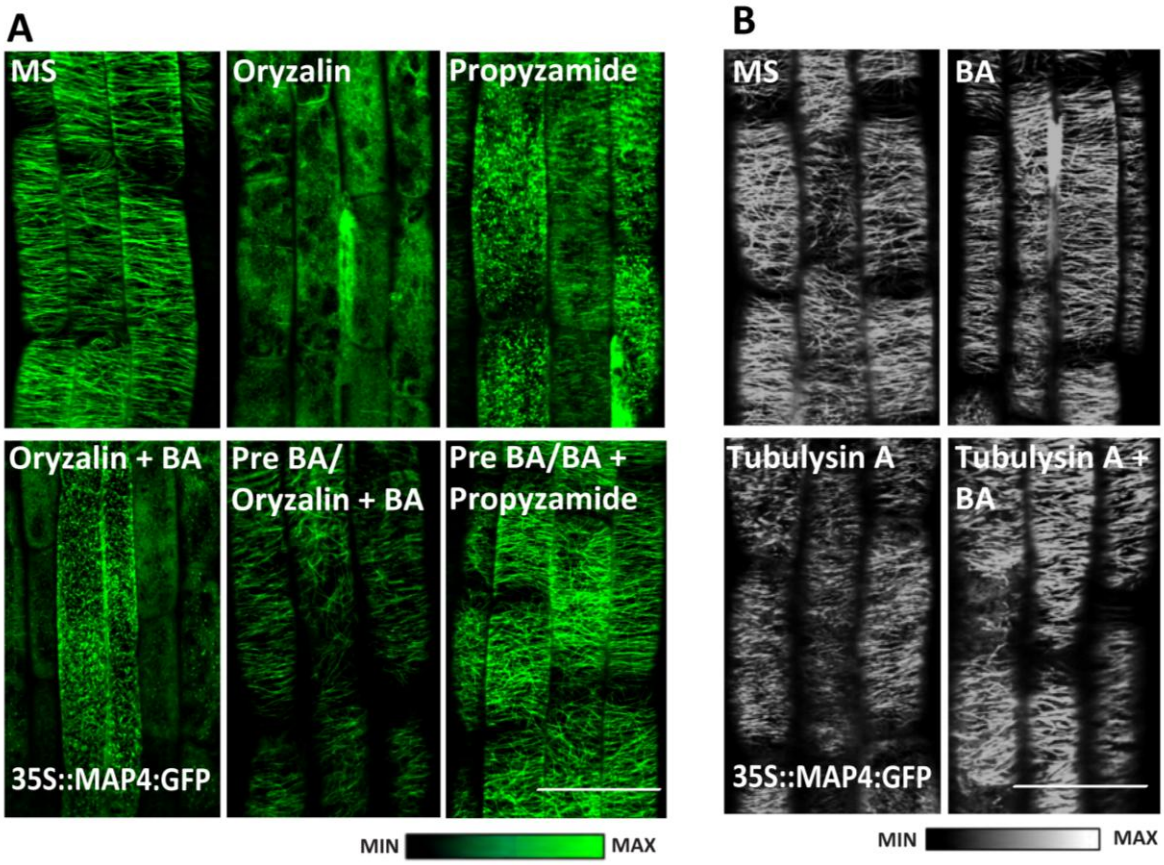

Figure S3: Cytokinin partially stabilizes MTs (Related to Figure 2).

(A and B) Pharmacological treatments of primary root of *Arabidopsis* with the 35S::MAP4-GFP reporter. Cytokinin (BA) counteracts the effect of the depolymerization drugs (10 µM oryzalin and 50 µM propyzamide) (A), Seedlings were treated with Oryzalin or Propyzamide for 30 min. 10 µM cytokinin pretreatment for 30 min followed by a double treatment prevents MT depolymerization by the drugs. Cytokinin (BA) shows a more organized orientation of MTs in the elongation zone, and rescues the effect of the depolymerized MTs caused by 0.5 µM tubulysine A (B). (n= 15 roots), (Scale bars= 10 µm). MS, Murashige and Skoog; BA, 6-benzylaminopurine.

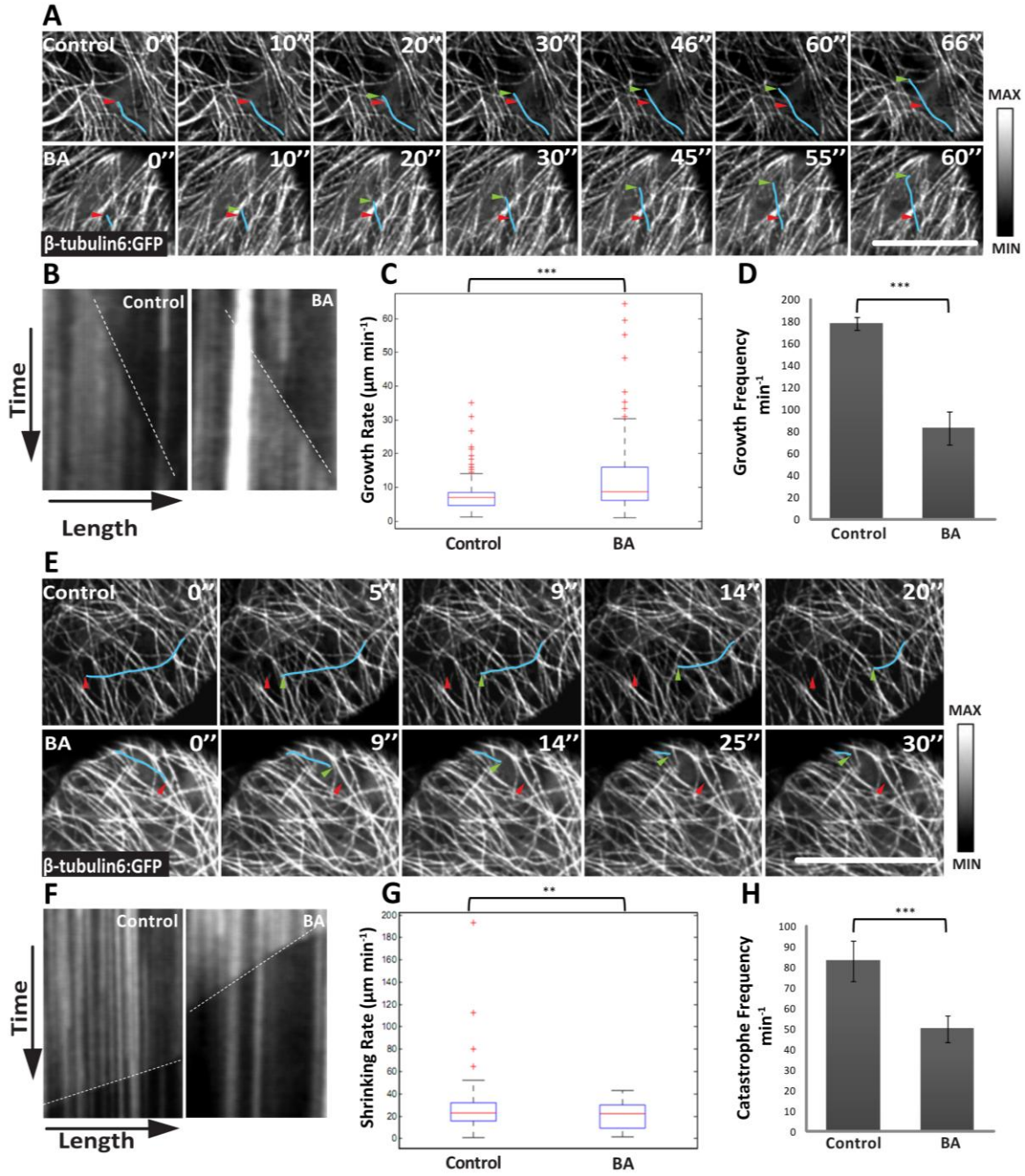

Figure S4: Cytokinin affects the MT instability (Related to Figure 4).

(A-D) Spinning-disk analysis of the growth rate at the plus ends with tobacco BY-2 FABD2 (fimbrin actin-binding domain 2):mCherry/(inducible) β-tubulin:GFP. Time-lapse images of the spinning disk show a faster growth of tubulin after application of 10 µM cytokinin for 1.5 h (A). Kymograph, generated from time-lapse microscopy images in (A) (B). Box plot showing that cytokinin enhances the growth rate of β-tubulin6 (C). Quantifications of growth frequency per cell per minute show a reduced number of events after treatment with 10 µM cytokinin for 1.5 h (D). (E-H) Spinning-disk analysis of the shrinking rate with cells of tobacco BY-2 FABD2 (fimbrin actin-binding domain 2):mCherry/(inducible) β-tubulin:GFP. Time-lapse images of spinning disk show a slower shrinking rate of tubulin after application of 10 µM cytokinin for 1.5 h (E). Kymograph, generated from time-lapse microscopy images in (E) (F). Box plot showing that cytokinin reduces the shrinking rate of β-tubulin6 (G). Quantifications of catastrophe frequency per cell per minute show a reduced

number of events after treatment with 10 µM cytokinin for 1.5 h (H). Red arrows indicate the initial position of the MTs where growth or shrinking starts. Green arrows indicate the new position of MTs at each time point (A and E). Error bars for (D and H) mark ±Standard Deviation (± SD). Significant values were evaluated using *One-way ANOVA* and Kruskal–Wallis test using MATLAB software. Box plots indicate the 25th percentile (bottom boundary), median (middle line), 75th percentile (top boundary), nearest observations within 1.5 times the interquartile range and outliers (red plus). Blue lines were used for better visualization of the MT and represent the MT growth or shrinkage. (***p<0.001 n=250 independent MT) (Scale bars= 5 µm).

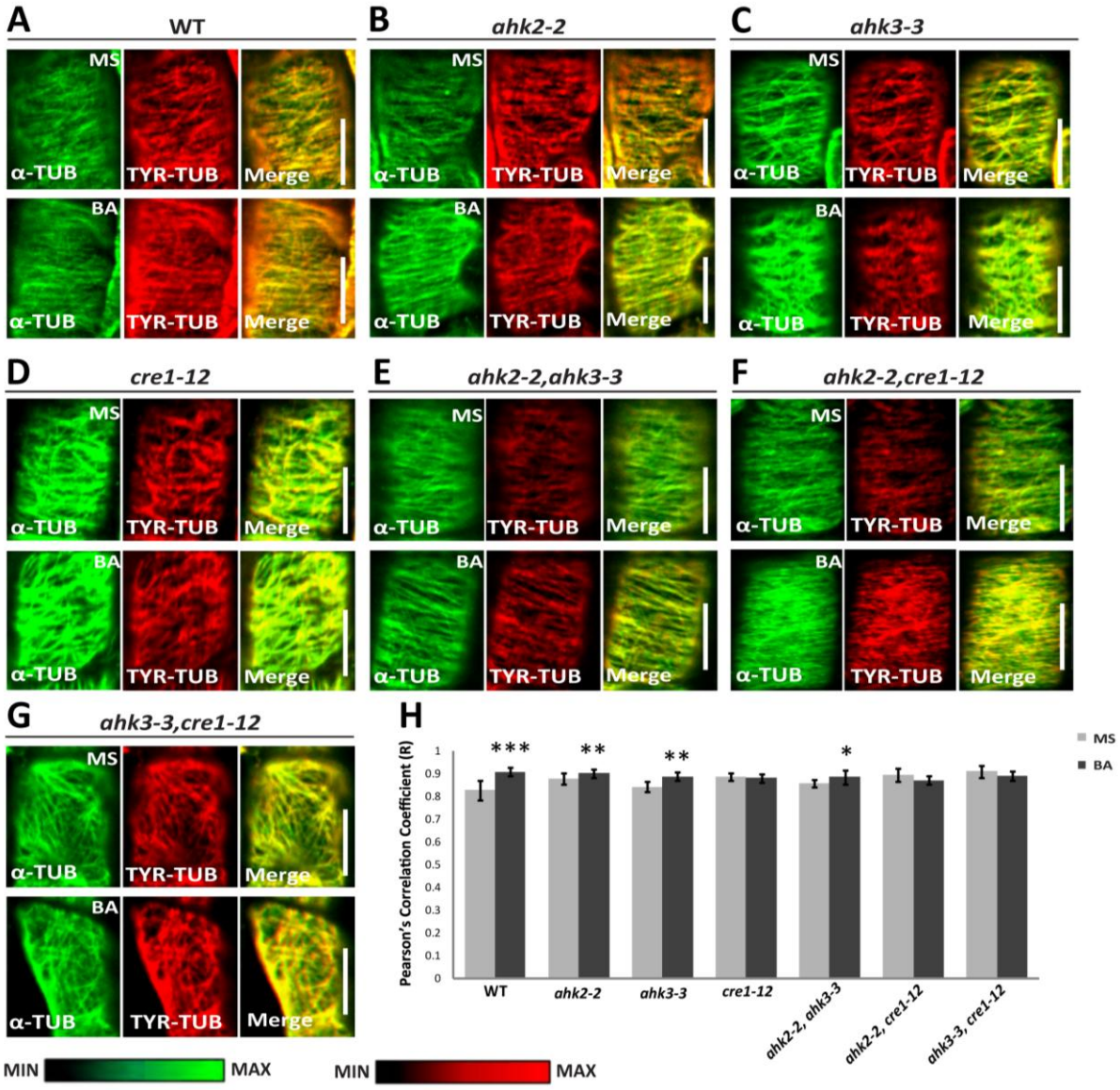

Figure S5: Analysis of tyrosination in cytokinin receptor mutants (Related to Figure 5).

(A-H) Tyrosination level with or without treatment with 10 µM cytokinin for 1.5 h in the dark of wild type (Col0), *ahk2-2* (B), *ahk3-3* (C), *ahk4/cre1-12* (D), *ahk2-2ahk3-3* (E), *ahk2-2 ahk4/cre1-12* (F), and *ahk3-3 ahk4/cre1-12* (G) mutants revealed that the mutation in the *AHK4/CRE1* receptor interferes with the tyrosination pattern when compared to the control

and the *ahk2* and *ahk3* mutants. In the *ahk4/cre1* mutant, the Pearson's correlation coefficient increased (H). (*p<0.05, **p<0.01, ***p<0.001 n= 15 roots). Error bars mark ±Standard Error of the Mean (±SE). Significant values were evaluated using *One-Way ANOVA* test and confirmed by *Post-hoc* analysis. (Scale bars= 5 µm).

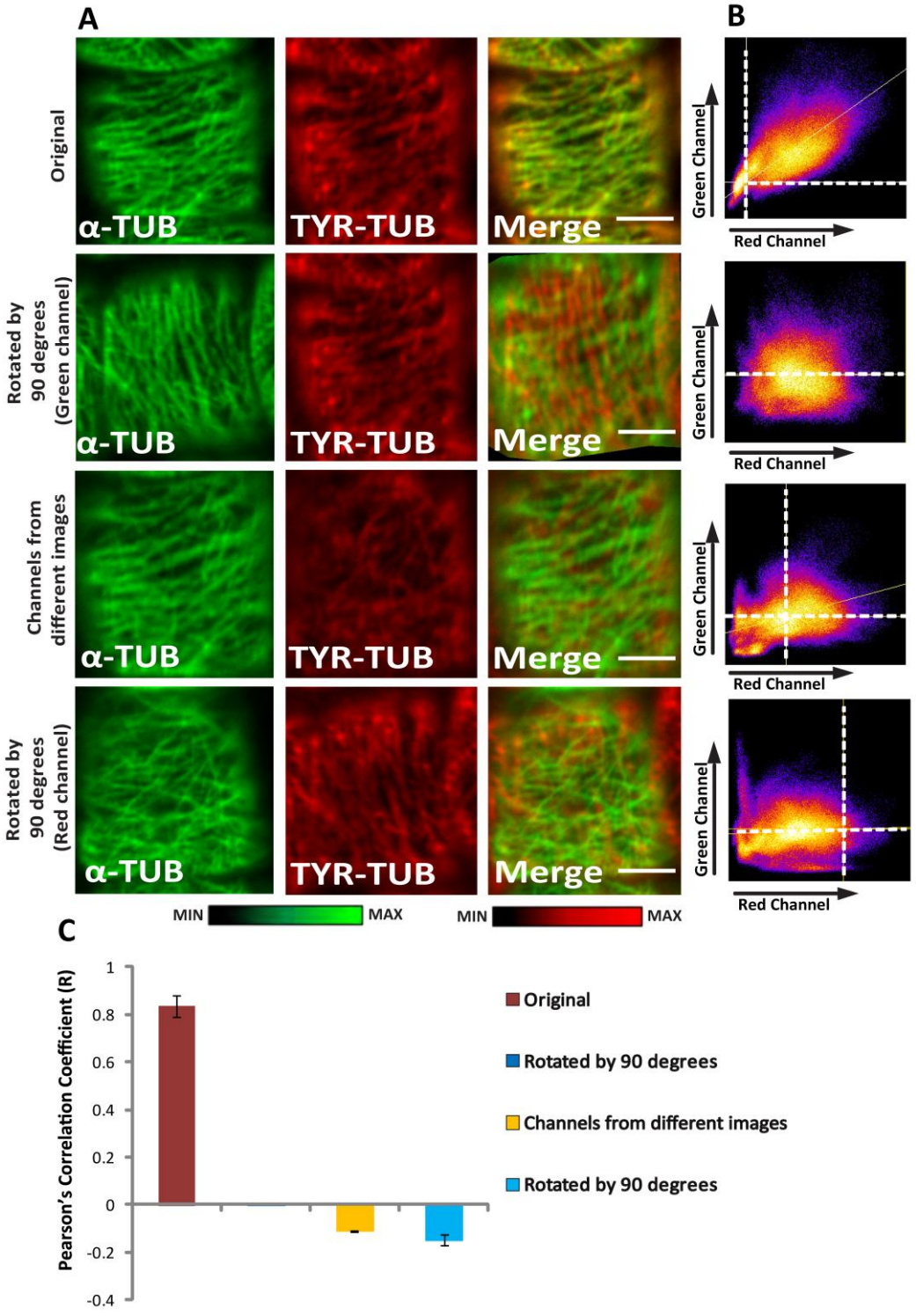

Figure S6: Validation of Pearson's correlation coefficient (R) as *in vivo* estimate of α-tubulin tyrosination (Related to Figure 5).

Immunodetection of α-tubulin (green channel) versus tyrosinated tubulin (red channel) of root epidermal cells (meristematic zone) on wild-type seedlings (Col0) (A). In the image with the original orientation (A; 1st panel) there is co-localization between α-tubulin and tyrosinated tubulin: Pearson's correlation coefficient is greater than 0.8 (C; 1st column) and values on the scatter plot of co-localization between the two channels show high positive correlation (B; 1st panel). When the green channel is rotated by 90 degrees (A; 2nd panel), no co-localization is observed: Pearson's correlation coefficient is almost zero (C; 2nd column) and the scatter plot of co-localization shows randomized values (cloud shape which indicates no correlation) (B; 2nd panel). Combining two channels from different images (A; 3rd panel), also shows no co-localization: Pearson's correlation coefficient has a negative value close to -0.1 (C; 3rd column) and the scatter plot of co-localization between channels again indicates very low to zero correlation (all values are again organized in cloud shape and close to the intersection of the x- and y- axis) (B; 3rd panel). When combining two channels from different images and also rotate the red channel 90 degrees (A; 4th panel), we observe no co-localization: Pearson's correlation coefficient is again negative and close to -0.15 (C; 4th column) and the scatter plot of co-localization between channels indicates very low correlation (values are mainly distributed in the 3rd quadrant of the x-y axes) (B; 4th panel). All these findings suggest that indeed the co-localization of the original image is not due to an artifact nor found by chance. Dashed white lines in scatter plots represent the pixel intensity of the red channel (x-axis) and of the green channel (y-axis). (n= 10 roots). We used 1 cell as 1 ROI; in every image (or root) approximately 5 cells (5 ROIs) were measured. Error bars mark ±Standard Deviation (±SD). (Scale bars= 2 μm).

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Cytokinin controls postembryonic plant development through actin-dependent lytic degradation of PIN-FORMED1

Adapted from:

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Author contribution:

A.A and E.B initiated the project and designed most of the experiments, A.A carried out all the experiments related to figures 1, 2, 3, 4, 5, S1, and S2. M.V, P.M, and J.P. helped in the discussion of the data. A.A and E.B wrote the manuscript. All authors saw and commented on the manuscript.

Cytokinin Controls Postembryonic Plant Development through Actin-Dependent Lytic Degradation of PIN-FORMED1

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The endocytosis process that involves formation and internalization of the plasma membrane (PM) vesicles plays an important role in the positioning of PM auxin transporters, namely the PIN-FORMED (PIN) auxin efflux carriers. Although some evidence supports the idea that differential PIN protein trafficking depends on actin and microtubule cytoskeletons, information on the components linking the cytoskeleton dynamics with the endocytic trafficking of auxin transporters in particular and endocytosis in general is still lacking. Recently, the plant hormone cytokinin has been demonstrated to interfere with the cellular trafficking of the auxin membrane efflux carrier PIN1 and to direct it for lytic degradation. For cytokinin to affect the PIN1 levels in the PM, intact actin was proposed to be required. To investigate the molecular mechanism underlying the cytokinin-dependent PIN1 lytic degradation, mutants were analyzed that were defective in genes controlling actin cytoskeleton formation and activity, including actin, components of the Actin-related protein, the Wiskott-Aldrich Syndrome Protein complex, and small Rho-like GTPases of plants. Our findings suggest that the actin cytoskeleton and actin-associated proteins play an important role in cytokinin-regulated root growth via cytokinin-controlled PIN cellular trafficking.

Introduction

Plant morphogenesis is highly dependent on endocytosis for regulated and constitutive uptake and recycling of PM proteins, nutrient uptake, hormone and pathogen signaling, and other cellular processes (Baisa et al., 2013). PIN auxin efflux carriers are prominent examples of PM proteins of which the subcellular dynamics directly impact on the distribution of the plant hormone auxin. Abundance of PIN proteins at the PM determines the auxin flow efficiency and direction, thereby regulating the formation of auxin gradients and the availability of the hormone in cells and tissues (Geldner et al., 2001; Kleine-Vehn et al., 2006). This graded polar auxin distribution is critical for development, pattern formation, and morphogenesis in plants (Geldner et al., 2001; Kleine-Vehn et al., 2006; Tanaka et al., 2006, 2013).

Membrane-located PIN proteins undergo a dynamic subcellular transport, involving constitutive cycles of endocytosis and recycling back to the PM (Geldner et al., 2001; Dhonukshe et al., 2007). The endocytic recycling is required to establish the polar PIN localization and presumably underlays the rapid polarity changes that are observed during root gravitropic responses and embryogenesis (Friml et al., 2002, 2004). Alternatively, after internalization, PIN proteins can be targeted to the vacuole for lytic degradation (Malenica et al., 2007; Kleine-Vehn et al., 2008b; Löfke et al., 2013). Importantly, PIN cycling provides a mean for feedback regulation and modulation of PIN intracellular trafficking can directly affect the PIN abundance at membranes as well as its polarity. For instance, endocytosis of membrane vesicles is inhibited by auxin itself, promoting its own efflux (Paciorek et al., 2005). Recently, not only auxin has been demonstrated to shape the capacity and directionality of its transport, but also other plant hormones, such as jasmonate, gibberellin (GA), salicylic acid (SA), and cytokinin to modify the activity of the PIN-dependent auxin distribution network (Sun et al. 2011; Willige et al., 2011; Du et al., 2013; Růžička et al., 2009; Yoshida et al., 2011; Zhang et al., 2011a), thereby regulating plant growth and development. Most of the plant hormones have been shown to control transcription of numerous genes downstream of their corresponding signaling pathways and, thus, to influence directly or indirectly the transcription of *PIN* genes (Swarup et al., 2007; Růžička et al., 2007, 2009; Dello Ioio et al., 2008; Bishopp et al., 2011a, 2011b; Zhang et al., 2011b; Liu et al., 2013). Besides transcriptional control, plant hormones modulate the PIN activity at the protein level. For example, PIN proteins at the PM are stabilized when plants are subjected to pharmacologically or genetically induced accumulation of the plant hormone SA (Du et al., 2013). In contrast, GA deficiency observed in GA biosynthesis mutants promote the lytic degradation of PIN proteins (Willige et al., 2011; Löfke et al., 2013). The plant hormone cytokinin modulates the PIN1 endocytic trafficking and redirects this membrane protein for lytic degradation into the vacuoles. In this case, the cytokinin activity is transduced through the Arabidopsis Histidine Kinase4 (AHK4) receptor (Marhavý et al., 2011). However, the mechanism by which cytokinin interferes with

the PIN1 endocytotic trafficking remains poorly understood. On the whole, the PIN endocytotic recycling seems to be targeted by various plant hormones to modulate rapidly auxin fluxes and distribution. This still largely unexplored hormonal interaction with the cellular protein trafficking machinery might represent an important generic mechanism used by hormones to regulate plant development.

Endocytotic recycling of PIN auxin efflux carriers depends on the functional actin cytoskeleton (Geldner et al., 2001). Similarly to auxin, also other plant hormones, including cytokinin, acting through modulation of PIN trafficking might be functionally dependent on the actin cytoskeleton. Indeed, studies involving a number of cell types have highlighted the importance of the actin cytoskeleton in protein subcellular trafficking, in particular during the early steps of endocytosis. Actin can be considered to play an active role in assisting different stages of the endocytosis, including (i) assembly of the endocytotic coat complex, (ii) invagination, and (iii) vesicle scission and movement from the PM (Baisa et al., 2013).

To get insights into the role of the actin cytoskeleton during the hormonal crosstalk, we focused on the role of actin in the cytokinin-induced retargeting of PIN proteins to vacuoles and on the significance of this regulation for the root system establishment. We demonstrate that molecular components controlling nucleation and polymerization of actin filaments at the PM are required for rapid modulation of the PIN1-mediated auxin transport and for shaping the root system architecture in response to cytokinin. Our results indicate that lack of a functional actin cytoskeleton might interfere with the PIN1 endocytosis and, thus, compromise plant responses to hormonal regulation.

Results

The actin cytoskeleton is involved in the cytokinin-regulated root development

Cytokinin is one of the key hormonal regulators of the root system architecture. Increases in either cytokinin levels or in signaling attenuate lateral root initiation and promote root meristem differentiation, resulting in a dramatically reduced root meristem size (Laplaze et al., 2007; Dello Ioio et al., 2008; To and Kieber, 2008; Mason et al., 2005; Moubayidin et al., 2009). Cytokinin control of the root system development involves rapid modulation of the auxin flow (Bishopp et al., 2011b). Cytokinin has been found to interfere with the PIN1 subcellular trafficking and to redirect PIN1 for lytic degradation to vacuoles. This rapid decrease in the PIN1 membrane upon cytokinin treatment negatively impacts on the availability of auxin for lateral root organogenesis and root meristem maintenance (Marhavý et al., 2011). Inhibition of actin filament polymerization by treatment with latrunculin B (LatB) prevents PIN1 trafficking into the vacuoles in response to

cytokinin (Figure 1A, 1B; Marhavý et al., 2011), suggesting that cytokinin-induced PIN1 lytic degradation requires actin polymerization. However, so far, no genetic evidence has been provided to support the role of the actin cytoskeleton in this process.

To gain insights into the contribution of the actin cytoskeleton to the cytokinin-regulated root growth, we analyzed mutants in actin-encoding and actin-related genes. In particular, we focused on the cytokinin sensitivity of the root growth, root meristem size, lateral root initiation and development. Actin filaments consist of actin monomers encoded by 10 isoforms in the *Arabidopsis thaliana* genome (McKinney and Meagher, 1998). According to the gene expression profiling data obtained by Genevestigator (Hruz et al., 2008), *ACTIN2* (*ACT2*), *ACTIN7* (*ACT7*) and *ACTIN8* (*ACT8*) are expressed in the roots (Kandasamy et al., 2009) and their role in root hair development has been demonstrated (Kandasamy et al. 2009). Therefore, mutants in *ACT2, ACT7* and *ACT8* were selected for closer observation. Mutations in either *ACT2* or *ACT8* did not affect significantly root growth, lateral root initiation, and their sensitivity to cytokinin (Figure S1A, S1B). In contrast, the *act7* mutant had a significant effect on root growth (Figure S1A). Due to the severe retardation of the primary root growth, it was impossible to conclude unequivocally on the role of *ACT7* in the cytokinin-regulated lateral root organogenesis.

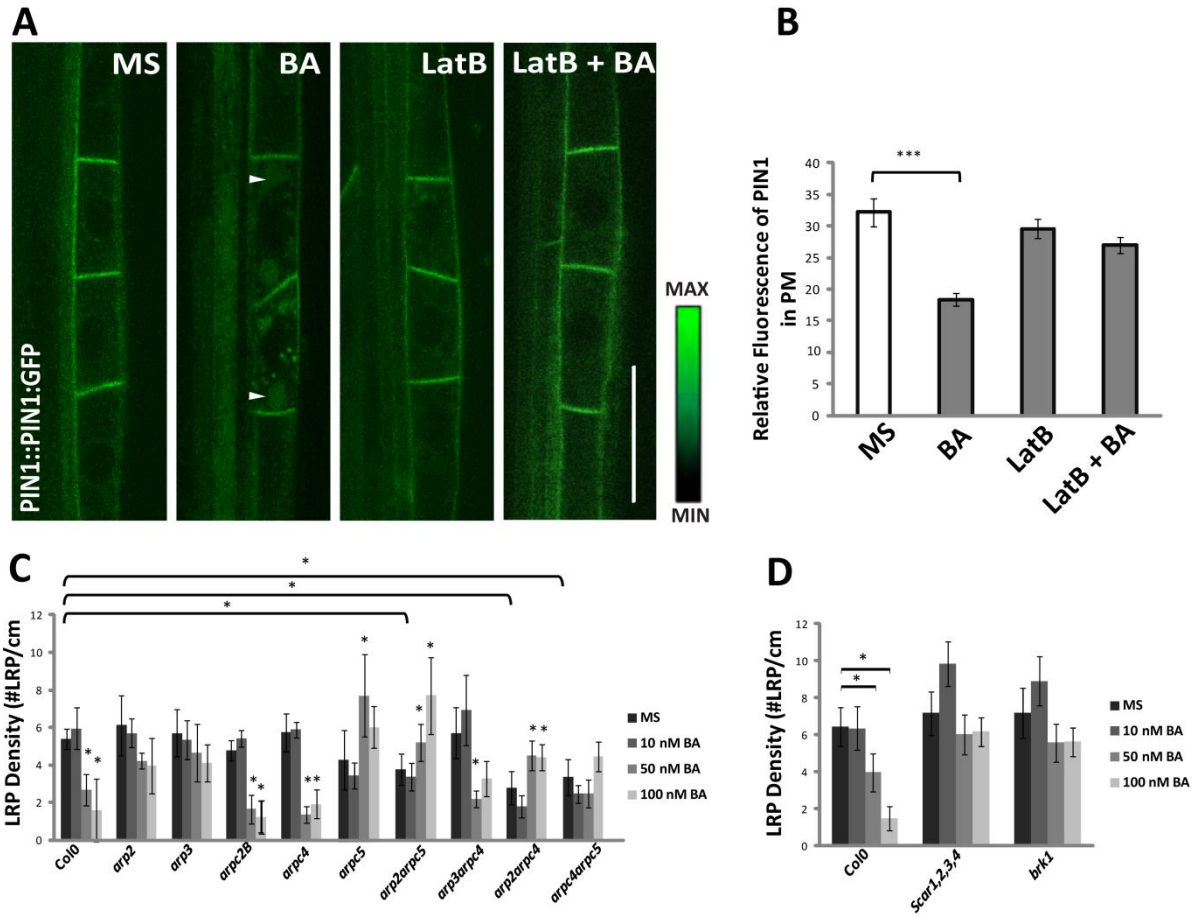

Figure 1: Cytokinin-mediated root growth requires a functional actin cytoskeleton.

(A, B) Depolymerization of actin by LatB interferes with the PIN1-GFP trafficking to the vacuoles in response to BA. White arrows indicate PIN1-GFP accumulation in vacuoles. (A) Membrane PIN1-GFP was quantified at anticlinal membranes of stage-I lateral root primordia untreated or treated with 0.1 µM BA and 20 µM LatB for 1.5 h in the dark (***p<0.01; n=15 LRP) (B). (C and D) Mutants in the ARP2/3 complex subunits (C) and in the WAVE complex subunits *brk1* and quadruple *scar1,scar2,scar3,scar4* mutant (D) exhibit cytokinin-insensitive lateral root density. Seedlings were grown on 10, 50 and 100 nM BA-containing media for 7 days (*p<0.05; n= 15 roots). Error bars are ± Standard Deviation (±SD). Significant values were evaluated using Student's *t*-test. MS, Murashige and Skoog media; BA, 6-benzylaminopurine; LatB, latrunculin B. (Scale bar= 10 µm).

The Actin-related protein (Arp2/3) complex has a major function in the regulation of the actin cytoskeleton and stimulates actin polymerization by creating a new nucleation core (Staiger et al. 2010; Yanagisawa et al. 2013). Mutants in the Arp2/3 subunits (*arp2* and *arp3* (Dyachok et al., 2011), *arpc2B*, *arpc4, arpc5, arp2arpc4, arp2arpc5, arp3arpc4* and *arpc4arpc5* (Szymanski, 2005) did not exhibit a significant effect on root growth except for *arpc4, arpc5, arp2arpc4,* and *arpc4arpc5*. In contrast, the double mutants *arp2arpc4, arpc4arpc5* and *arp2arpc5* affected the density of lateral root primordia, indicating their specific functions in the lateral root initiation (Figure 1C, Figure S1C). When exposed to cytokinin, the overall root elongation , which is primarily under the control of ethylene overproduced in response to cytokinin (Cary et al., 1995; Růžička et al., 2009), remained largely unaffected in most of the tested *arp* mutants except for *arp2arpc4*, and *arpc4arpc5* (Figure S1C). On the contrary, the root meristem size that is reduced specifically by cytokinin and independently of ethylene (Růžička et al. 2009), was unaffected in *arp2* and *arp3* mutants, implying that the ARP2/3 complex contributes to the cytokinin-specific regulation of the root meristem size (Figure S1E and S1F), Accordingly, in the majority of the *arp* mutants, except for mutants in the *ARPC4* and *ARPC2B* subunits, the sensitivity of the lateral root initiation to the cytokinin inhibition was reduced significantly, strongly supporting a role of the ARP2/3 complex in the cytokinin-regulated root branching.

The Arp2/3 complex activation depends on the activity of the Wiskott-Aldrich syndrome Protein (WASP) family verprolin homologous (WAVE)/Suppressor of cAMP receptor (SCAR) complex consisting of proteins that are stabilized by the interaction with BRICK1 (BRK1) (Yanagisawa et al., 2013 ;Dyachok et al., 2011). Close examination of the mutants in components of the WAVE complex, including *brk1* and the quadruple *scar1scar2scar3scar4* mutants revealed that in both lines the sensitivity of the lateral root initiation to cytokinin is severely reduced (Figure 1D, Figure S1A-1D). Neither the *brk1* nor the *scar1scar2scar3scar4* mutants were affected in the overall root growth by cytokinin, hinting at a specific

role of this protein complex in mediating cytokinin, but not ethylene-related developmental responses (Figure S1D).

Altogether, analyses of mutants in the ARP2/3 and WAVE complex subunits indicate that cytokinin requires a functional actin to control root development. Our results support primary mechanisms regulating nucleation and branching of an actin network in the cytokinin-regulated root growth.

The actin cytoskeleton is required for cytokinin-stimulated PIN1 lytic degradation

Previously, the inhibitory cytokinin effects on the lateral root organogenesis and the enhanced differentiation of the root meristem had been correlated with rapid PIN1 degradation (Marhavý et al., 2011). The PIN1 decrease prevents proper auxin distribution with defective lateral root organogenesis and reduced root meristem size as a consequence. The reduced cytokinin sensitivity observed in mutants of the ARP2/3 and WAVE/SCAR complex subunits prompted us to test whether the lack of the ARP2/3 and WAVE/SCAR complex activities impacts on the cytokinin-stimulated PIN1 lytic degradation. Immunolocalizations by means of PIN1-specific antibodies were performed to monitor the PIN1 sensitivity to the cytokinin treatment in control and mutant roots (see Materials and Methods).

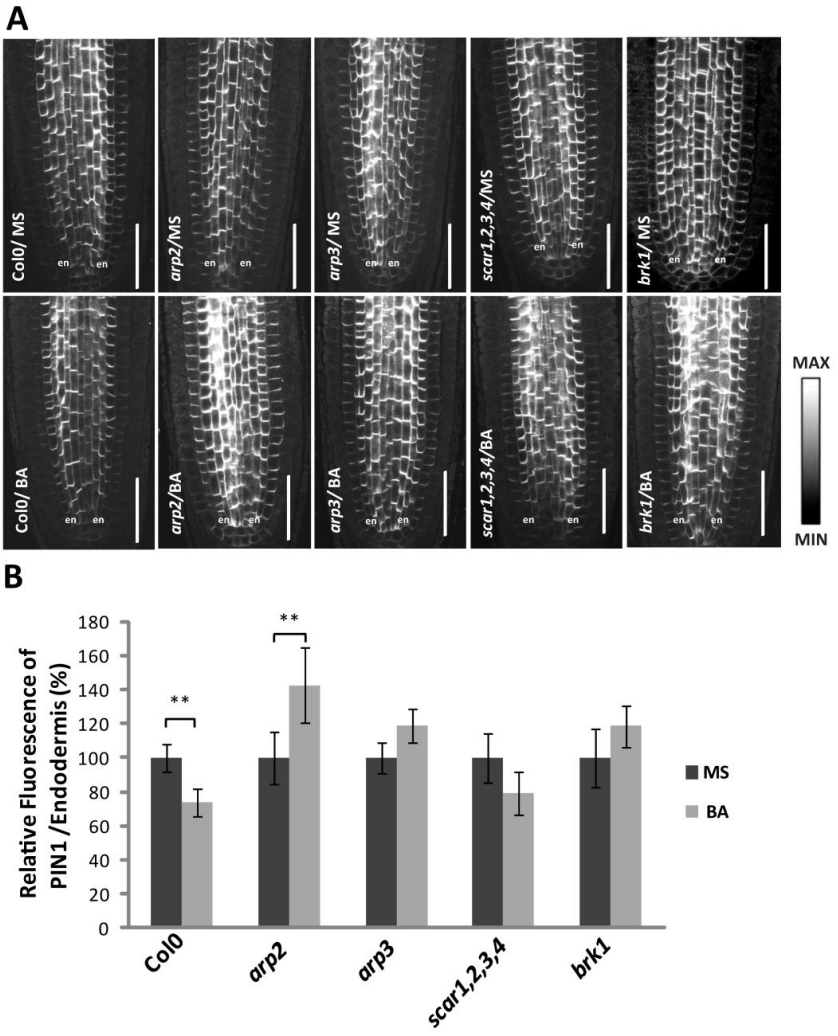

Figure 2: ARP2/3 complex activity is required for cytokinin-mediated PIN1 vacuolar targeting.

(A and B) PIN1 is cytokinin insensitive in the endodermal cells (en) of *arp2*, *arp3, sacr1,2,3,4* and *brk1* mutants. The PIN1 signal was detected by immunolocalization in endodermal cells of the control (MS)-grown seedlings and in root meristems treated with 0.1 μM BA for 1.5 h in the dark (A) for details, see Materials and Methods. Quantification of the PIN1 signal intensity in the basal membrane of the endodermis (B) (**p<0.001, n=10 roots, 10 cells per root). Error bars mark ± Standard Deviation (±SD). (Scale bars= 30 μm). Significant values were evaluated using Student's *t*-test. MS, Murashige and Skoog media; BA, 6-Benzylaminopurine.

We found that the lack of either the *ARP2* or *ARP3* subunits of the ARP2/3 complex affected, besides lateral root initiation and root meristem cytokinin sensitivity, also the PIN1 sensitivity to cytokinin-stimulated targeting to vacuoles (Figure 2A and 2B). Similarly, the absence of the BRK1 activity strongly interfered with the cytokinin impact on the root meristem size and lateral root initiation as well as on the PIN1 lytic degradation (Figures 2A and 2B, Figures S1E and S1F). Altogether, these data indicate that a functional actin cytoskeleton is required for

both cytokinin-regulated root development and PIN1 trafficking to vacuoles in response to cytokinin (Figure 2A).

The ROP2 signaling pathway is involved in cytokinin-regulated root growth

Rho GTPases are conserved molecular switches that link signal transduction to actin organization in various eukaryotic organisms (Zheng and Yang, 2000; Fu et al. ,2002; Yang, 2002). Once activated, Rho GTPases bind to a variety of effectors, including protein kinases (Zhao and Manser, 2005) and some actin-binding proteins (Mucha et al., 2011; Craddock et al., 2012) that directly or indirectly affect the local assembly or disassembly of filamentous (F)-actin (Gu et al., 2004). ROP is a plant-specific subfamily of the Rho family of small GTPases (Zheng and Yang, 2000; Yang, 2002), of which the activity has been linked to actin-dependent tip growth in pollen tubes and root hairs and to anisotropic growth in hypocotyls, roots and leaf epidermal cells (Kost et al., 1999; Li et al., 1999; Molendijk et al., 2001; Fu et al., 2002; Foucart et al., 2009; Fu, 2010). To investigate the role of actin in cytokinin-regulated root growth, we examined transgenic plants with an altered ROP signaling activity. For this purpose, transgenic lines expressing either constitutively active ROP2 (CA-ROP2) or dominant-negative ROP2 (DN-rop2) alleles were analyzed in detail (Li et al., 2001). CA-ROP2 promoted lateral root initiation, but neither CA-ROP2 nor DN-rop2 alleles significantly affected root growth (Figure 3A and 3B). Distinct differences in their cytokinin sensitivity were observed between roots expressing either CA-ROP2 or DN-ROP2. Whereas CA-ROP2 signaling dramatically reduced the cytokinin sensitivity of root meristems, root growth and lateral root initiation, attenuation of the ROP2 signaling in DN-rop2 lines did not change the sensitivity of roots to cytokinin (Figure 3A and 3B, Figure S2A-S2C). Because ROP2 promotes the accumulation of the fine cortical actin microfilaments through its downstream target ROP-INTERACTIVE CRIB MOTIF-CONTAINING PROTEIN4 (RIC4) (Fu et al., 2005), we also assessed whether the root cytokinin sensitivity depends on the RIC4 activity. Detailed analyses of the *ric4-1* root growth revealed that, similarly to the DN-rop2 lines, roots of the *ric4-1* mutant were fully sensitive to the cytokinin treatment (Figure 3A and 3B, Figure S2A-S2C). Together, the data demonstrate that constitutive activation of the ROP2 pathway severely interferes with cytokinin-regulated root growth.

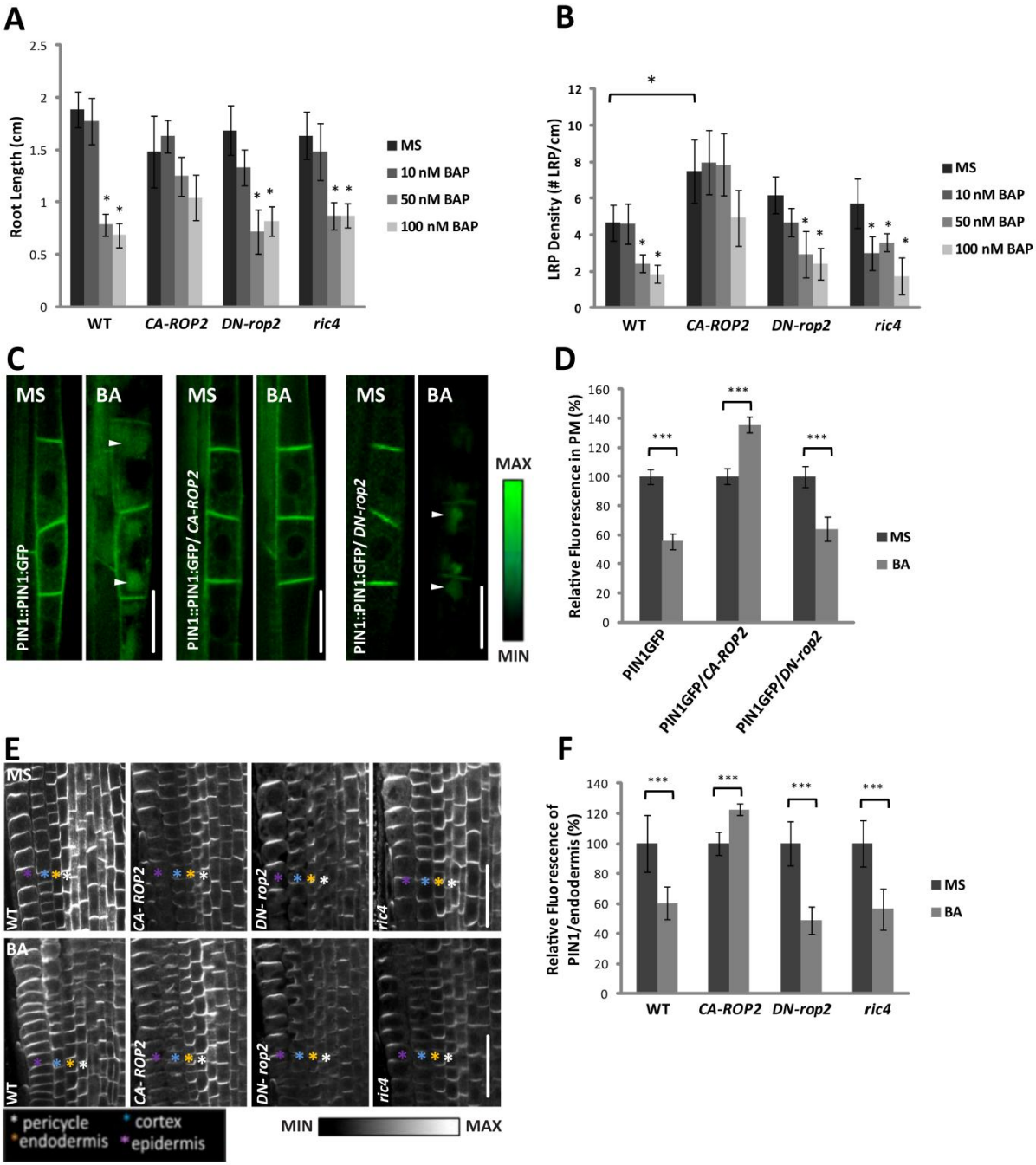

Figure 3: ROP2 and RIC4 are required for cytokinin-mediated PIN1 vacuolar targeting.

(A, B) *CA-ROP2* reduces the cytokinin sensitivity of the root length (A) and lateral root initiation (B) in contrast to *DN-rop2* and *ric4-1*. Seedlings were grown on 10, 50, and 100nM BA-containing media for 7-days (*p<0.05; n= 15 roots). (C, D) The membrane PIN1-GFP is insensitive to BA in *CA-ROP2* but normal sensitivity to *DN-rop2* (C). Membrane PIN1-GFP signal was quantified at anticlinal membranes of stage-I lateral root primordia untreated and treated with 0.1µM BA for 1.5 hr in the dark (D). (***p<0.001: n=10 LRP). (Scale bar= 10 µm). (E, F) Immunodetection of α-PIN1 and α-PIN2 in ROP2 signaling mutants. PIN1 is cytokinin insensitive in the root endodermal cells of *CA-ROP2,* in contrast to *DN-rop2* and *ric4-1* (E). Quantification of the PIN1 signal intensity in the basal membrane of the

endodermis (F). The PIN1 signal was detected by immunolocalization in endodermal cells of the control (MS)-grown seedlings and in root meristems treated with 0.1 μM BA for 1.5 h in the dark. (***p<0.001; n=10 roots, 10 cells per root). (Scale bars= 10 μm). White arrows indicate PIN1-GFP accumulation in vacuoles. Error bars mark ± Standard Deviation (±SD). Significant values were evaluated using Student's *t*-test. MS, Murashige and Skoog media; BA, 6-benzylaminopurine.

Activated ROP2 signaling prevents cytokinin-stimulated PIN1 degradation

To examine whether, besides controlling root sensitivity to cytokinin, the ROP signaling pathway is required for cytokinin-stimulated PIN1 degradation, we monitored the PIN1 sensitivity to cytokinin in both the root meristem and stage I lateral root primordia by either immunodetection or by the PIN1::PIN1-GFP reporter, respectively. Whereas cytokinin targeted PIN1 for lytic degradation in both *DN-rop2* and *ric4-1* lines to levels comparable to those of the control (Figure 3C-3F), constitutive activation of the ROP signaling pathway in CA-ROP2 roots fully prevented the cytokinin-induced PIN1 lytic degradation (Figure 3C-3D). These data suggest that the cytokinin regulation of both the root development and as the PIN1 subcellular trafficking depends on the ROP2 signaling pathway. The constitutive activation of the ROP2-mediated signaling desensitizes the PIN1 trafficking to cytokinin and, as a consequence, the root system sensitivity to cytokinin might be attenuated. Thus, our results suggest that ROP GTPases contribute to the proper cellular PIN1 trafficking that is essential for the cytokinin-regulated root system establishment.

Actin-related mutants exhibit defective endocytosis

The actin cytoskeleton has been shown to play an important role in control of the auxin transport through regulation of the PIN recycling between PM and endosomal compartments (Geldner et al., 2001; Voigt et al., 2005; Dhonukshe et al., 2008b). Our experiments show that the intact actin cytoskeleton is required for both cytokinin-mediated redirecting of PIN1 to vacuoles as well as for root development. Therefore, we explored whether PIN trafficking and, consequently, the cytokinin insensitivity of the root system might result from nonfunctional endocytosis in actin-related mutants. Recently, activated ROP2 signaling at the lobe tips of leaf pavement cells has been shown to inhibit clathrin-mediated endocytosis (Nagawa et al., 2012). To test whether a similar mechanism is employed in roots, we examined endocytosis in root cells of mutants with a modulated ROP2 signaling pathway. As a proxy for the rates of clathrin-mediated endocytosis, we monitored the intracellular PIN1 accumulation in response to Brefeldin A (BFA) that inhibits GNOM-ADP ribosylation factor-GTPase-guanine nucleotide exchange factor (ARF-GEF)-dependent exocytosis, but allows endocytosis (Geldner et al., 2003). In control roots, BFA leads to reversible accumulation of PIN1 in the endosomes and to rapid decrease of the membrane-localized PIN1 (Geldner et al., 2001; Dhonukshe et al., 2007). In the

constitutively active CA-ROP2, no decrease in the membrane PIN1 could be detected in the presence of BFA, indicating that PIN1 is not properly endocytosed from PMs. In contrast, DN-ROP2 did not interfere with the PIN endocytosis and the membrane PIN1 decreased significantly after 1.5 h of treatment with 50 μM BFA (Figure 4A and 4B). Next, we examined whether the modified BFA-sensitive cycling caused by the CA-ROP2 pathway modulation interfered with the cytokinin-mediated PIN1 trafficking. Indeed, in CA-ROP2, PIN1 at membranes did not decrease in response to cytokinin either in the absence or presence of BFA. In contrast, roots expressing *DN-ROP2* exhibited a BFA sensitivity comparable to that of the control (Figure 4A and 4B).

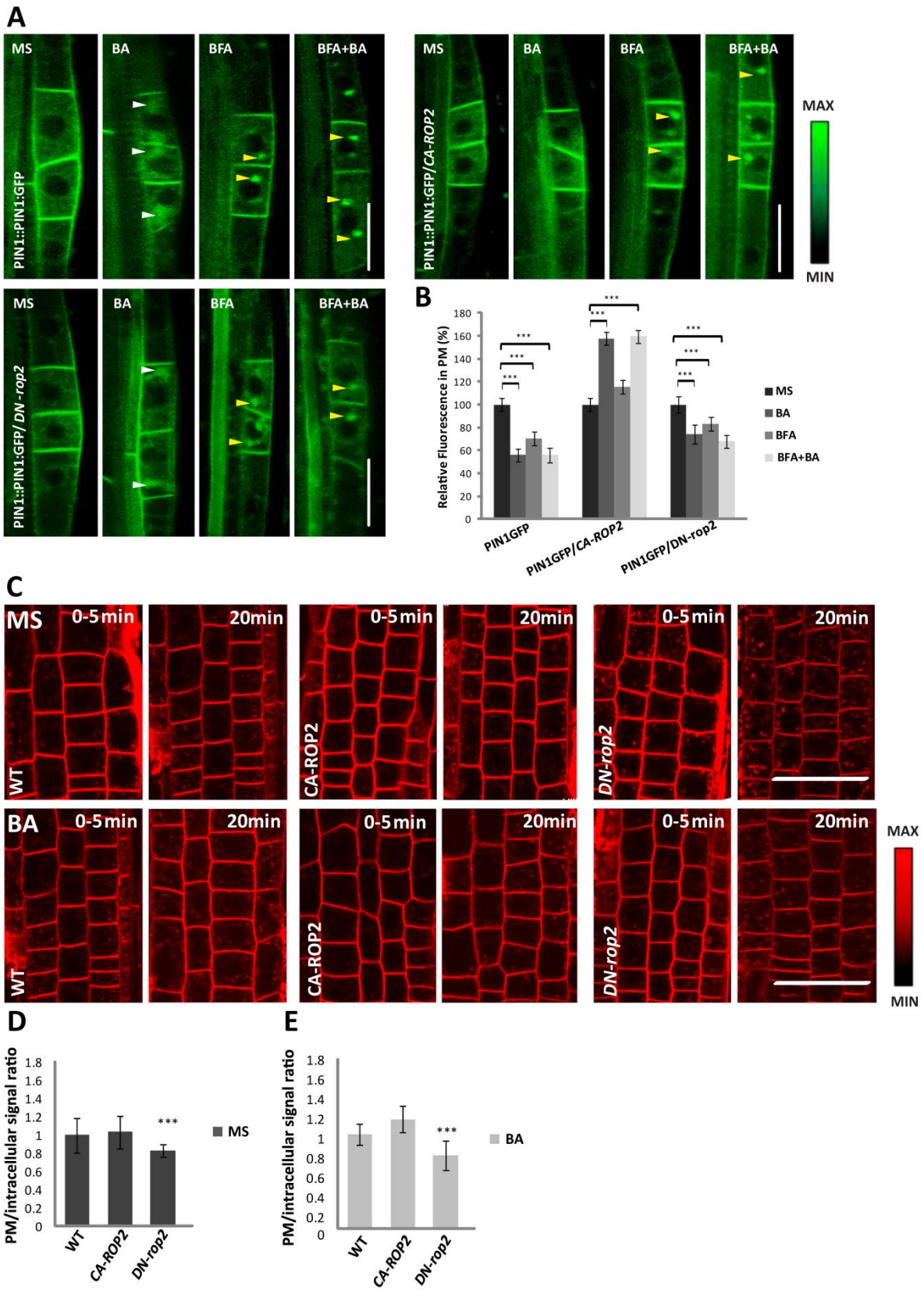

Figure 4: Endocytosis depends on the functional ROP2 signaling pathway.

(A, B) PIN1-GFP endocytosis assay using Brefeldin A (BFA) with and without BA, *CA-ROP2* shows defect in PIN1 internalization, but not in *DN-rop2* roots (A). Membrane PIN1-GFP

signal was quantified at anticlinal membranes of stage-I lateral root primordia untreated and treated with 0.1 μM BA, 50 μM BFA and simultaneously with BA and BFA for 1.5 h in the dark (***p<0.001; n=15 lateral root primordia) (B). Error bars mark ±Standard Deviation (±SD). (C-E) Enhanced FM4-64 uptake in *DN-rop2* root epidermal cells (C). Measurements of the FM4-64 uptake after 20 min. Quantifications of signal intensity of FM4-64 dye in the PM divided by the intracellular signal on MS and 0.1 μM BA, respectively (D and E). (Ratio = 1, represents the normalized absolute value to 100%. (***p<0.001; n= 5, 10 cells per root were measured). (Scale bar= 10 μm) for (A) (Scale bar= 20 μm) for (C). Error bars mark ±Standard Error of the Mean (±SE). Significant values were evaluated using Student's *t*-test. MS, Murashige and Skoog media; BA, 6-benzylaminopurine; BFA, Brefeldin A.

To explore the role of ROP2 signaling in the endocytic trafficking, we used the vesicle trafficking and endocytosis tracker *N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide) (FM4-64) (Kleine-Vehn et al., 2008b; Robert et al., 2010; Chen et al., 2012). Roots of control and transgenic lines were treated with FM4-64 for 20 min and the ratio of the endocytosed (intracellular) and the membrane FM4-64 signal was monitored. The FM4-64 uptake was significantly enhanced in root expressing the *DN-rop2* allele. However, no significant change in FM4-64 uptake could be detected in CA-ROP2 transgenes when compared to the control, except for a slight, but reproducible, inhibition of the FM4-64 uptake in cytokinin-treated roots (Figure 4C-4E). These results indicate that ROP2-dependent signaling is important for the control of the PIN1 subcellular trafficking and balance between endocytosed and exocytosed PIN1. In contrast, unaffected FM4-64 uptake hints at a specific role of the ROP2 signaling in PIN1 trafficking rather than in regulation of the endocytotic pathway in general.

To examine the contribution of the actin cytoskeleton function in the control of endocytosis, we analyzed mutants in genes encoding for the ARP2/3 complex subunits and found that the *arp2* mutant and combination of *arp2* with mutants of the *arpc4*, *arpc5* subunits exhibited a significant delay in the uptake of the FM4-64 endocytosis tracker (Figure 5A, 5B). These results suggest that the ARP2/3 complex is involved in the regulation of the endocytosis. However, more detailed analyses are needed to confirm and better understand the functions of the individual subunits of the ARP2/3 complex.

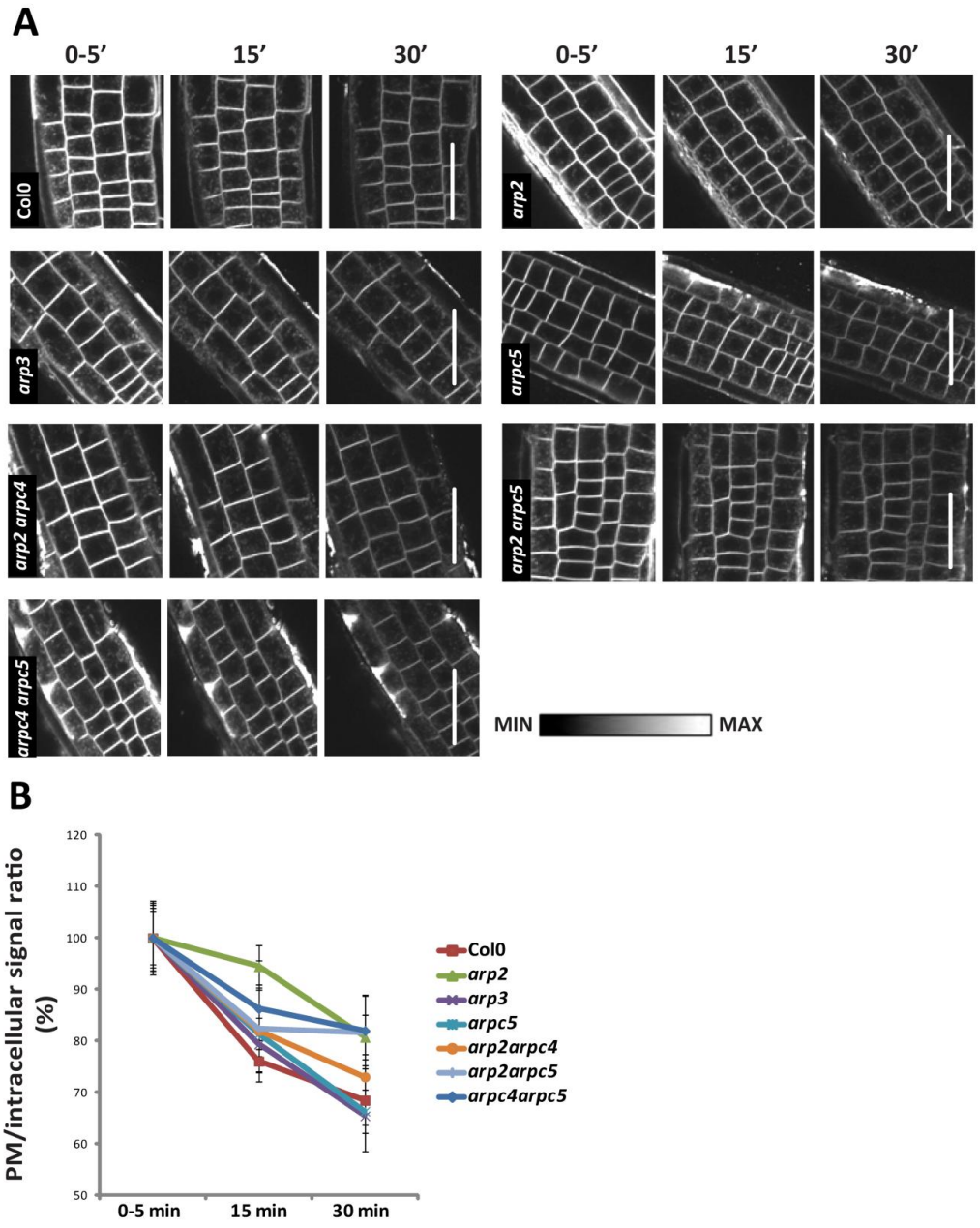

Figure 5: ARP2/3 mutant subunits exhibit inhibition of endocytosis.

(A, B) Inhibited uptake of the endocytotic tracer dye FM4-64 in mutants during the first 15 min (A). Measurements of the FM4-64 signal ratio at (0-5), 15 and 20 min. Quantifications of signal intensity of FM4-64 dye in the PM divided by the intracellular signal on MS in the wild-type (Col-0) and in the *arp* mutant subunits (B). (Ratio = 1, represents the normalized absolute value to 100%. Two independent replicas (n= 5, 10 cells per root were measured). Error bars are ± Standard Deviation (±SD). (Scale bar= 20 µm).

Discussion and Conclusions

In plants, the coordinated polarization of the auxin efflux carriers of the PIN family within plant tissues is required for the polar auxin transport and the

formation of auxin gradients that regulate a wide range of morphogenetic and growth patterns in plants. The majority of the PIN family members function on the cell surface to control rate and direction of the auxin flux (Petrášek et al., 2006; Wiśniewska et al., 2006). Conceptually, the polar localization of any protein can be considered as the sum of processes that regulate delivery, maintenance and removal of the protein from its polar domain. Mechanistically, the abundance of PIN proteins on the cell surface results from continuous cycles of endocytosis and exocytosis. To date, various mechanisms for the generation of the PIN polarity have been proposed (Geldner et al., 2001; Dhonukshe et al., 2007; Kleine-Vehn et al., 2011), but the signals for conditional endocytosis and subsequent degradation of PIN proteins remain elusive.

Noteworthy, diverse hormonal signals are known to influence the stability of the PIN proteins at the cell surface. Fluctuations in auxin concentration above or below a certain native threshold have been shown to target PIN proteins for the late endocytic pathway (Paciorek et al., 2005; Malenica et al., 2007). Pharmacologically or genetically induced cellular SA accumulation by interference with endocytosis stabilizes PIN proteins at the PM (Du et al., 2013). GA deficiency, observed in GA biosynthesis mutants, promotes degradation of auxin efflux carriers, whereas treatment with GA increases the PM abundance of PIN proteins by inhibiting their vacuolar trafficking (Willige et al., 2011; Löfke et al., 2013). In contrast to these stabilizing effects, cytokinin triggers the vacuolar trafficking of PIN proteins (Marhavý et al., 2011). Not only hormonal, but also environmental, signals, such as light, have been shown to influence the stability of PIN proteins at the PM. As demonstrated experimentally, prolonged dark treatments promote PIN relocation to the vacuolar compartments (Laxmi et al., 2008).

Previously, actin filaments have been considered to provide guidance for vesicle trafficking and polar growth in plants (Voigt et al., 2005) and to play important roles in auxin transport, PIN endocytotic recycling (Dhonukshe et al., 2008b; Geldner et al., 2001) and polarity (Baluška et al., 2003; Kleine-Vehn et al., 2008a). Although an intact actin cytoskeleton is necessary for endocytic uptake (Baluška et al., 2002, 2004) and PIN recycling (Geldner et al., 2001) and, thus, for both apical and basal PIN localization in root cells, it is the apical delivery route that is more sensitive to actin filament disruption by the actin polymerization inhibitor LatB. On the contrary, the basally localized PIN1 in the stele and PIN2 in young cortex cells are sensitive to microtubule disruption by oryzalin, whereas the apical PIN2 in the epidermis is largely insensitive to disruption of the microtubule arrays (Kleine-Vehn et al., 2008a). These data suggest that delivery and maintenance of polar cargos to apical or basal cell domains depend on different arrays of cytoskeletal components. We hypothesized that if the PIN1 trafficking mechanism to the vacuole relied on the intact actin cytoskeleton, genetic interference with actin components would modulate PIN trafficking and root system sensitivity to

cytokinin. Analysis of mutants in genes controlling the actin cytoskeleton, including subunits of the ARP2/3 and WAVE complex, revealed that the functional actin cytoskeleton plays an important role in the cytokinin-dependent root system establishment, whereas the unchanged cytokinin sensitivity of the *act2*, *act7* and *act8* mutants might either be the consequence of the functional redundancy of actin genes or of the unessential role of actin bundles in this process. Although most of the actin-related mutants did not exhibit any dramatic defects in root growth, root meristem size and lateral root initiation, the strikingly reduced sensitivity to cytokinin imply an important role for the functional actin cytoskeleton in developmental responses to the hormone.

Our data show that cytokinin-specific regulations of developmental processes, such as lateral root initiation and root meristem differentiation, depend on the actin cytoskeleton, in contrast to the overall root growth elongation that apparently was not so strongly dependent on the actin activity. These variations might be due to different molecular mechanisms underlying the cytokinin effects on root meristem differentiation and lateral root initiation in comparison to root elongation. Whereas first two are under direct cytokinin control, root elongation inhibition involves the ethylene pathway (Cary et al., 1995). Moreover, both root meristem maintenance and lateral root initiation rely primarily on the equilibrium between cell division and differentiation, but overall root growth is determined by rapid anisotropic cell growth (Szymanowska-Pułka et al., 2012). In this context, the dramatically reduced root growth in *act7* mutants, but not in *act2* and *act8*, indicate that specific components of the actin cytoskeleton might be required for rapid cell elongation.

Reduced cytokinin sensitivity of the root system in the *act*-related mutants correlates with the cytokinin inability to target PIN1 for lytic degradation and defects in endocytosis. The role of actin in the regulation of endocytosis is well documented in yeast and mammalian cells (Mooren et al., 2012) and has recently been shown in plants as well (Nagawa et al., 2012). In general, the role of actin at different stages of endocytosis, including assembly of the endocytotic coat complex, invagination and vesicle scission of endosomes has been demonstrated. In yeast and mammalian cells, the ARP2/3-WAVE complex acts at the membrane level to control nucleation of actin filaments when affected vesicles are unable of budding (Baisa et al., 2013). Considering that the function of this ARP2/3-WAVE complex is conserved in plant cells, mutations in the ARP2/3 subunits might prevent proper vesicle budding and this defect might interfere with the cytokinin-stimulated PIN1 targeting to vacuoles and the reduced cytokinin sensitivity of the root system.

Interestingly, subunits of the ARP2/3 complex seem to have specific functions; whereas the ARP2 and ARP3 components are apparently essential for cytokinin-regulated root development, loss of either the ARPC4 or ARPC2B activities

does not affect the root cytokinin sensitivity. The ARP2 subunit was detected exclusively in the membrane fraction, in contrast to ARPC4 that was found to integrate in a non-membrane fraction of the ARP2/3 complexes (Kotchoni et al., 2009). Hence, the dramatic difference between the *arp2* and *arpc4* mutants indicates that the functional membrane-associated ARP2/3 complex is crucial for cytokinin-mediated root development. This assumption is supported by the severely reduced cytokinin sensitivity of mutant in the ARP3 subunit that has been shown to be involved in phospholipid recruitment during vesicle formation in yeast cells (Martin et al., 2006). Unexpectedly, no change in cytokinin sensitivity of actin mutants could be observed, possibly due either to their functional redundancies or to the transport of endocytosis vesicles through pathways different from the actin cables reported for yeast cells (Mooren et al., 2012).

As mentioned, ROP is a plant-specific subfamily of the Rho family of small GTPases (Zheng and Yang 2000; Yang, 2002) and its activities has been linked to actin-dependent tip growth in pollen tubes and root hairs and to anisotropic growth in hypocotyls, roots and leaf epidermal cells (Kost et al., 1999; Li et al., 1999; Fu and Yang, 2001; Molendijk et al., 2001; Fu et al., 2002; Foucart et al., 2009; Fu, 2010). In leaf epidermal cells, ROP2 has been shown to modulate the actin network (Fu et al., 2002, 2005). More recently, stabilization of the cortical F-actin by overproduction of the ROP2 GTPase or its effector RIC4 has been correlated with endocytosis inhibition in pavement cells of *Arabidopsis* leaves (Nagawa et al., 2012). Accordingly, we found that constitutive activation of the ROP2 pathway inhibits the PIN1 endocytosis, linking the reduced PIN1 sensitivity to cytokinin-stimulated lytic degradation. In contrast, ROP GTPases are proposed to interfere with the Steroid Receptor RNA Activator1 (SRA1)-dependent inhibition of the SCARs, thereby activating the ARP2/3 complex (Yanagisawa et al., 2013). In view of the ROP2-positive function in the ARP2/3 complex activation, the cytokinin-insensitive phenotype observed in CA-ROP2 and mutants of the ARP2/3 subunits is counterintuitive. This apparent discrepancy raises the question on the role of ROP2 and individual ARP2/3 components in the control of the actin cytoskeleton and PIN1 subcellular trafficking, which need to be investigated. In conclusion, we demonstrate that the molecular components that regulate the actin cytoskeleton are required for the rapid modulation of the PIN1-mediated auxin transport and for shaping the root system architecture in response to cytokinin.

Materials and Methods

Plant Material

The transgenic *Arabidopsis thaliana* (L.) Heynh. lines have been described elsewhere: PIN1::PIN1-GFP (Benková et al., 2003). ROP gain- and loss-of-function *Arabidopsis* lines include constitutively active (*CA-ROP2*), dominant negative (*DN-*

rop2) and its RIC4 downstream effector. The *ric4-1* mutant is in the *Wassilewskija* (WS) background, the other lines were in the Columbia (Col-0) background (Nagawa et al., 2012) as well as the the *scar1,scar2,scar3,sacr4, brk, arp2, and arp3* (Dyachok et al., 2011), and the*, arpc2B, arpc4, arpc5, arp2arpc5, arp3arpc4, arp2arpc4,* and *arpc4arpc5* mutants (Szymanski, 2005). For the actin full knockout mutants, GABI-Kat lines (University of Bielefeld, Germany) were selected, namely *act2* (GK481G03.01)*, act7* (GK498G06.02)*, act8* (GABI_480C07.04) (Kandasamy et al., 2007).

Mutant Confirmation and Plant Crosses

Offsprings of the crosses with PIN1::PIN1:GFP were genotyped with the primers: forward 5'-CTATCCTTCGCAAGACCCTTC-3' and reverse 5'-CAACAAGGATAATGGGAACACC-3' for *DN-rop2;* for the *ric4-1* mutant, the T-DNA flanking sequence was genotyped by the forward 5'-CACCATGAGAGATAGAATGGAGAGACTTGTG-3' for RIC4 CDS-F, reverse 5'-TTATAAAGTTGGATGAAGATGAGGG-3' for RIC4 CDS-R and 5' cgacggatcgtaatttgtcg 3' for pSKI015-LB1n.

Growth Conditions

Seeds of *Arabidopsis* (accession Col-0) were plated on half-strength Murashige and Skoog (1/2MS) medium (Duchefa) with 1% sucrose and 1% agar (pH 5.7) and stratified for 2 days at 4°C. Seedlings were grown on vertically oriented plates in growth chambers under a 16-h light/8-h dark photoperiod at 18°C.

Pharmacological and Hormonal Treatments

Five- to 6-day-old seedlings were transferred onto solid MS media with or without the indicated chemicals and incubated for 1.5 to 2 h in the dark at 18°C. Drugs and hormones used were: 50 µM BFA, 20 µM LatB and 0.1 µM and 2 µM 6-benzylaminopurine (BA). For double treatments, a 30-min pretreatment with BFA and LatB was done prior to the BA application.

Analysis of Primary Root and Lateral Root Primordia Organogenesis

For phenotypic analyses of root growth, lateral root initiation, at least 15 seedlings were processed. The lateral root primordia density was measured on 7-day-old seedlings as described (Malamy and Benfey, 1997). By scoring the number of lateral roots (1st stage-LR stage) and divide them by the root length of that specific root. Root growth parameters (root length and root meristem) were analyzed with the ImageJ software package (NIH;http://rsb.info.nih.gov/ij) as described (Růžička et al., 2009).

Immunodetection

Immunofluorescence in *Arabidopsis* roots was analyzed as previously described (Sauer et al., 2006). The anti-PIN1 antibody (1:1000), anti-PIN2 antibody (1:1000) (Wiśniewska et al., 2006) were used as primary antibodies, and anti-rabbit Cy3 (1:600) was used as secondary antibody. Fluorescence was observed with a Zeiss LSM710 confocal microscope (543 nm excitation, 560 nm emission). Relative fluorescence of membrane PIN1-GFP signal intensity was quantified at anticlinal membranes of endodermal cells in untreated or treated with 0.1 μM BA for 1.5 h in the dark.

FM4-64 Staining and Confocal Microscopy Observation

Four-day-old seedlings were incubated with FM4-64 (2 μM for 5 min in liquid 1/2MS as solution on ice) as described (Kleine-Vehn et al., 2008b) and washed out three times at 4°C (on ice). As some of the lines were in WS and Col-0 backgrounds, we first established that no endocytosis differences occurred between the two *Arabidopsis* accessions (wild type represents either WS or Col-0) (data not shown). After incubation for 5, 15, 20 and 30 min, fluorescence was observed with a confocal microscope (543 nm excitation, 515-650 nm emission). The green fluorescent protein (GFP) signal of the membrane was quantified at anticlinal membranes of stage-I lateral root primordia untreated or treated with 0.1 μM BA for 1.5 h in the dark. Pictures were taken by a Zeiss 710 confocal microscope) with a 40x or 63x (water immersion) objective. Criteria for quantification of membrane signals in particular tissues and microscope settings were specified as for the enhanced GFP (EGFP) at excitation wavelength of 488 nm and 505-550 nm emission. All the experiments were repeated at least three times. The fluorescence signal and intensity of the membrane PIN-GFP signal were quantified with ImageJ (NIH; http://rsb.info.nih.gov/ij) as described (Žádníková et al., 2010). The statistical significance was evaluated with Student's *t*-test.

Quantification of FM4-64 Uptake

The mean fluorescence intensity of vesicles inside the cell, excluding the PM, was measured with the ImageJ software (http://rsb.info.nih.gov/ij). The average value of the signal intensity at the 4 membranes of the cell was measured and then divided by the intracellular signal intensity at the surface area; the value generated by this division was then normalized to 100%. Quotients of PM and intracellular fluorescence intensities were calculated for 10 cells from at least five different roots as described (Robert et al., 2010).

Supplementary Data

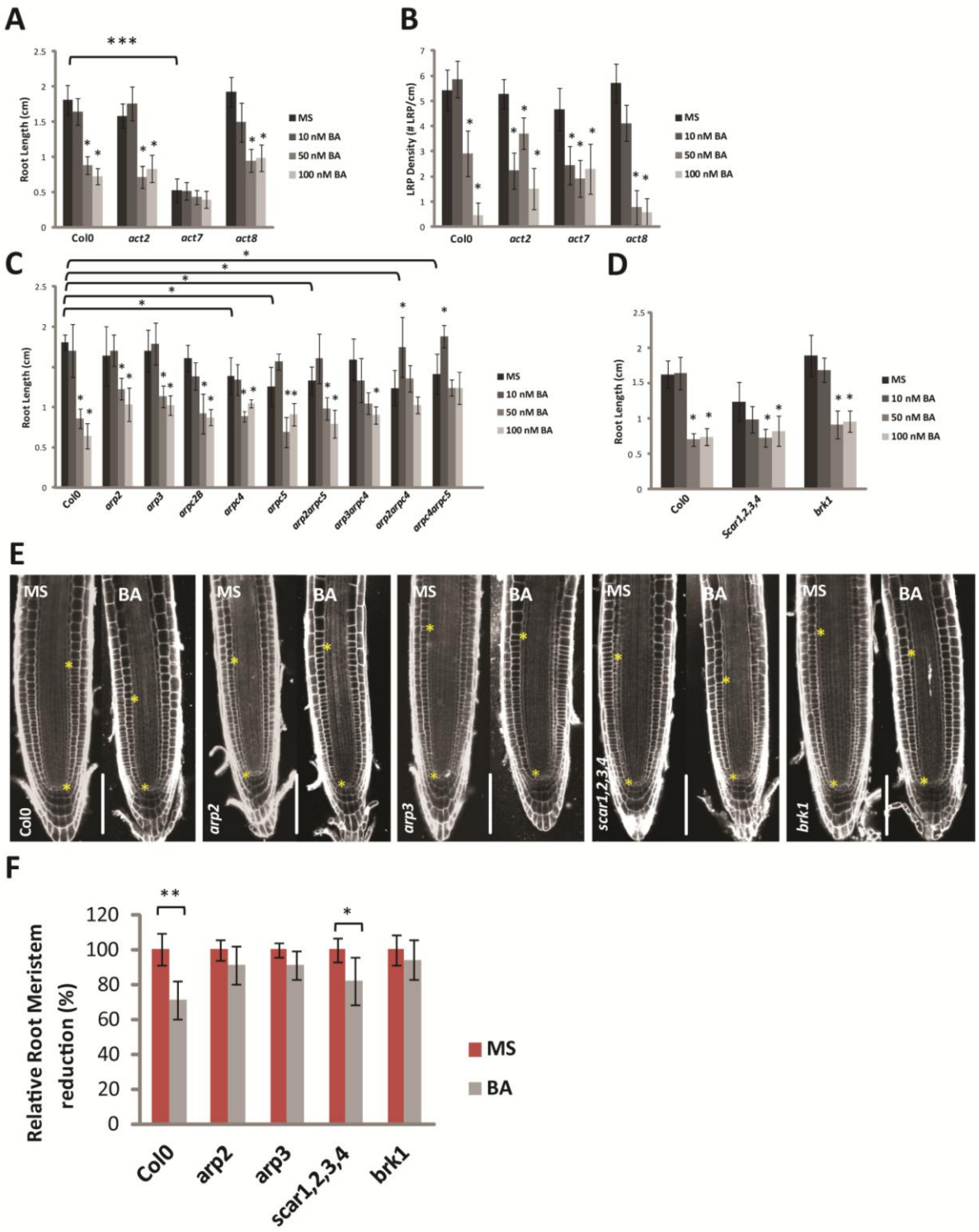

Figure S1: Cytokinin-mediated root growth requires functional actin cytoskeleton (Related to figure 1).

(A-D) Root growth and lateral root initiation sensitivity to cytokinin (BA) of ACTIN mutants (A and B). Seedlings were grown on 10, 50, and 100 nM BA-containing media for 7-days (*p<0.05; ***p<0.001, n= 15 roots). In mutants of the ARP2/3 complex subunits (C) and the *brk1* and *scar* mutants of the WAVE complex subunits (D), root growth was affected in mutants of *arpc4, arpc5, arp2arpc5, arp2arpc4,* and *arpc4arpc5*, root growth remained unaffected by the cytokinin sensitivity, except for *arp2arpc4* and *arpc4arpc5* double mutants. Seedlings were grown on 10, 50, and 100 nM BA-containing media for 7-days, (*p<0.05; n= 15 roots). (E-F) Root meristem size of *arp2, arp3, scar1scar,2,scar3,scar4* and *brk1* mutants exhibited cytokinin insensitivity when grown on MS media and media supplemented with 0.1 μM BA for 7-days, then stained with propidium iodide (*p<0.05, **p<0.001, n=15 roots). Yellow asterisks indicate the distance between the quiescent center (QC) and the cortex transition zone. Error bars mark ± Standard Deviation (±SD). (Scale bars= 50 μm). Significant values were evaluated using Student's *t*-test. MS, Murashige and Skoog media; BA, 6-benzylaminopurine.

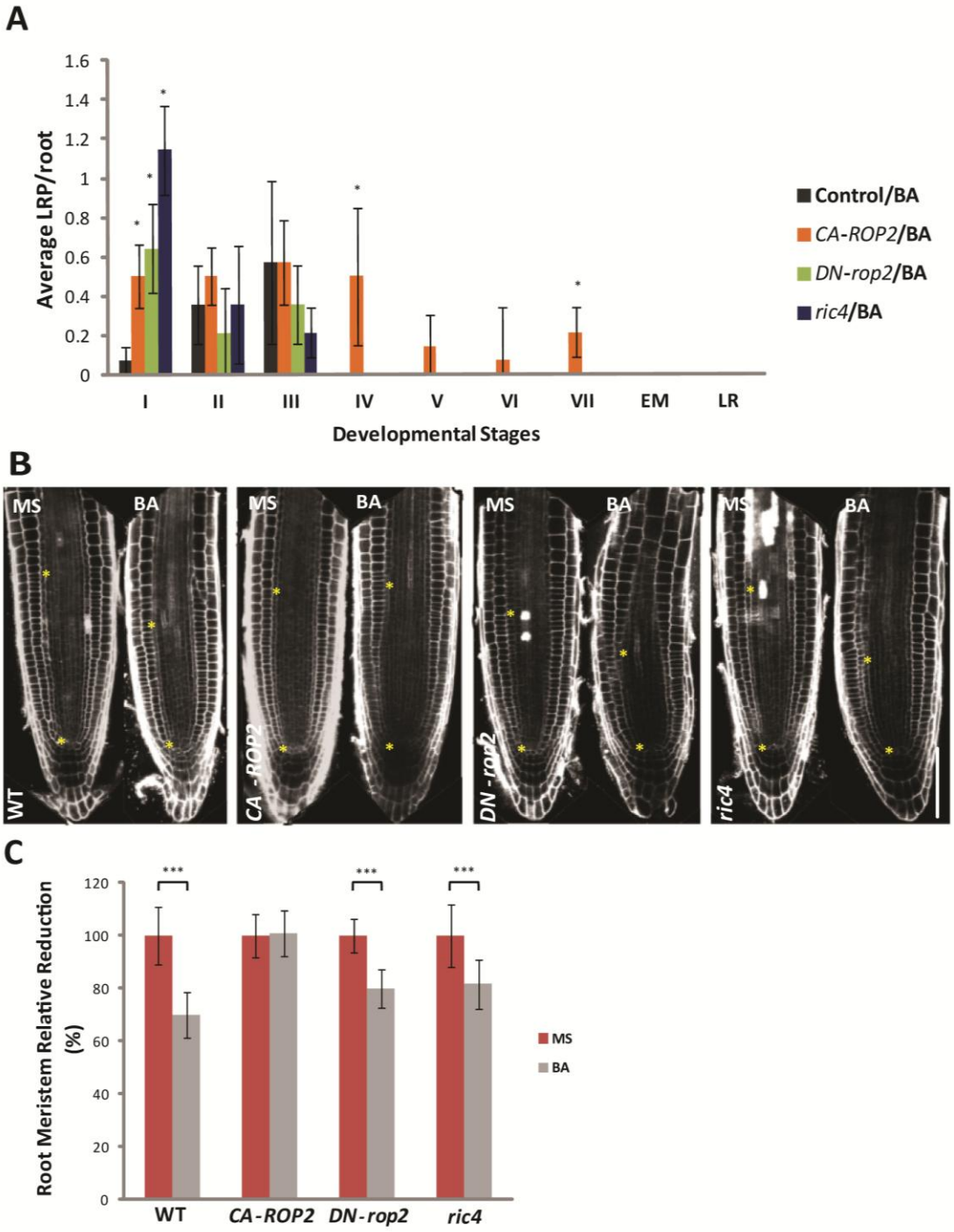

Figure S2: ROP2 and RIC4 are involved in cytokinin regulation of root development (Related to Figure 3).

(A-C) *CA-ROP2* reduces cytokinin sensitivity of lateral root initiation and development, in contrast to *DN-rop2* and *ric4-1*. Seedlings were grown on 0.1 µM BA for 7-days (A) (*p<0.05; n= 15 roots), Error bars mark ± Standard Error of the Mean (±SEM). (B-C) The root meristem size of *CA-ROP2* exhibited cytokinin-insensitivity but not in *DN-rop2*, and *ric4-1* mutants when grown on MS media and media supplemented with 0.1 µM BA for 7-days, then stained with propidium iodide (***p<0.001; n= 15 roots). Error bars are ± Standard

Deviation (±SD). Significant values were evaluated using Student's *t*-test. Yellow asterisks indicate the distance between the quiescent centre (QC) and the cortex transition zone. (Scale bars= 50 μm). MS, Murashige and Skoog media; BA, 6-benzylaminopurine**.**

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PAC7 encoding a putative GARP-G2 type transcription factor regulates cytokinin-mediated PIN1 recruitment to the vacuoles

Adapted from:

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Author contribution:

A.A and E.B conceived the study and designed the experiments, A.A carried out most the experiments. A.B carried out the analysis of the mutagenized EMS population. E.D analyzed the SHOREmap data, A.V and M.V analyzed the data by the GenomeMapper. A.A and E.B wrote the manuscript.

PAC7 Encoding a Putative GARP-G2 Type Transcription Factor Regulates Cytokinin-Mediated PIN1 Recruitment to the Vacuoles

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Plant hormones are important endogenous signaling molecules that control plant growth and development. Typically, more than one hormone is implicated in certain developmental processes and the coordination of their overlapping activities is crucial for a correct developmental output. Auxin and cytokinin are central hormones involved in the regulation of various aspects of plant development, including processes that determine root architecture, such as root pole establishment during early embryogenesis, root meristem maintenance, and lateral root organogenesis. Hence, to control root development, both pathways put special demands on the mechanisms that equilibrate their activities and mediate their interactions. To identify new molecular components required for balancing the auxin/cytokinin activities, we used a forward genetics screen to look for mutants that produce lateral roots when auxin is applied simultaneously with cytokinin at inhibiting concentrations. Based on the screen, 22 novel mutant alleles, designated primordia on auxin and cytokinin (*pac*) had been recovered (Bielach et al., 2012). Among them, the *pac7* mutant showed a severely reduced lateral root initiation sensitivity to inhibitory cytokinin effects. Detailed examination, of *PAC7* revealed that it plays a role in the development of the cytokinin-regulated root system and that it presumably functions in the regulation of the PIN-FORMED1 auxin transporter trafficking. Combination of positional cloning and SHOREmap strategies indicated that *PAC7* encodes the *AT3G19070* gene that contains a plant-specific MYB-related DNA-binding motif, predicted to bind to cytokinin-regulated genes.

Introduction

Lateral root formation is governed by a complex network of hormonal interactions. The plant hormone auxin dominates this process and has been shown to positively regulate lateral root initiation and development. In *Arabidopsis thaliana*, lateral roots (LRs) originate in the pericycle cell layers of the xylem poles (Dubrovsky et al., 2000; Beeckman et al., 2001). After certain pericycle cells have acquired founder cell properties, LR primordia (LRP) are initiated by several anticlinal divisions and they develop as a result of coordinated cell division and differentiation. Later on, the LRP emerge from the parent root, mainly by cell elongation, and then the LR meristem is activated, of which the structure and function are similar to those of the primary root meristem (Malamy and Benfey, 1997). The positive role of auxin at all stages of the LR organogenesis, including initiation and development, is well established. Accumulation of auxin and activation of auxin responses in individual pericycle cells stimulate founder cell specification and LR initiation (LRI) (Laskowski et al., 1995; Dubrovsky et al., 2008). This positive impact of auxin on LRI was evidenced by modulation of auxin levels (Laskowski et al., 1995; Laskowski et al., 2008) as well as of auxin responses (Dharmasiri et al., 2005; Fukaki et al., 2005; Vanneste et al., 2005). In the later developmental phases of the LRP formation, auxin distribution gradients with maxima at the primordia tips determine the proper primordia organogenesis (Benková et al., 2003). This auxin gradient is generated by the concerted action of the AUXIN RESISTANT (AUX)/LIKE AUX (LAX) auxin influx carriers (Bennett et al., 1996), the PIN-FORMED (PIN) auxin efflux carriers (Gälweiler et al., 1998; Luschnig et al., 1998; Friml et al., 2002a, 2002b, 2003), and members of the multidrug-resistant/P-glycoprotein subfamily of ATP-binding cassette proteins (Blakeslee et al., 2007). Besides auxin, several other plant hormones have been found to regulate the LR organogenesis (Sponsel et al., 1997; Bao et al., 2004; De Smet et al., 2006; Mouchel et al., 2006), among which cytokinin exhibits one of the strongest inhibitory effects. Any increase in cytokinin activity, either by exogenous manipulation of cytokinin levels (Li et al., 2006; Laplaze et al., 2007) or endogenous modulation of the activity of genes involved in cytokinin metabolism, results in changed LRI frequencies and developmental defects (Werner et al., 2003; Laplaze et al., 2007). The opposite contributions of both the auxin and cytokinin pathways to regulate the LR organogenesis put special demands on the mechanisms that balance their activities and mediate their interaction. The auxin control over the transcription of the *ARABIDOPSIS RESPONSE REGULATOR7* (*ARR7*) and *ARR15* genes and the cytokinin signaling repressors appears to be critical for proper early embryo root pole establishment (Müller and Sheen, 2008). Similarly, auxin and cytokinin activities in the shoot apical meristem are counterbalanced through transcriptional regulation of *ARR7* and *ARR15* by the *AUXIN RESPONSE FACTOR5/MONOPTEROS* (Zhao et al., 2010). Besides crosstalk between auxin and cytokinin signaling pathways, cytokinin also interferes with the auxin distribution by modulating the polar auxin transport activity. This mode of

interaction is particularly important for root apical meristem maintenance and LR organogenesis (Laplaze et al., 2007; Dello Ioio et al., 2008; Pernisová et al., 2009; Růžička et al., 2009; Zhang et al., 2011). In the root apical meristem, the auxin-cytokinin circuit is mediated through the ARABIDOPSIS HISTIDINE KINASE3 (AHK3) receptor and the downstream transcription factor ARR1 that adjusts the expression of the *INDOLE ACETIC ACID3/SHORT HYPOCOTYL2 (SHY2)* auxin signaling repressor and attenuates the expression of several *PIN* genes as a consequence (Dello Ioio et al., 2008). Besides transcriptional regulation, cytokinin also controls the PIN protein levels at the posttranscriptional stage (Marhavý et al., 2011; Yoshida et al., 2011; Zhang et al., 2011). This regulatory mode is important in view of the control of leaf initiation and positioning and of the LR organogenesis (Marhavý et al., 2011; Yoshida et al., 2011). During the early stages of LRP formation, PIN1 is rapidly degraded in lytic vacuoles after CK treatment and prevents the formation of an auxin gradient, which is required for normal LRP patterning (Laplaze et al., 2007, Marhavý et al., 2011). The cytokinin signal is transduced through the CRE1/AHK4 receptor that modulates the endocytic trafficking of PIN1 and redirects this membrane protein for lytic degradation into the vacuoles. Stimulation of the lytic PIN1 degradation is not a default effect of the overall downregulation of proteins from plasma membranes, but a specific mechanism that rapidly modulates the auxin distribution in cytokinin-mediated developmental processes (Marhavý et al., 2011).

In the field of experimental plant biology, new genes are discovered continuously, thus expanding our understanding of processes underlying plant development. To identify new molecular components that are required for balancing the auxin/cytokinin activities, we decided to use the LR organogenesis as a suitable model system. By means of a forward genetic screen, we searched for mutants that produce LRs when auxin is applied simultaneously with cytokinin at inhibiting concentrations (Bielach et al., 2012). The screen yielded 22 mutants, designated *p*rimordia on *a*uxin and *c*ytokinin (*pac*) that were resistant to auxin and cytokinin (Bielach et al., 2012). These mutant lines were divided into four subgroups (A, B, C, and D) based on their LRI density and sensitivity to hormonal treatment. The LRI density remained unchanged in the mutants of subgroup D, but their cytokinin sensitivity was significantly reduced. Among them, *pac7* with the most pronounced change in cytokinin sensitivity was selected for further analysis (Bielach et al., 2012). By using map-based cloning techniques and the SHOREmap platform analysis, we identified the *PAC7* gene and verified the mutation with a complementation test and its structure and localization by *in silico* investigation.

Results

PAC7 mediates hormonal control of LR organogenesis

The antagonistic auxin-cytokinin interaction is strongly visible in the regulation of the LR organogenesis. Whereas auxin promotes both LRI and LR development, cytokinin exhibits inhibitory effects (Laskowski et al., 1995; Himanen et al., 2004; Li et al., 2006; Laplaze et al., 2007). A detailed analysis of *pac7* revealed that the LRI sensitivity to either auxin or cytokinin changed when applied separately (Figure 1A and 1B), indicating that PAC7 might control LR organogenesis through balancing the input from individual hormonal pathways. In contrast, the sensitivity to cytokinin of the *pac7* root growth remained unchanged, hinting at the specific role of PAC7 in the hormonal regulation of the LR organogenesis, but not of the primary root growth (Figure 1C, and 1D).

PAC7 mediates hormonal control of light-regulated seedlings growth

Next, we examined whether the *PAC7* gene plays a role exclusively in the auxin-cytokinin-controlled LR organogenesis or is involved also in other developmental processes that require the activity of both hormonal pathways. Such a process is seedling development in response to light. In the dark, seedlings undergo skotomorphogenesis and develop long hypocotyls, an apical hook, and closed cotyledons. Under light, they adopt photomorphogenesis, resulting in short hypocotyls, open cotyledons, and chlorophyll accumulation (Lau and Deng, 2010). Exogenous cytokinin promotes light-grown phenotypes in dark-grown seedlings (Chory et al., 1994), but auxin enhances hypocotyl elongation, whereas suppressed auxin responses seem critical in the regulation of photomorphogenic responses (Lee et al., 2007; Lau and Deng, 2010). When wild-type (WT) and *pac7* mutant seedlings were grown in the dark, the *pac7* mutant seedlings exhibited strong defects in skotomorphogenesis, with reduced hypocotyl length (Figure 1E), premature opening of the apical hook, and cotyledon development. Moreover, the *pac7* mutant showed a cytokinin-resistant phenotype for the hypocotyl length (Figure 1E and 1F). Taken together, these data indicate that, besides the cytokinin-regulated LRI, *PAC7* is involved in light-dependent growth regulation (Bielach et al., 2012).

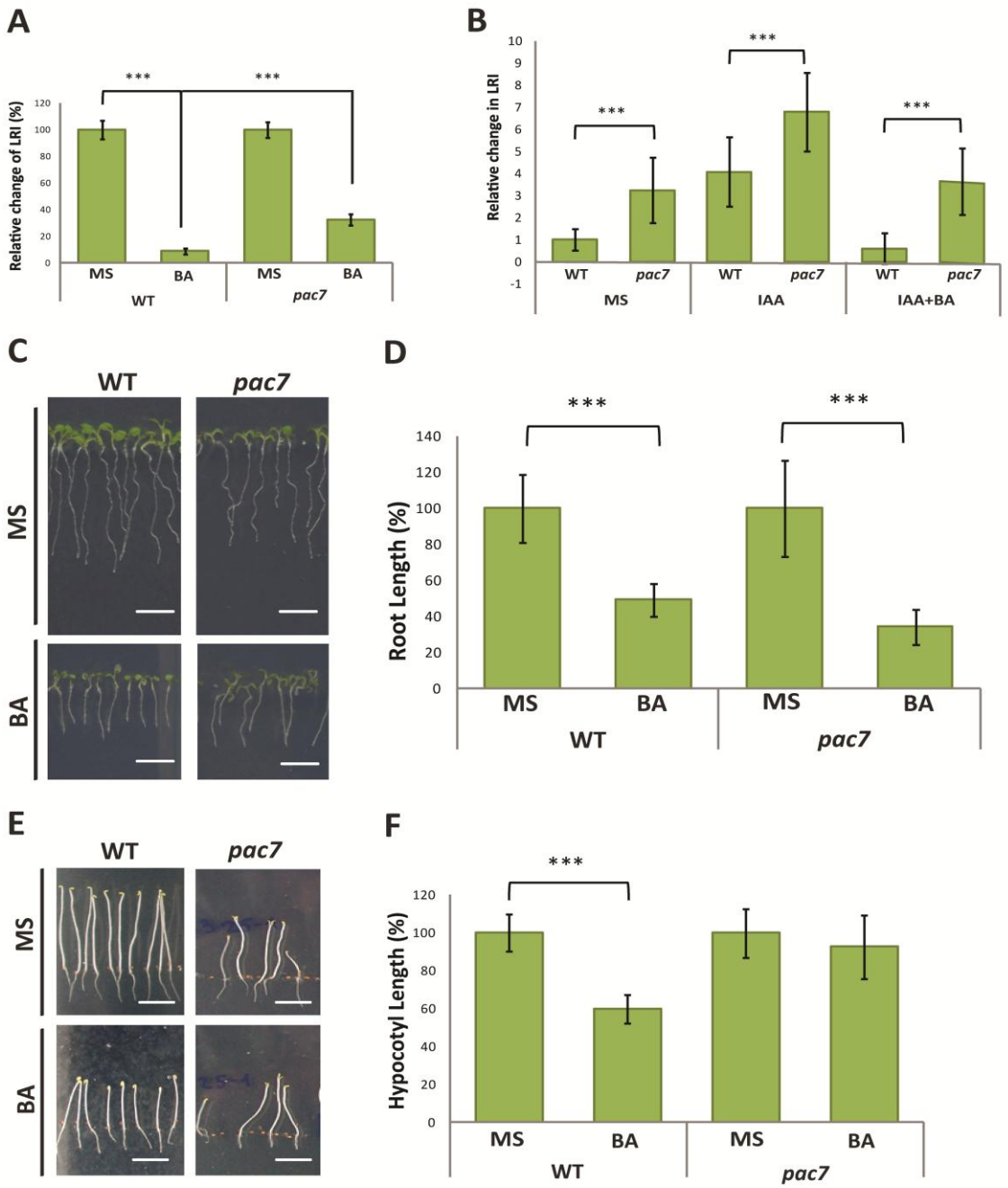

Figure 1: The phenotypical analysis of the *pac7* mutant.

(A, B) Quantification of the relative change in LRI of 7-day-old seedlings. Quantification of the LRI phenotype of the *pac7* mutant with and without BA in three biological replicas and at least 15 seedlings evaluated for each replica (A). Quantification of the relative change in LRI in the presence of 1 µM IAA, 0.1 µM BA, and double auxin-cytokinin treatment in WT and *pac7* mutant (B). Light-grown, 7-day-old WT Col-0 and *pac7* mutant seedlings germinated on MS media and media supplemented with 0.1 µM 6-benzylaminopurine (BA) (C). Quantification of the *pac7* mutant root growth with and without BA in three biological replicas and at least 20 seedlings evaluated for each replica (D). (E, F) Dark-grown, 3-day-old WT Col-0 and *pac7* mutant seedlings germinated on MS media and media supplemented with 0.1 µM 6-benzylaminopurine (BA). Quantification of the *pac7* mutant hypocotyl growth with and without BA in three biological replicas and at least 15 seedlings evaluated for each

replica (F) (***P<0.001). Error bars mark ± Standard Deviation (±SD). Significant values were evaluated using Student's *t*-test. (Scale bar= 0.5cm).

PAC7 is required for the cytokinin-stimulated degradation of PIN1 auxin efflux carriers

One of the recently revealed mode of action of the auxin and cytokinin pathways is the cytokinin-mediated modulation of the polar auxin transport (Dello Ioio et al., 2008; Pernisova et al., 2009; Růžička et al., 2009; Marhavý et al., 2011; Yoshida et al., 2011; Zhang et al., 2011). By modifying the expression of the PIN auxin efflux carriers, cytokinin was shown to control the cell-to-cell auxin transport and, thus, the auxin distribution required for the regulation of LR organogenesis and root meristem activity (Laplaze et al., 2007; Dello Ioio et al., 2008; Růžička et al., 2009; Zhang et al., 2011). Cytokinin was found to interfere with the PIN1 subcellular trafficking and to redirect it to vacuoles for lytic degradation. Genetic interference with cytokinin-stimulated PIN1 lytic degradation resulted in a strongly reduced LRI cytokinin sensitivity (Marhavý et al., 2011). To investigate whether *PAC7* might be involved in this regulatory pathway, we examined the cytokinin-mediated *PIN1* lytic degradation in the *pac7* mutant background. Expression of the *PIN1-GFP* in the primary root meristem provasculature (Figure 2A and 2B) and stage-I LRP (Figure 2C and 2D) was monitored after 1.5 h of 0.1 μM of cytokinin treatment in control and *pac7*. As previously described (Marhavý et al., 2011), cytokinin dramatically reduced the abundance of the membrane-located *PIN1-GFP* and, simultaneously, enhanced the accumulation of the *PIN1-GFP* signal in vacuoles of control seedlings. However, in the *pac7* mutant, the *PIN1-GFP* signal remained at the membranes in the presence of cytokinin and did not accumulate in the vacuoles (Figure 2C- 2E). Altogether, these results imply that PAC7 plays a role in the pathway that mediates the cytokinin-dependent PIN1 lytic degradation.

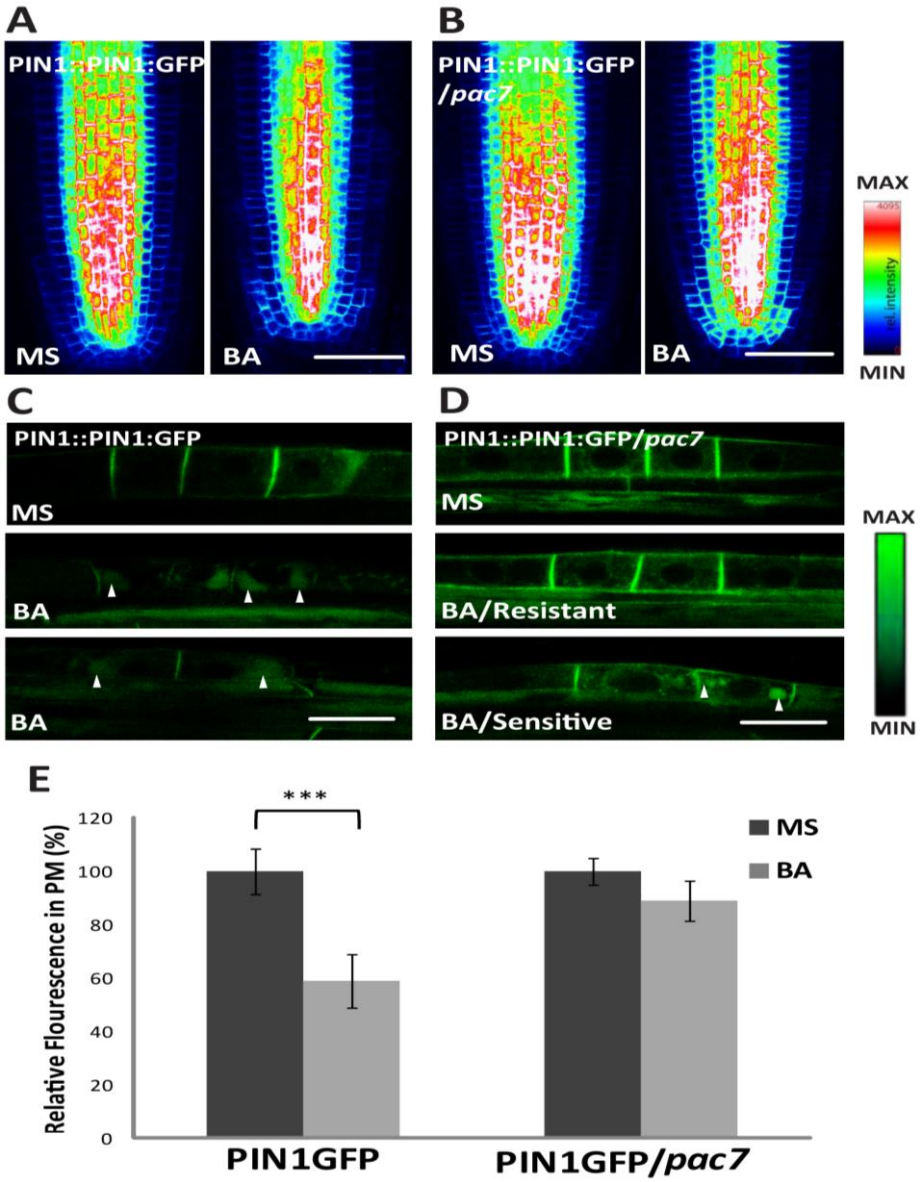

Figure 2: Cytokinin-mediated repression of the *PIN1* expression reduced in the *pac*7 mutant.

(A-E) *PIN1::PIN1:GFP* expression in the root meristem (A and B) and stage-I LRP (C-D) of the WT and *pac7* mutant in response to cytokinin. The *pac7* mutant exhibits cytokinin insensitivity in *PIN1-GFP* compared to the WT in the primary root vasculature (A and B) after treatments with 0.1 µM BA for 1.5 h in the dark. (C-E) PIN1 stability at the plasma membrane in the *pac7* background. Cytokinin induces PIN1 lytic degradation in WT (C) but not in *pac7* (D). (D) F2 generation of *pac7* crossed with Landsberg *erecta* (*Ler*) shows a ratio of 3:1 (Resistant:Sensitive) phenotype of PIN1 lytic degradation upon 0.1 µM 6-benzylaminopurine (BA) for 1.5 hr in the dark, resistant seedlings were chosen for mapping analysis. Relative fluorescence of membrane PIN1-GFP signal intensity was quantified at anticlinal membranes of stage-I lateral root primordia untreated or treated with 0.1 µM BA for 1.5 h in the dark. White arrows indicate the PIN1 signal in the vacuoles. Error bars mark ± Standard Deviation (±SD), (***P<0.001, n=15 LRP (E)). Significant values were evaluated using Student's *t*-test. [Scale bar = 100 µm (A, B), 10 µm (C, D)].

Map-based cloning for the identification of the pac7 mutation

To characterize the *pac7* mutation, we analyzed it by means of a map-based cloning. The F2 progeny was screened that was derived from the cross between the *pac7* line and the WT accession Landsberg *erecta* (L*er*). Considering the role of PAC7 in the control of the PIN1 lytic degradation in response to cytokinin, we used a fluorescence-based approach to select the mapping population from the F2 generation and selected only individuals in which the PIN1-GFP signal was insensitive to cytokinin-induced vacuolar targeting (Figure 2C and 2D). The progeny of this cross segregated with a 3:1 ratio in the F2 generation, inferring that one single mutation is responsible for the mutant phenotype.

Positional (polymerase chain reaction [PCR]-based) cloning was done on the mapping population consisting of 70-80 individuals selected from the F2 seedlings. Rough mapping revealed that the mutation was positioned on chromosome 3 between the bacterial artificial chromosomes (BACs) T19F11 (3.678 Mb) and MLD14 (6.748 Mb) (Figure 3), with a 13.48% recombination frequency. For fine-mapping of the mutation, 70 additional F2 seedlings were used in the 2.1 Mb interval between BACs MIE1 (4.8 Mb) and MLD14 (6.748 Mb) with a 7.38% recombination frequency (Figure 3). Although a total of 601 genes were present in this region, we decided to apply a SHOREmap approach because of the difficulties to narrow down the interval region.

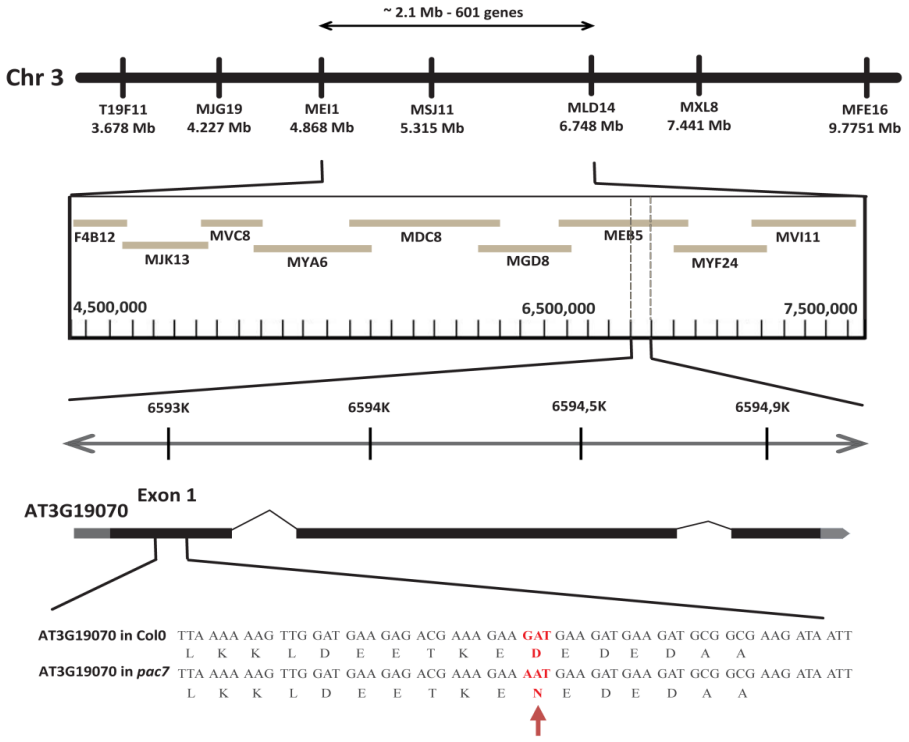

Figure 3: *pac7* correlation with the AT3G19070 locus mutation.

Physical map of the chromosomal region in which the mutation is located that causes the cytokinin resistance in *pac7*. The illustration shows the analyzed polymorphic markers, the recombination frequency, and the BAC map of the 2.1-Mb candidate region, the genomic structure of AT3G19070 and the location of the mutation (red arrow).

SHOREmap analysis for the identification of the pac7 mutation

As first step to analyze the data, we used the SHORE pipeline that is a mapping analysis application for short DNA/RNA reads produced by the Illumina Genome Analyzer platform. In principle, reads are aligned to the reference sequence (Columbia-0 [Col-0] accession). Based on the alignment, base counts per position and single-nucleotide polymorphisms (SNPs) are determined as well. The candidate zone is narrowed down depending on the relative allele frequencies of the two parents (Col-0 and L*er*) by SHOREmap that is an analysis pipeline for one single-step mapping and mutant identification (Figure S1) (Schneeberger et al., 2009). For the SHOREmap analysis, the mapping population was selected based on the LRI phenotype, i.e. sensitivity to auxin and cytokinin (Bielach et al., 2012) (see Materials and Methods). A pool of genomic DNA was extracted from all F2 plants that exihibited the mutant phenotype (i.e. homozygous for the mutation). After a genomic DNA library had been constructed, 23,785,650 reads (fragments) with 50 bases in length were sequenced, resulting in ~11 Mbp. Candidate mutations in the predicted interval were ranked based on the distance to the allele distribution peak. Command line programs and parameters used in this study are listed in Table S1. Results of SHOREmap revealed a strong peak on chromosome 3 (Figure 4), overlapping with the interval identified by the classical map-based strategy.

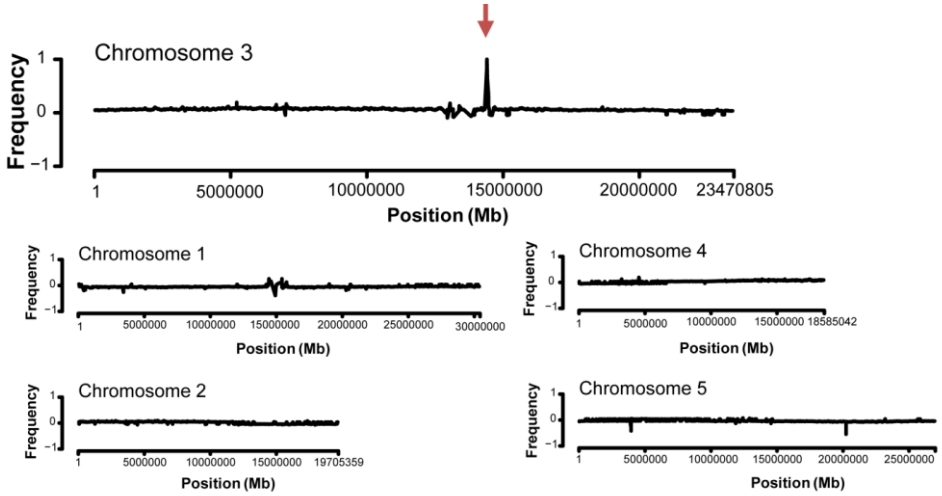

Figure 4: Relative frequency of the reference allele (winstep:10000, winsize:100000).

Red arrow indicates the mutation site in chromosome 3.

The 14,000,000 to 15,000,000 interval on chromosome 3 was used as input for ANNOTATE in SHOREmap (Figure 4) and the output of the top eight ranked mutations is listed in table S2. Additionally, the GenomeMapper was used to check the 600 genes that had resulted from the classical mapping. Surprisingly, a SNP change in the codon sequence was found in four genes (AT3G17880, AT3G18670, AT3G18970, and AT3G19070) (Figure 5), due to the existence of two additional small peaks in this interval (Figure 4), by which AT3G17880 and AT3G18670 underlie the other peaks and are not mentioned in table S2, but without reaching the mutation frequency threshold. Next, we examined which of these four genes is responsible for the pac7 phenotype by PCR experiments and sequence analysis. The flanking region of each SNP in the four genes resulting from the GenomeMapper was sequenced (data not shown). Sequencing and alignment of these genes with the reference allele (Col-0) revealed that only the AT3G19070 gene has a single G-to-A substitution in the first exon, resulting in a change of the amino acid sequence from Asp38 to Asn38. These analyses confirm the results obtained from the SHOREmap output (Table S2). In the generated SNP file, a single nucleotide in the coding region of AT3G19070 was substituted at first exon (Table S2). The first detected mutation is a guanine to adenine transition in the codon position 112 in the gene, with a change of an aspartic acid D38 to an asparagine N38 (approximately 38 kb from the peak) (Table S2).

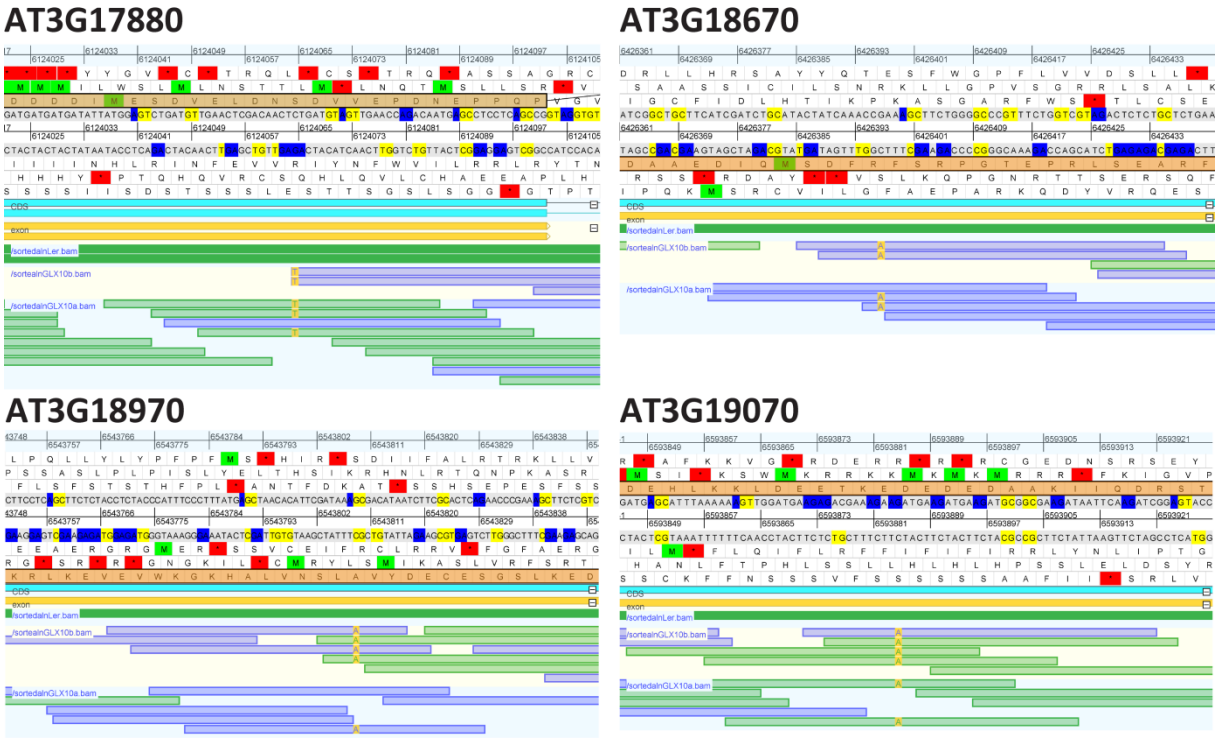

Figure 5: Alignment of DNA fragments generated from the SHOREmap analysis with the GenomeMapper tool.

Nucleotides of the DNA fragments highlighted in yellow indicate the SNP in each candidate gene.

Genetic complementation of the pac7 mutant by allelic tests

To confirm that the mutation in AT3G19070 initiates the observed *pac7* phenotype, we analyzed the AT3G19070 loss-of-function mutant. The mutant line with a T-DNA insertion in the second exon of AT3G19070 (*salk_082481*) (N582481) was obtained from the NASC collection (Figure S3). Reverse-transcription (qRT)-PCR analysis confirmed that the T-DNA disrupts the gene expression and that *salk_082481* is a full knockout allele (Figure 6F and 6G). The *salk_082481* line was crossed with *pac7* and a complementation analysis in the F1 generation was carried on to confirm the *pac7*/*at3g19070* allelism.

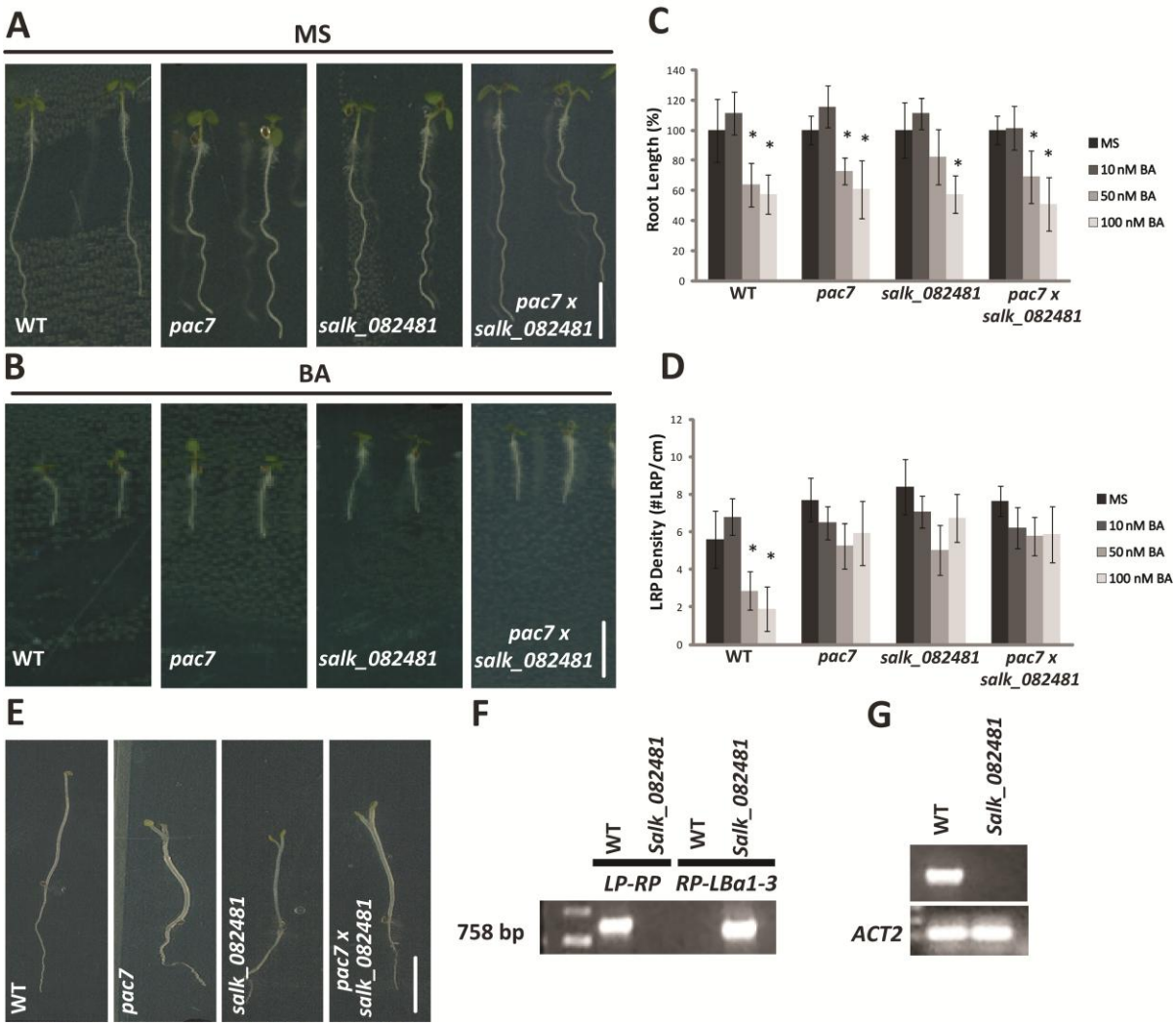

Figure 6: Genetic complementation of *pac7* by allelic test.

(A-C) Root length phenotype of 7-day-old seedling grown on MS (A, C) and with 0.1 µM BA (B) for control (Col-0), *pac7*, *Salk_082481*, and offspring of *pac7*×*Salk_082481* (F1). (D) Cytokinin-resistant phenotype for lateral root density (LRD) in *pac7*, *Salk_082481*, and

pac7×*Salk_082481* (F1) compared to the WT (*P<0.05, n=15). Error bars mark ± Standard Deviation (±SD). Three-day-old seedlings grown in the dark show earlier opening of the apical hook in the mutants background but not in WT on MS. Homozygosity test for *salk_082481* using combinations of WT (LP-RP) and mutant (RP-LBa1-3) primers. Product size is 758 bp (F). RT-PCR from RNA extracts of the of WT and *salk_082481* mutant seedlings, confirming a knockout allele of *salk_082481* (G). Significant values were evaluated using Student's *t*-test. [Scale bars= 1 cm (A and B), Scale bars= 0.5 cm (E)].

The phenotype in the F1 seedlings of the *pac7/salk_082481* cross was thoroughly analyzed with special focus on the root cytokinin sensitivity and the dark growth. Plants of the F1 generation were compared with both parental lines and relevant control. No root growth phenotypes were detected in the mutants compared to the WT, when grown on Murashige and Skoog (MS) medium and three different cytokinin concentrations (10, 50, and 100 nM). Most importantly, similarly to *salk_082481* and *pac7,* the LRI was significantly less sensitive to the cytokinin inhibitory effects in *pac7*×*salk_082481* than in the control (Figure 6D). Furthermore, when grown in the dark, *salk_082481* and the F1 offspring of the *pac7*×*salk_082481* mutant exhibited premature apical hook opening and cotyledon development, as noticed previously for the *pac7* mutant (Figure 6E, Figure 1B). Taken together, these analyses corroborate the conclusion that the mutation in AT3G19070 underlies the observed *pac7* mutant phenotype.

The PAC7 *gene encodes a putative GARP-G2-type transcription factor*

The *PAC7* gene was mapped to a genomic interval of ~38 kb away from the peak annotated in the SHOREmap analysis. The *AT3G19070* gene is relatively small, consists of three exons and two introns (Figure S3), and encodes a protein of 346 amino acids in length with a molecular mass of 37.7 kDa. *In silico* analysis of the protein domains predicted by the Plant membrane protein database (ARAMEMNON 8.0) revealed that this protein has a highly conserved domain, designated the GARP domain with the plant MYB-related DNA-binding motif located at the C terminus and is a putative GARP-G2-type transcription factor (Figure 7A and 7B). The GARP domain had previously been shown to optimally bind to the core sequence 5'-(A/G)GAT(T/C)-3' *in vitro* (Sakai et al., 2001; Hosoda et al., 2002; Imamura et al., 2003). Such short sequence motifs are enriched in primary cytokinin target genes and are, therefore, likely important *in vivo* (Rashotte et al., 2003). Additionally, all B-type ARRs shared a conserved DNA-binding domain of approximately 60 amino acids, designated the GARP/SCOP domain (Figure 7A), such as ARR10 that belongs to the GARP family of the plant MYB-related DNA-binding motif. Moreover, predictions for the protein localization by the Cell eFP browser (Heazlewood et al., 2007), hint at the localization of this protein in the nucleus, cell walls, and mitochondria (Figure 7C). Taken together, these data suggest that this protein is involved in cell wall functionality and in transcriptional regulation of cytokinin-related genes.

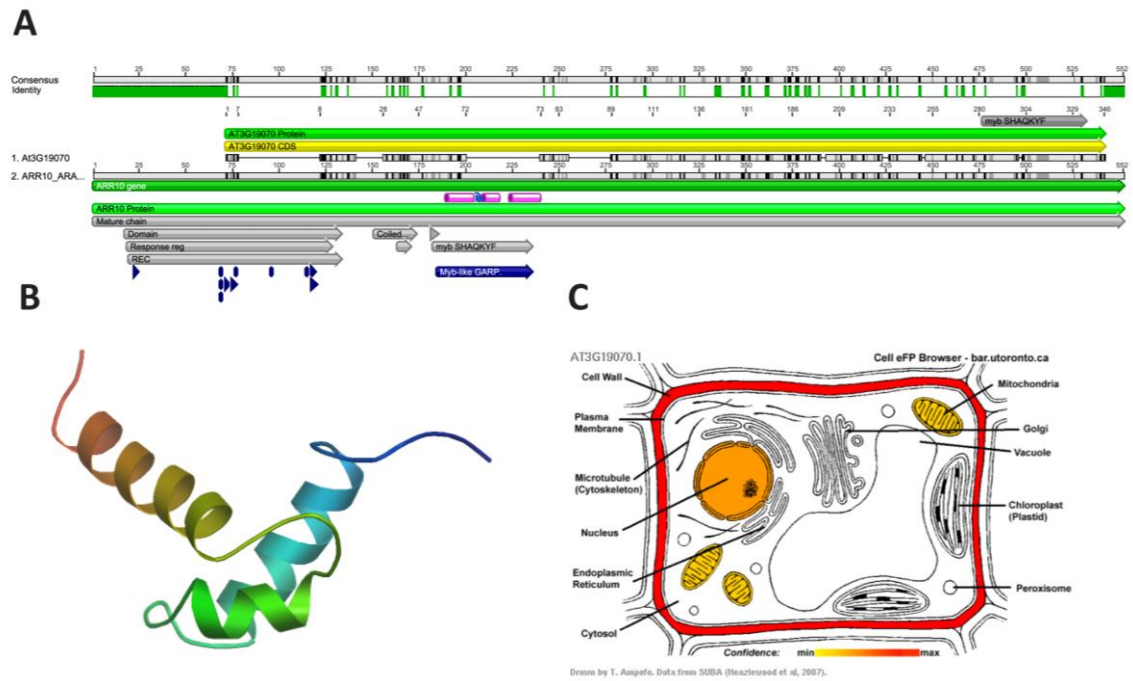

Figure 7: Predicted protein structure and localization of AT3G19070.

(A, B) MYB-like domain motif structure in AT3G19070 and ARR10**.** Alignment of the protein domains of AT3G19070 and B-type ARR10, showing the homology in the MYB-like GARP motif in both proteins (A). Three-dimensional structure of the ARR10 MYB-like DNA-binding motif which is common with AT3G19070 (B). Predicted AT3G19070 protein localization at the cellular level with the highest confidence and dynamicity (C). Red and orange signals mark the highest and intermediate confidence at the cell wall and in the nucleus and mitochrondria, respectively. Data were generated by Geneious for (A) (Drummond et al., 2011) (http://www.geneious.com/), PDB accession, 1irz (Hosoda et al., 2002) (B) and the Cell eFP browser for (C) (Heazlewood et al., 2007).

Discussion and Conclusions

Arabidopsis thaliana is a simple plant model system that has been exploited as a tool for forward genetic screens. In the last decade, several fluorescently tagged reporters have been used for diverse screenings with the aim to map intracellular trafficking, compartments integrity, and protein function and enhance our knowledge in plant cell biology (Zwiewka and Friml, 2012). In forward, reverse, and chemical genetic studies, one is often interested in a specific phenotype based on changes in the subcellular morphology, signal intensity, or location. Current standard approaches to these screens include manual microscopy, which is slow (Jorgensen and Mango, 2002), whereas high-throughput microscopy systems (Salomon et al., 2010) are insufficiently automated and/or have inadequate resolutions (Furlong et al., 2001; Crane et al., 2009). Recent developments in high-throughput technologies in life sciences provide quantitative profiling data for

numerous biomolecules, such as gene transcripts, proteins, and metabolites from single cells, tissues, or whole organs. However, these technologies do not provide spatial information about these biomolecules. In contrast, confocal laser microscopy and the use of fluorescent tags can reveal *in vivo* dynamics of molecules at cellular and subcellular resolutions (Held et al., 2008; Salomon et al., 2010). Nevertheless, forward genetic screens proved to be very powerful tools to dissect the molecular components and mechanisms of the different hormonal signaling pathways, including those of auxin and cytokinin (Hobbie and Estelle, 1994; Kakimoto, 1996; Leyser, 1997; Inoue et al., 2001). To assess the hormonal crosstalk and to identify the molecular components that mediate the pathway interactions, genetic screens have to be designed accurately by taking into account the activities of both hormonal pathways in the regulation of common developmental processes. The forward genetic screen that resulted in the identification of the *pac* mutants was aimed at finding genes that balance the auxin and cytokinin actions during LR organogenesis (Bielach et al., 2012). The LR organogenesis is a very suitable model for such screens because both auxin and cytokinin contribute to its regulation from the earliest stage on (Fukaki and Tasaka, 2009).

As both auxin and cytokinin interact antagonistically, proper crosstalk is particularly important for the LR organogenesis to proceed and any deficiency in their interaction might be manifested by a defective LR organogenesis. The common feature of all *pac* mutants is the reduced LRI sensitivity to the simultaneous auxin/cytokinin treatment (Bielach et al., 2012). According to the initial phenotypical results of our study, the *pac7* mutant might contribute to the general cytokinin signal transduction in view of its LRI insensitivity to cytokinin. Interestingly, further detailed characterization of *pac7* and other *pac* mutants revealed that *PAC7*, along with *PAC5*, *PAC12*, and *PAC20* (Bielach et al., 2012), but not other ones, might represent the molecular components that control the cytokinin-dependent regulation of the expression of the *PIN* auxin efflux carriers (this work and unpublished results).

The specific aspects of the *pac7* mutant phenotype (such as LRI cytokinin insensitivity and reduced sensitivity of PIN1 to cytokinin-stimulated degradation) prompted us to identify gene affected by the *pac7* mutation. Map based cloning linked the *pac7* mutation to chromosome 3. Due to difficulties with the fine mapping, we applied the SHOREmap approach in the next step. Hence, the Next Generation Sequencing (NGS) technologies have accelerated the genetic mapping by providing high-resolution genetic information. Once a mapping population is available, the short read analysis pipeline (SHOREmap) method allows a single investigator to identify a mutation within a little more than a week with the Illumina Genome Analyzer sequencing (Figure S2) (Schneeberger et al., 2009).

We described the genomic context of the mutation and confirmed that a single substitution in the *AT3G19070* gene encoding a cell wall-related protein with

the GARP-G2 type transcription factor motif is responsible for the *pac7* mutant phenotype. Results from the SHOREmap analysis and complementation by allelic test revealed that indeed the causative gene is *AT3G19070*. The mutation identified is a G-to-A substitution that causes a change in the amino acid sequence from the negatively charged Asp38 to an uncharged Asn38 (Table S1). This change might interfere with the conformational structure of the protein and lead to a nonfunctional protein. Surprisingly, the prediction of phosphorylation hotspots with the PhosPhAt 4.0 database (Durek et al., 2010; Zulawski et al., 2013) revealed a phosphorylation site at Thr35 with the highest score of 1.53 (Figure 8). The mutation on Asp38, which is close to Thr35, might affect the phosphorylation status of the protein, which might explain the cause of the substitution mutation in the gene.

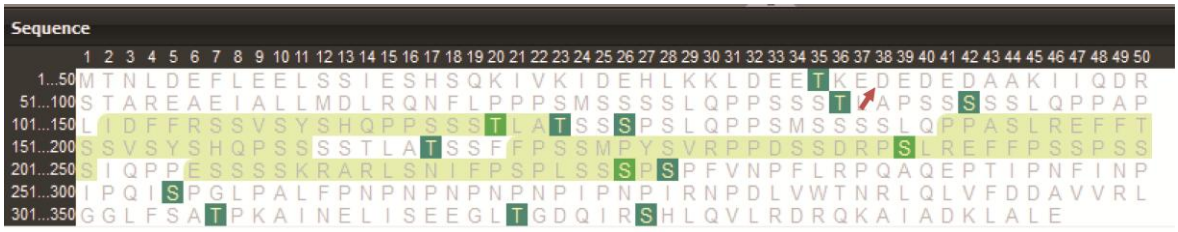

Figure 8: Predicted phosphorylation hotspots by PhosPhAt 4.0.

Red arrow marks the mutation in D38, close to T35 which has the highest score as phosphorylation site.

The predicted protein localization analysis of AT3G19070 by the eFP browser shows that the protein localize in the nucleus, cell walls, and mitochondria; however, this localization should be proved experimentally to confirm the true localization. Interestingly, evidence from the literature suggested several proteins with a dual localization under specific conditions, such as BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL) that controls asymmetric cell division, accumulates in a polarized crescent at the cell periphery before division, and then localizes differentially to the nucleus and a peripheral crescent in self-renewing cells and their sisters after division (Dong et al., 2009). Moreover, The BREVIS RADIX (BRX) protein with an unknown biochemical function maintains a rate-limiting production of the brassinosteroid biosynthesis enzyme to keep it above a critical threshold (Mouchel et al., 2006) and is localized in the plasma membrane and translocate to the nucleus upon auxin treatments to modulate cellular growth (Scacchi et al., 2009). Nevertheless, the *brx-2* mutant has been shown to exhibit insensitivity to exogenous cytokinin-induced inhibition of LRI, which can be restored by embryonic brassinosteroid treatment (Li et al., 2009). Due to the plant MYB-like binding domain, *PAC7* has a predicted nuclear localization that might give this protein an important function in gene expression regulation, more probably at the

transcriptional level, perhaps through the interaction of common sets of transcription factors. Moreover, the homology of the MYB motif sequence of AT3G19070 with B-type ARRs, such as ARR10 (Figure 7A) prompt us to predict a new function for AT3G19070 in the cytokinin-mediated signaling pathway to modulate the cytokinin-related gene expression. The similar expression pattern of *BASL* and *BRX* in the nucleus and the resistant phenotype for the cytokinin-induced inhibition of LRs in the *brx-2* mutant let us speculate that AT3G19070 might balance the cytokinin homeostasis level intracellularly, through the expression regulation of some cytokinin genes, and, thus, might control the LR formation in response to cytokinin. In view of the cytokinin-insensitive phenotype of the *pac7* mutant that stimulates PIN1 to the vacuoles and the cell wall localization of *PAC7,* this protein might interfere with the PIN1 trafficking from the plasma membrane, but this connection should be tested experimentally. Taken together, PAC7 interferes with the PIN1 stabilization in accordance to the structural cell wall integrity. Although possible explanations are provided for the role of PAC7 in the cytokinin response during root development and for its localization and function, further investigations are needed to confirm them and its modulation of the PIN1 degradation upon cytokinin during LRP organogenesis as well.

Materials and Methods

Plant Materials and Growth Conditions

Seeds of transgenic *Arabidopsis thaliana* (L.) Heyhn. plants (accession Columbia-0 [Col-0]) harboring *PIN1::PIN1-GFP* were plated on half-strength Murashige and Skoog (MS) media and stratified for 2 days at 4°C. Seedlings were grown on vertically oriented plates in growth chambers under a 16-h-light/8-h-dark photoperiod at 18°C for 4 days. The plants were subsequently transferred to liquid media containing 1 μM IAA plus 7 μM BA for 2 days as described (Bielach el al., 2012).

Analysis of Primary Root and Lateral Root Primordia Organogenesis

For phenotypic analyses of root growth, lateral root initiation, at least 15 seedlings were processed. The lateral root primordia density was measured on 7-day-old seedlings as described (Malamy and Benfey, 1997). By scoring the number of lateral roots (1st stage-LR stage) and divide them by the root length of that specific root. Root growth parameters (root length and root meristem) were analyzed with the ImageJ software package (NIH;http://rsb.info.nih.gov/ij) as described (Růžička et al., 2009).

Screening and Mapping of Mutant

For the positional PCR-based cloning screen was done on F2 generation and the cytokinin-resistant plants were selected using a fluorescence stereomicroscope MZ16F (Leica Microsystems) as described in (Bielach et al., 2012). For PCR-based positional cloning, we used simple sequence length polymorphism (SSLP) markers. We mapped *pac7* on the upper arm of chromosome 3 between MIE1 (4.8 Mb) and MLD14 (6.748 Mb) (cleaved amplified polymorphism sequence [CAPS] and SSLP markers). For information on the Col-0/L*er* polymorphisms, we used the collection of SNPs and small insertions and deletions (INDELs) provided by the Monsanto Arabidopsis Polymorphism, L*er* Sequence Collection (Cereon Genomics), and TAIR (http://www.arabidopsis.org).

Confocal Microscopy Screening

For the SHOREmap analysis we used a fluorescent-based screening. Six-day-old F2 seedlings were incubated for 1.5 h on half-strength MS media supplemented with 0.1 or 2 μM BA, and then stage-I primordia were observed for PIN1 degradation to the vacuoles as described (Marhavý et al., 2011) with a Zeiss CLSM 510 confocal microscope. Enhanced green fluorescent protein (EGFP) was observed at the excitation wavelength of 488 nm and 505-550 nm emission. Relative fluorescence of the PIN1 at the anticlinal plasma membrane in stage-I LRP was analyzed with the ImageJ software (NIH;http://rsb.info.nih.gov/ij) as described (Růžička et al., 2009).

SHORmap Analysis

Nuclear DNA was extracted from a pool of 100 resistant F2 seedlings and used for this analysis as described (Schneeberge et al., 2009). Samples were first analyzed for the absence of chloroplast DNA, and then sent for sequencing to the FASTERIS SA HiSeq service. Results were analyzed with the procedure explained in Figure S1. Primers used for chloroplast and genomic DNA amplification, were 5'-ggcctgatggtcaaggatcttc-3' and 5'-gcacctgcaaatccaatcaca-3' and 5'-taccggtctgtctgtcacca-3' and 5'-acgccttggctaaccctatt-3', respectively.

Confirmation of the Mutation by Resequencing

To confirm the change from guanine to adenine, four individual mutant plants were used to extract genomic DNA. The candidate gene was amplified with specific primers (twice independently with a proofreading polymerase). The purified reactions were sent for sequencing with another pair of specific primers, flanking the changed position. All the eight sequenced samples showed a guanine (G) to adenine (A) modification at the first exon of codon 112 (data not shown). Primers of

the candidate genes resulting from GenomeMapper analysis were: for AT3G17880_F, 5'-AGCTCCGTACGATCACAAGGA-3'; AT3G17880_R, 5'-GCAGTAGGGTCTCCCATCTAAGA-3'; for AT3G18670_F, 5'-ATCCCAGAGGTTTCAGAGCA-3'; for AT3G18670_R, 5'-ACCTTATGCGTTTCCTAGCG-3'; for AT3G18970_F, 5'-GCCATTTCTCAGACGGGTTTG-3'; and for AT3G18970_R, 5'-CGAACTTGACAAGATTCTACACGGT-3'.

Complementation Test

Seeds of *Arabidopsis* (accession Col-0), *pac*7, *Salk_082481*, and F1 (*pac7×Salk_082481*) were plated on half-strength MS medium (Duchefa) with 1% sucrose and 1% agar (pH 5.7) supplemented with 10, 50, and 100 nM BA and stratified for 2 days at 4°C. Seedlings were grown on vertically oriented plates in growth chambers under a 16-h-light/8-h-dark photoperiod at 18°C. Primers used for PCR amplification and sequencing were: for AT5G19070_F1, 5'-GAAAATCGTGAAGATAGATGAGC-3' and for AT5G19070_F2, 5'-CGATGACGGTGGTTGAAGAGAG-3'. LBa1-3: 5'- ATTTTGCCGATTTCGGAAC-3'.

Supplementary Data

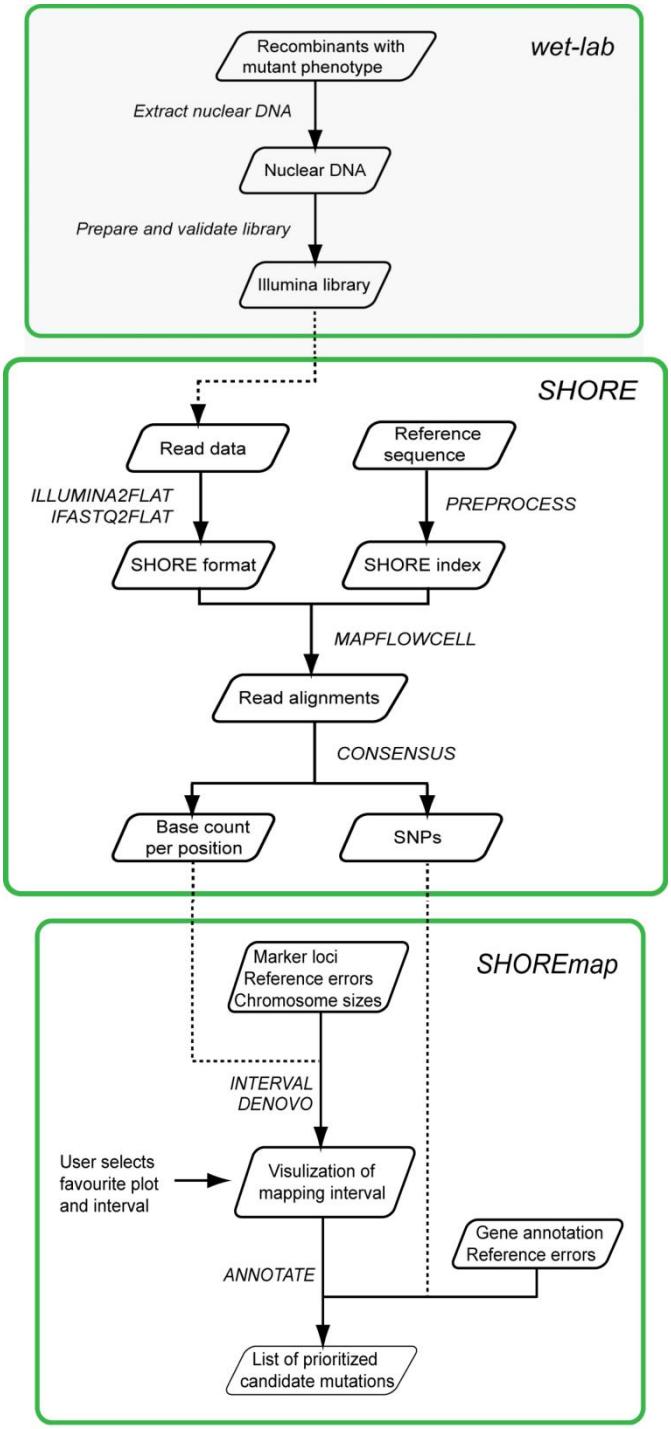

Figure S1: SHOREmap workflow.

In the wet lab, the Illumina library was prepared with genomic DNA extracted from a pool of recombinants and the Illumina reads were aligned to the reference sequence with SHORE. Based on the alignments, base counts per position and SNPs were defined. The candidate region was delimited by means of SHOREmap with (INTERVAL) or without (DENOVO) marker position information. Finally, SNPs corresponding to candidate mutations were prioritized and annotated through SHOREmap ANNOTATE to allow the identification of the causal mutation (adapted from Schneeberger et al., 2009).

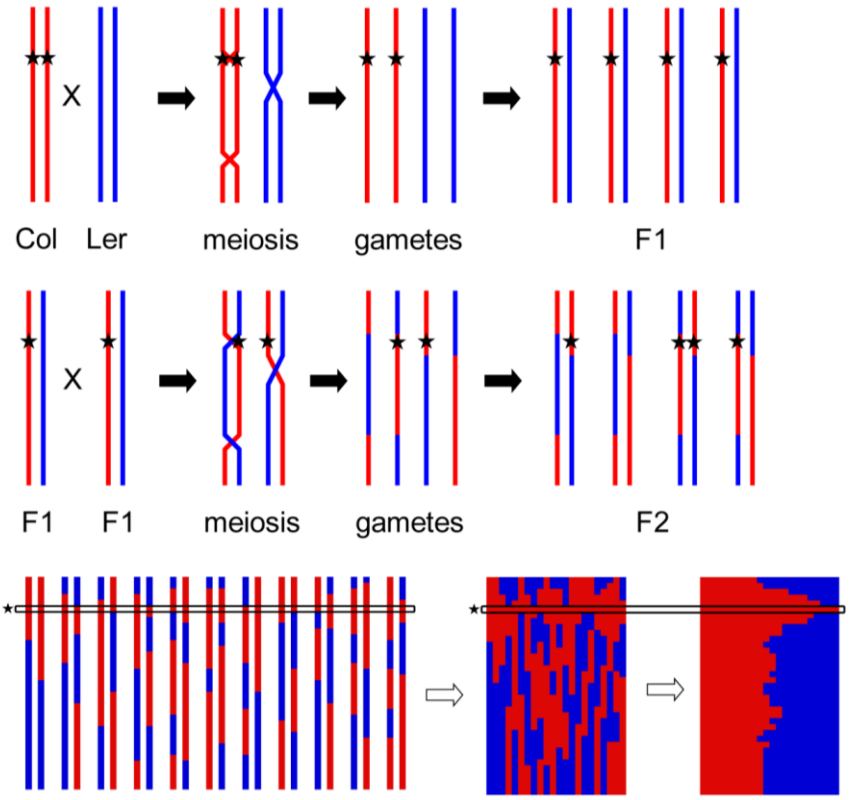

Figure S2: Representative scheme of the principle of mutant identification by Next Genaration Sequencing (NGS).

For mapping of the mutations, mutants in the Columbia-0 (Col-0) reference background are crossed to the wild-type Landsberg *erecta* (L*er*) accession plants. The heterozygous F1 plant is self-fertilized to make a pool of F2 plants that segregate for the mutation. Genomic DNA of F2 plants that display the mutant phenotype (i.e. homozygous for the mutation) are pooled and their DNA is deep-sequenced. Comparing the density of SNPs of the two ecotypes identifies a region on the chromosome in which only Col-0 marker SNPs are present. The sequencing data in this interval are then invistigated for the possible mutation (adapted from Lister et al., 2009).

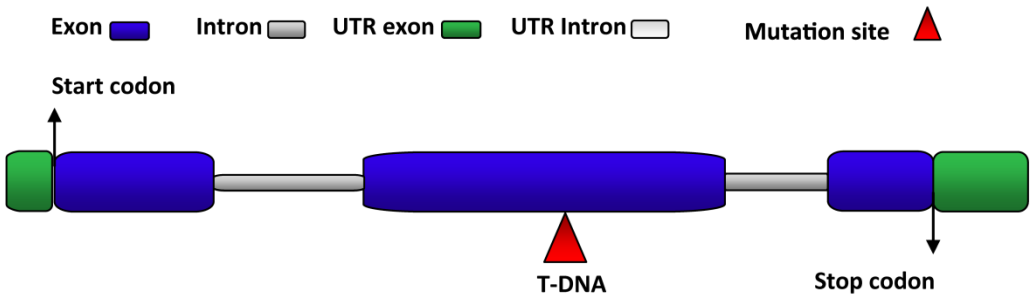

Figure S3: Structure of the candidate gene (AT3G19070) by means of the PLAZA 2.5 platform.

Blue and gray rectangles represent the exons and introns of the gene, respectively. The red triangle indicates the mutation site of *Salk_082481*.

Table S1: SHOREmap commands.

Program	Parameters
SHORE preprocess	-f TAIR8_ch_all.fa -i IndexFolder
SHORE import	-v Fastq -a genomic -e shore -x Fastq File (Illumina reads file) -o Run_Triffid1
SHORE mapflowcell	-n 4 -f Run_Triffid1 -i IndexFolder/TAIR9_ch_all.fa.shore -z
SHORE merge	-m Run_Triffid1 -o AlignmentFolder_Triffid1
SHORE consensus	-n Triffid1 -f IndexFolder/TAIR8_ch_all.fa.shore -o AnalysisFolder_Triffid1 -i AlignmentFolder_Triffid1/map.list -v -r
SHOREmap_interval	--consensus=consensus_summary.txt --marker=ler.marker_pos.txt --chrsizes=At.chrsizes.txt --referrors=At.ref.errors.txt --agg=afreq --parent1=Col --parent2=L*er* --min=100
SHOREmap_annotate	--snp=homozygous_snps.txt --dist=SHOREmap_INTERVAL.output.txt --chrom=3 --start=12,000,000 --end=15,000,000 --genome=TAIR8.v1.fa --gff=TAIR8.gff --referr=At.ref.errors.txt

Table S2: Top 8 ranked mutations from the SHOREmap ANNOTATE output.

Chr number	*Position (bp)*	*Ref. allele*	*Mut. Allele*	*Distance from the peak*	*Type*	*Seq. type*	*Gene ID*	*Codon position*	*a.a type*	*Ref. a.a*	*Mut. a.a*	*Predicted function*
3	6593890	G	A	**38904**	NEWSNP	CDS	AT3G19070	112	Nonsyn	D	N	cell wall protein-related
3	7122883	C	G	**60300**	NEWSNP	CDS	AT3G20430	601	Nonsyn	L	V	expressed protein
3	13495703	T	G	**64675**	NEWSNP	CDS	AT3G38940	923	Nonsyn	K	T	expressed protein
3	6543814	G	A	**88980**	NEWSNP	CDS	AT3G18970	1310	Nonsyn	A	V	pentatricopeptide (PPR) repeat-containing protein
3	14385974	C	T	**127283**	NEWSNP	CDS	AT3G42160	268	Nonsyn	P	S	pectinesterase-related
3	6504431	C	G	**128363**	NEWSNP	CDS	AT3G18860	836	Nonsyn	T	S	transducin family protein WD-40 repeat family protein
3	12954331	C	G	**181220**	NEWSNP	CDS	AT3G31950	1119	Nonsyn	Y	*	hypothetical protein
3	12954345	G	A	**181234**	NEWSNP	CDS	AT3G31950	1133	Nonsyn	G	E	hypothetical protein

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5

Concluding remarks and future perspectives

Microtubules (MTs) and the actin cytoskeleton play important roles during plant development in which they regulate the cell division orientation, cell growth and pattern formation. The MTs and actin cytoskeleton have crucial functions in controlling responses to the environment. A common theme for the MT and F-actin regulation is their spatial reorganization. The evidence presented in this thesis provides further insights into the crosstalk between auxin and cytokinin in controlling the cytoskeleton activity and dynamics, and, hence, their influence on plant development. In this chapter, we will try to give an integrative view of the results described in the previous chapters. Furthermore, we will present some future perspectives that emerged from the observations of this research.

During plant morphogenesis, the orientation of cell division planes and the cell expansion direction must be strictly controlled because, unlike mammalian cells, plant cells are constrained within rigid cell walls and are unable to rapidly change shape or migrate. In this thesis, we focused on the role played by the plant cytoskeleton during root growth, and more precisely, on lateral root formation as a model system in *Arabidopsis thaliana*. We also investigated how growth and development are regulated by phytohormones, specifically on the plant hormones auxin and cytokinin. In plants, the cytoskeleton consists of two major protein polymers: microtubules (MTs) and actin filaments (AFs) that form dynamics tracks and scaffolds within the cells (Kueh et al., 2009).

In the first part of this thesis, we gave an overview of the recent knowledge on auxin and cytokinin perception, signaling and transport and summarized their crosstalk to regulate root architecture and development. Although the interactions of most plant hormones have been studies extensively, the molecular basis for the hormonal crosstalk between auxin and cytokinin is still largely unknown, especially regarding the actin and MT cytoskeleton to control plant development (see **Chapter 1** for details).

The effects of phytohormones on the spatial organization and localization of MTs are well studied. Auxins, gibberellins, cytokinins, ethylene, abscisic acid, and brassinosteroids exert a regulatory action on MTs. They influence the MT patterns and, thus affect growth since MTs navigate cellulose deposition in the cell wall (Paredez et al., 2006). Cellulose microfibrils prevent stretching along their length direction and determine the direction of growth which is perpendicular to their orientation. As shown in epidermal cells of the root elongation zone, cortical MTs mostly lie in a parallel pattern perpendicular to the cell elongation axis, which are called transverse MTs, whereas a switch to a longitudinal alignment accompanies growth inhibition (Sedbrook and Kaloriti, 2008). In **Chapter 2,** the crosstalk between auxin and cytokinin and their effect on the MT organization and dynamics is investigated, and thus, their role on the regulation of plant growth and development. Recently it was reported the effect of the plant hormones auxin and gibberellin on cortical MTs orientation, results showed that application or auxin or gibberellin promote the transverse orientation in Arabidopsis hypocotyl cells (Vineyard et al., 2013). In this context, we have tested the effect of auxin and cytokinin in MT orientations in Arabidopsis root cells. Results indicated that after 10 min of auxin application the MT orientation in root epidermal cells changed from transversal to random (Chen et al., unpublished), whereas cytokinin had no effect on the MT orientation; when both hormones were applied simultaneously, cytokinin was able to counteract auxin, suggesting an antagonistic effect of auxin and cytokinin on the MT arrangement. As a conclusion, cytokinin may contribute to the

elongation zone by influencing MT patterns and stimulate growth. The high cytokinin levels here would impede the auxin-induced longitudinal pattern and maintain the transversal pattern and thus, regulate growth may be by affecting the cellulose pattern. Also, it could be hypothesized that a transversal pattern is like a default pattern and that an actively signaling by auxin induces a longitudinal pattern (Chen et al., unpublished). How does auxin affect MT patterns? What are the targets and the molecular players involved? How are they coordinated? And most importantly how does cytokinin interfere with this mechanism? These questions can be answered by identifying new players with altered MT structure and orientation. Taking in consideration the *abp1-5* mutant, which has defect on MTs orientation (Chen et al., unpublished), performing an EMS mutagenesis experiment on this mutant and looking for suppressors that rescue the phenotype and have normal MT orientation, would allow us to link auxin to MT arrangement.

Furthermore, it is demonstrated that cytokinin was able to interfere with the MT dynamics by enhancing the MT growth rate and reducing the shrinking rate as well as reducing the growth and catastrophe frequencies, but auxin did not affect growth rate, however, enhanced the catastrophe frequency. We conclude that cytokinin promotes stabilization, while auxin enhances shrinkage. This fits nicely with the idea that cytokinin stabilizes the default transversal pattern while auxin promotes reorganization of the MTs. Collectively, these observations provide a new action mode for the auxin-cytokinin antagonism, which might be part of the regulatory auxin-cytokinin mechanism to control root elongation and development. The question that emerged here is how cytokinin interferes with the MT growth at the plus ends. Is it by regulating the function of specific plus-end-binding proteins that recruit α/β tubulin heterodimers to the plus ends? This issue needs to be further investigated by examining the molecular components at the cytokinin-affected plus-ends.

Another observation concerned the tyrosination level that had been measured by co-localization analysis between α-tubulin and tyrosinated α-tubulin. When compared to control plants, cytokinin enhanced the proportion of the tyrosinated α-tubulin, whereas auxin reduced it, hinting at an auxin-cytokinin antagonistic effect. However, to quantitatively measure the amount of tyrosinated versus detyrosinated tubulin, western blot analysis should be performed. The cytokinin effect on α-tubulin tyrosination was tested in mutants defective in cytokinin perception, biosynthesis and degradation that confirmed the initial observation. Moreover, it is revealed that the cytokinin regulation of lateral root organogenesis might depend on the activity of the tubulin tyrosine ligase (TLL) enzyme, which is the main enzyme that adds tyrosine residues on the α-tubulin (Westermann and Weber, 2003), because a lack of functional *AtTTL*

results in cytokinin-insensitive lateral root initiation. This observation opens the question on how this enzyme is involved in lateral root formation.

How does the TTL activity affect MT stability and lateral root organogenesis in response to cytokinin? By modulation of the TTL activity that leads to MT stiffness and reduced cell division? However, the stability of MTs is not affected by tyrosination/detyrosination, since the stability parameters are equal for both conditions, but they merely 'mark' a subpopulation of MTs, with different function or regulation. Moreover, it's important to map the activity and role of MT interacting proteins to see how they take part in the stabilization of MTs (cytokinin-promoted) and reorganization into a longitudinal pattern (auxin-promoted). They are the key players to understand spatial organization of MTs. These are the questions to be answered in the future. Nevertheless, the tyrosinated α-tubulin in the *ttl* mutant should be tested.

Additionally, it's shown that endogenous and exogenous cytokinin is crucial for maintaining the proper MT orientation, as confirmed by analysis of mutants defective in cytokinin biosynthesis and perception. So how is this accomplished? I think that a transversal pattern is like a default pattern due to the geometry of the cell (Besson and Dumais, 2011), supported/promoted/facilitated by the cytokinin dependent tyrosination. It can be hypothesized that stabilizing molecules such as MOR1, severing molecules like KATANIN, or nucleation proteins like γ-tubulin contribute the final MT structure within the cell. These 3 candidates would be therefore promising candidates for future analysis to get a better understanding of the molecular events necessary for organ formation.

In plant cells, the actin cytoskeleton is required for organellar movements, cytoplasmic streaming, vesicle transport and endoplasmic reticulum organization. The F-actin filaments are highly dynamic and this dynamic organization seems to be important for proper cell growth (Deeks and Hussey, 2009). Actin appears in specific organizational patterns. For example, during tip growth in growing pollen tubes and root hairs, thicker F-actin microfilaments span the length of the cell but do not reach the growth tip. Actin appears as a fine F-actin ring subapically to the tip in the pollen tube, whereas in root hairs fine F-actin is found at the tips (Fu et al., 2001; Jones et al., 2002). When root hairs cease to grow, the fine actin mesh is replaced by thicker microfilaments at the root hair tips (Jones et al., 2002; Bloch et al., 2005, 2011). In leaf epidermis pavement cells, which are characterized by diffuse growth, fine F-actin accumulates at the growing lobes (Fu et al., 2002). Moreover, actin is involved in the regulation of the auxin distribution via its effect on the vesicle transport. The polar PIN localization depends on constitutive recycling to and from the plasma membrane and the PIN recycling is compromised by treatment with actin

disruption drugs (Geldner et al., 2001). Accumulating evidence suggests that F-actin affects the PIN polarization by the localized regulation of clathrin-dependent endocytosis (Lin et al., 2012; Nagawa et al., 2012).

Although the past decade has yielded a wealth of new information regarding plasma membrane trafficking mechanisms, many outstanding questions are still to be addressed. The mechanisms underlying the polar targeting of proteins to the plasma membrane are yet to be fully elucidated. A more substantive elaboration of the transcytotic redirection events and the evaluation of the contribution of this phenomenon to distinct polar trafficking pathways are required. The hormone cytokinin has been demonstrated to play an important role in the mechanism of the PIN1 auxin efflux trafficking and targeting for lytic degradation (Marhavý et al., 2011).

In **Chapter 3**, it is shown that the molecular components and regulators of the actin polymerization and nucleation (ARP2/3) subunits are crucial for proper PIN1 cellular trafficking and targeting for lytic degradation in response to cytokinin, with modulation of the PIN1-mediated auxin transport what ultimately will result in proper root architecture. Intact actin is essential for proper endomembrane trafficking, endocytic vesicle formation and dynamics that, in turn, are part of the PIN1 protein targeting and cycling. The results indicate that the functional actin cytoskeleton is involved in PIN1 endocytosis and, thus, in the control of plant responses to hormones. Therefore, the intact actin might be essential for vesicle trafficking events in response to cytokinin. In addition, our data suggest a link between the plant family of Rho G proteins, known as Rho of Plants (ROPs) and the cytokinin-induced PIN1 lytic degradation during lateral root formation. However, several experiments are required to investigate the interaction between cytokinin, ROP signaling and actin, by checking whether the actin cytoskeleton is modified in *rop* mutants and whether it is changed by cytokinin. Also one of the future tasks will be the study of the ROP effector function in the actin organization and dynamics and how they are regulated by ROPs during plant development, in particular during lateral root initiation and organogenesis. Moreover, to further analyze the cytoskeletal requirements for vesicle trafficking and protein targeting in *planta*, a phenotypic microscopical analysis of the PIN1 localization in actin mutants of the *Arabidopsis* root apex needs to be investigated.

In **Chapter 4**, the aim was to identify possible molecular mechanisms for the cytokinin-auxin crosstalk during lateral root organogenesis. Therefore, the ethylmethylsulfonate mutant allele *pac7*, generated from a previously described screen (Bielach et al., 2012), was further characterized and mapped. This *pac7* mutant was selected based on two criteria, its lateral root initiation (LRI) sensitivity to auxin and cytokinin, and also for its insensitivity for the cytokinin-

induced PIN1 auxin efflux carrier to the vacuoles. PAC7 is predicted to localize in the cell wall and the nucleus, where it might have a GARP-G2 type transcription factor activity to control the expression of the cytokinin-related genes. It is unlikely that both localizations are true, so further experiments are needed to verify the precise localization and function of this protein. The main question here is where PAC7 acts in the auxin-cytokinin network to regulate lateral root formation. Is it only at the transcriptional or also at the posttranscriptional level?

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SUMMARY

Interaction with the surrounding environment is an elementary part of every-day existence of each living organism. In the animal kingdom, evolution typically promoted behaviorally based solutions for adjusting to an ever-changing habitat (Davies, 2004), whereas, plants, due to the stationary nature of their lifestyle, developed alternative mechanisms, mediating the fascinating plasticity of their development (Tejos and Friml, 2012).

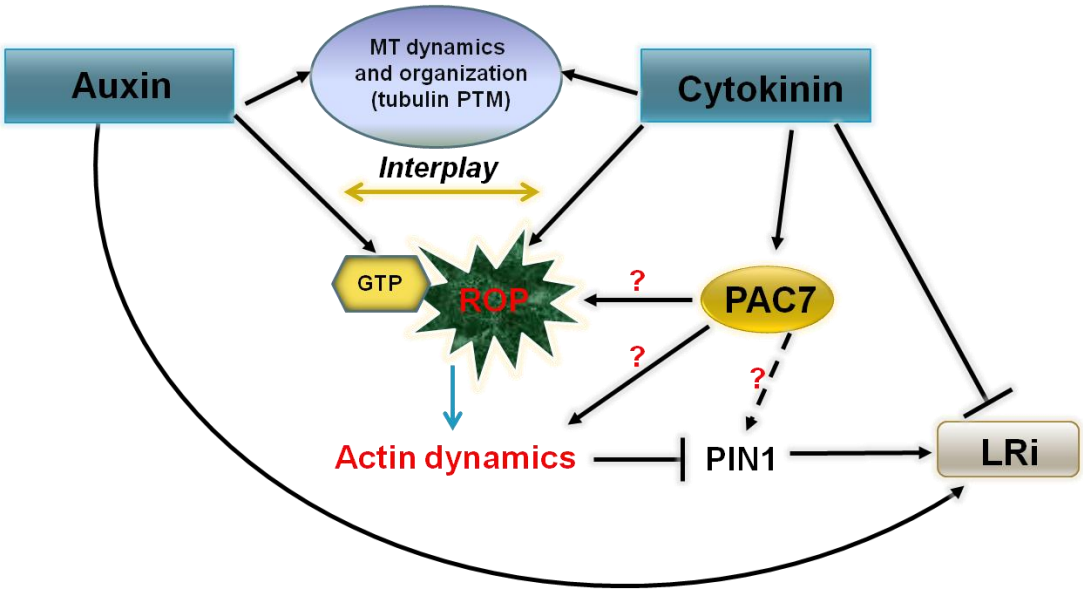
Plant hormones shape the plant by modulating growth in response to internal and environmental signals. They regulate every aspect of the plant's life, from pattern formation during development to responses to biotic and abiotic stresses. Evidence for hormonal interactions can be found in all developmental processes. The main regulators of root growth and meristem maintenance are auxin, cytokinin (CK), ethylene and gibberellins (Depuydt and Hardtke, 2011; Su et al., 2011). The plant hormones auxin and CK are the major hormonal regulators of the lateral root (LR) organogenesis and significantly contribute to shape the root system architecture (Laplaze et al., 2005, 2007; Péret et al., 2009; Benková and Bielach, 2010). The plant root system is a complex structure consisting of the main root and LRs that, besides from anchoring the plant in the soil, mediates a vitally important interaction between plant and soil to provide water and nutrients. Thus, understanding of molecular mechanisms that control the root architecture is of high agronomical importance (Fitter et al., 2002; Fukaki et al., 2007; Osmont et al., 2007; MacGregor et al., 2008).

In plants, microtubules (MTs) and F-actin microfilaments serve as spatial organizers during development and stress responses. Consequently, the study of the organization and dynamics of MTs and actin in plants is fundamental for agriculture and might potentially lead to the discovery of superior traits and small molecules that could be used as herbicides or growth enhancers. Furthermore, due to the growing interest in plant cell walls as potential biofuel resources (Somerville et al., 2010), investigation of the cytoskeleton and how it affects the cell walls could provide important insights into the development of sustainable biofuel crops.

In the first part of this PhD thesis, we show that the MT cytoskeleton is essential for LR initiation and development because mutants in the tubulin subunits had a reduced lateral root density (LRD) and were insensitive to cytokinin. By following the MT dynamics in response to auxin and cytokinin, we demonstrate that modulation of cytokinin levels in plants has an impact on the cytoskeleton dynamics and antagonize the auxin effects. We show that cytokinin interferes with the principal processes that determine the MT dynamics and that this antagonistic regulation of the MT dynamics by auxin and cytokinin might be part of the

regulatory mechanism underlying the opposite auxin–cytokinin control of root development and LR initiation (**Chapter 2**).

In the second part, we examine the role of the actin cytoskeleton and vesicle trafficking of the auxin efflux carrier PIN1 that targets to the vacuoles upon cytokinin treatment to regulate LR organogenesis, implying a mechanism in which the actin-regulated endocytic transport has a function in the cytokinin-controlled root development (**Chapter 3**). The fourth chapter of this thesis is dedicated to the identification of potentially novel players of the cytokinin-induced PIN1 auxin efflux carrier targeting to the vacuoles, by means of a forward genetic screen. This approach enabled us to identify the ***p**rimordia on **a**uxin and **c**ytokinin 7* (*pac7*) mutant that was mapped by means of classical mapping and by SHOREmap analysis. The *PAC7* gene codes for an unknown function with a MYB-like binding domain and a GARP-G2 type transcription factor activity, predicted to bind cytokinin-regulated genes (**Chapter 4**). The following scheme is a summarized model for my PhD thesis.



Altogether, this PhD research brings novel insights into the hormonal control mechanism on the plant cytoskeleton dynamics and their role on vesicle trafficking, thus regulating the LR formation and the root system architecture.

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Thank you!

Anas Abuzeineh

June 2014

Curriculum Vitae

Personal Information

Name: **Anas Abuzeineh**

Date of Birth: 7th November 1982

Place of Birth: Jerusalem, Palestine

E-mail: a_abuzeineh@hotmail.com

Education

2009_ 2014 ***Ph.D.*** Biochemistry and Biotechnology, VIB Department of Plant Systems Biology, Faculty of Sciences, Ghent University, Ghent, Belgium. Thesis title: "*The Role of the Cytoskeleton in Hormone-Regulated Root Development*"

2008_2009 ***M.Sc.*** Horticultural Genetics and Biotechnology, Mediterranean Agronomic Institute of Chania, Crete, Greece. Thesis title: "*Characterization of Arabidopsis thaliana Prolyl 4-hydroxylase 11 T-DNA insertional mutants"*

2007_2008 ***DSPU*** Diploma of Specialized Post-Graduate studies (DSPU), Horticultural Genetics and Biotechnology department, Mediterranean Agronomic Institute of Chania, Crete, Greece.

2001_2005 ***B.Sc.*** Biology and Biochemistry, Birzeit University, Birzeit, Palestine. Thesis title: "*Inhibition of tumor growth by DT-A expressed under the control of IGF-2 P3 and P4 promoter sequences"*

1999_2000 ***Tawjihi*** Tawjehi certificate (Al-Ummah secondary school) scientific stream, Jerusalem, Palestine

Research Experience

10/2009 – 05/2014: Predoctoral Researcher in Plant Cell and Developmental Biology, VIB Department of Plant Systems Biology, Gent University, Gent, Belgium

1/2006 - 11/2006: Research assistant of Biotechnology at Hebron University, Faculty of Agriculture and The Hebrew University of Jerusalem, Department of Plant Genetics and Breeding, Rehovot, Israel

Training and Special Courses

High Magnification Microscopy (Spinning-Disk Microscopy) (14 January – 4 February 2013)

Effective Writing for Life Sciences Research (12 – 13 October 2011)

Introduction to Biostatistics (18 – 19 January 2011)

Plant Cell Development course (9 – 13 February 2009)

Microbiology Techniques (September 2004 – February 2005)

Publications

Peter Marhavý, Agnieszka Bielach, Lindy Abas, **Anas Abuzeineh,** Jerome Duclercq, Hirokazu Tanaka, Markéta Pařezová, Jan Petrášek, Jiří Friml, Jürgen Kleine-Vehn, Eva Benková **(2011),** *Cytokinin Modulates Endocytic Trafficking of PIN1 Auxin Efflux Carrier to Control Plant Organogenesis.* ***Developmental Cell*** 10.1016/j.devcel.2011.08.014

Manuscripts in Preparation

Anas Abuzeineh, Xu Chen, Marleen Vanstraelen, Evangelia Dougali, Kateřina Schwarzerová, Jan Petrášek and Eva Benková: *Cytokinin Modulates Microtubule Cytoskeleton Dynamics in Arabidopsis Root* (Manuscript in Preparation)

Anas Abuzeineh, Marleen Vanstraelen, Peter Marhavý, Jan Petrášek and Eva Benková: *Cytokinin Controls Plant Postembryonic Development through Actin-Dependent PIN1 Lytic Degradation* (Manuscript in Preparation)

Peter Marhavý, **Anas Abuzeineh**, Ellie Himschoot, Steffen Vanneste, Jiří Friml and Eva Benková: *Ethylene through the ABP1-Dependent Signaling Inhibits Cytokinin Mediated PIN1 Recruitment to Lytic Vacuoles* (Manuscript in Preparation)

Jerome Declerq, Peter Marhavý, **Anas Abuzeineh**, Simon Rudiger, Jelle Van Leene, Geert De Jaeger, Jiří Friml and Eva Benková: *CLE Peptides Counteract Cytokinin-Induced PIN-FORMED 1 Degradation through Clathrin-Mediated Endocytosis Modulation* (Manuscript in Preparation)

Xu Chen, Grandont Laurie, Hongjiang Li, Robert Hauschild, Paque Sebastien, **Anas Abuzeineh**, Eva Benková, Catherine Perrot-Rechenmann, and Jiří Friml: *Cell Expansion by ABP1-mediated Binary Switch in Microtubule Arrangement* (submitted)

Evangelia Dougali, **Anas Abuzeineh**, Kathleen Marchal and Yves Van de Peer: *Inferring regulatory interactions in Arabidopsis thaliana* (Manuscript in preparation)

Meetings

16th European Plant Endomembrane Meeting (ENPER). Ghent, Belgium, 27 – 30 August 2013, Poster presentation

The spider's Web: How Microtubule Organize Cellular Space. Potsdam, Germany, 29 June & 1 July 2011, Poster presentation

International Symposium: Growth and Development of Roots. Louvain la Neuve, Belgium, 27 January, 2011

Frontiers in Developmental Cell Biology. Lausanne, Switzerland, 7 – 8 September, 2010

VIB Seminar. Blanckenberge, Belgium (2010, 2012, 2013)

Languages

Arabic: mother tongue

English: excellent

Hebrew: very good

Greek: good

French: basic

Technical Skills

-Molecular biology: techniques associated with DNA and RNA (extraction, purification, gel electrophoresis, restriction enzyme analysis etc.), PCR and qRT-PCR

-Cloning: Map based cloning, Gateway-based cloning (handling bacterial cultures)

-Cell biology: epi-fluorescence, laser scanning confocal microscopy visualization of GFP and fluorescent probes (FM4-64), developmental analysis of mutants, In vivo time lapse lateral root

development analysis using fluorescence microscopy, immuno-fuorescence, pharmacological trafficking inhibitors (BrefeldinA, Wortmanin, ConcanamycinA, etc.), biosynthesis inhibitors (L-Kynurenine, Cycloheximide etc.), phytohormones and analogs (auxin, cytokinin, *t*-zeatin, etc.)

-Microscopy: Excellent command of Olympus, Zeiss and Leica and spinning-disk microscopy, imaging analysis software, such as ImageJ package and Olympus FluorView

-Gene expression analysis using reporter genes (GFP, GUS)

-Experience with **robotized systems**: Biomek2000 (plasmid isolation), Janus (qRT-PCR), InsituPro Vsi (immuno-chemistry)

-Experience with *Arabidopsis thaliana* system

-Tissue Culture: sterile techniques for maintaining *Arabidopsis* cell cultures, somatic embryogenesis, callus cultures

-Genetics: cross pollination of *Arabidopsis*, generation of stable transgenic lines in *Arabidopsis* via floral dip, morphological and subcellular phenotypical characterization of the mutant *Arabidopsis* lines

-Bioinformatics: SHOREmap analysis, basic skills in gene and protein analyses using BLAST, ClustalW, Vector NTI

-Scientific writing and editing, graphical preparation of the manuscripts (Adobe Illustrator)