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Auxin – Cytokinin interactions during plant developmental processes

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Frequently used abbreviations

ABA: abscisic acid
ACC: 1-aminocyclopropane-1-carboxylic acid
ABP1: AUXIN BINDING PROTEIN1
AFB: AUXIN-RELATED F-Box protein
AHK: ARABIDOPSIS HISTIDINE PROTEIN KINASE
ARF: AUXIN RESPONSE FACTOR
ARR: ARABIDOPSIS RESPONSE REGULATOR
AVG: Aminoethoxy- vinylglycine
AuxRE: auxin responsive element
AUX1: AUXIN RESISTANT1
Aux/IAA: auxin/indole-3-acetic acid
AXR: AUXIN RESISTANT
BA: 6-benzylaminopurine
BFA: brefeldin A
CHX: cycloheximide
CK: cytokinin
CKX: oxidases/dehydrogenase
FC: founder cell
GA: Gibberellins acid
GFP: Green Fluorescent Protein
GUS: β-glucuronidase
IAA: indole-3-acetic acid
IPT: isopentenyltransferase
JA: Jasmonic acid
LAX: LIKE-AUX1
LOG: LONELY GUY
LR: lateral roots
LRD: lateral root density
LRI: lateral root initiation
LRP: lateral root primordium
MeJA: Methyl jasmonate
NAA: naphthalene-1-acetic acid

NLS: nuclear localization signal

NPA: N-1-naphthylphthalamic acid

PAT: polar auxin transport

PI: propidium iodide

QC: quiescent centre

Q-RT-PCR: semi-quantitative reverse transcriptase polymerase chain reaction

RFP: red fluorescent protein

TF: transcription factor

TIR1: TRANSPORT INHIBITOR RESPONSE1

WT: wild type

2,4-D: 2,4-dichlorophenoxy acetic acid

Ø: control seedlings

Scope

Plant growth and development are regulated by small signaling molecules called plant hormones. Plant hormones, including auxin, cytokinin (CK), ethylene, gibberellin, brassinosteroids, strigolactone, abscisic acid or jasmonic acid, collectively control the number of developmental processes such as embryogenesis, root and shoot growth and branching, or flowering. Over the past decades, studies that focused on mechanisms of phytohormone 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 which governs and coordinates proper plant development. Although the molecular principles of signal perception and transduction for most plant hormones were recognized, the molecular basis for hormonal crosstalk remains largely unknown. To get insights into the molecular mechanism underlying the hormonal crosstalk we focused on the interaction between auxin and CK. Auxin and CK are key hormonal coregulators in major developmental processes, such as shoot apical meristem activity (Reinhardt *et al*. 2000; Werner *et al*. 2003), shoot branching (Ongaro *et al*. 2008), root growth (Sabatini *et al*. 1999; Dello Ioio *et al*. 2008) or lateral root (LR) organogenesis (Benkova *et al*. 2003; Laplaze *et al*. 2007). The key principles of both the auxin and CK signal transduction pathways have been established (Hwang and Sheen 2001; Kepinski and Leyser 2005) and although mechanisms of their interaction have been proposed (Dello Ioio *et al*. 2008; Muller and Sheen 2008; Zhao *et al*. 2010) the crosstalk between auxin and CK is not fully understood yet.

In my PhD thesis I focused on the investigation of the molecular mechanisms of auxin and CK interaction using LR organogenesis as a suitable model system. Lateral root organogenesis is regulated by both hormones and accurate balancing is required between the promotive effect of auxin (Laskowski *et al*. 1995) and the inhibitory effect of CK (Laplaze *et al*. 2007).

Cytokinin modulation of polar auxin transport was found to represent an important mode of auxin and CK interaction (Dello Ioio, *et al*., 2008, Ruzicka *et al*., 2009; Pernisova *et al*., 2009). We further investigated the underlying molecular mechanisms and revealed that besides transcriptional regulation of auxin efflux carriers of the AtPIN family, CK interferes with cellular trafficking of the AtPIN1 protein and its stability. We found that CK regulates recycling of the auxin efflux carrier PIN1 to the plasma membrane by redirecting it for lytic

degradation in vacuoles independently of transcription. We proposed that such a rapid post-transcriptional regulation of PIN1 abundance provides a very efficient and precise mechanism to control auxin fluxes and distribution during CK-mediated developmental regulations, including lateral root primordia organogenesis and root meristem differentiation.

The results presented in the second part of my PhD demonstrate that the above described CK stimulatory effect on the lytic degradation of PIN1 not only allows overall regulation of auxin transport efficiency, but might be part of the mechanism determining directionality of the auxin stream. We reveal that CK interferes selectively with the trafficking pathway targeting PIN1 towards the basal membrane. Developmental and genetic modulations increasing the proportion of apically localized PIN1, due to an increased phosphorylation, dramatically reduce CK sensitivity. We demonstrate that during developmental processes such as LR organogenesis requiring redirection of the auxin flow, CK might contribute to PIN polarity re-establishment by targeting a specific subset of PIN1 membrane proteins for lytic degradation.

Last part of my PhD is focused on the role of the PIN3 (PIN-formed) auxin efflux carrier in the endodermis in the regulation of the early phases of lateral root initiation (LRI). We demonstrate that a local, developmentally specific auxin moves from endodermal to founder cells, so called auxin re-flux, and is required for the progress from founder cell to LRI phase. This auxin re-flux is mediated through the PIN3 auxin efflux carrier and its deficiency causes dramatic defects in the transition from founder cells to LRI. The data demonstrate that the endodermis plays an active role in the regulation of LRI and is part of the control circuit building up the auxin threshold in founder cells, which is required for the transition to the initiation phase.

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Chapter 1.

Introduction

Auxin-controlled plant growth and development

Phytohormones are crucial signaling molecules that coordinate a wide range of developmental processes. Among them, auxin is essential throughout the plant life cycle, involved in the control of axis formation during embryogenesis (De Smet and Jürgens, 2007; Friml *et al.*, 2003), root meristem maintenance, primary root growth and branching (Friml *et al*., 2002; Blilou *et al*., 2005; Peret *et al*., 2009; Heisler *et al.*, 2005), phylotaxis, (Reinhardt *et al.*, 2003), shoot branching (Leyser 2003), vascular tissue differentiation and regeneration (Sauer *et al*., 2006; Scarpella *et al*., 2006) and tropic responses (Abas *et al.*, 2006; Ding *et al.*, 2011; Friml *et al.*, 2002; Kleine-Vehn *et al.*, 2010).

The term auxin is derived from the Greek word *auxein* meaning ‘’to grow’’ or ‘’to increase’’. Charles Darwin described in his book "The Power of Movement in Plants" (1881) the process of phototropism, i.e. the effects of light on the movement of canary grass (*Phalaris canariensis*) coleoptiles and the predicted existence of a mobile signaling molecule, which allows the differential growth of plants. Later, the signaling molecule has been identified as indole-3-acetic acid (IAA) (Koegl and Kostermans, 1934; Went and Thimann, 1937), and subsequent efforts have illuminated numerous physiological effects of auxin.

Auxin-regulated plant development is orchestrated by single processes acting together: biosynthesis to create a source, transport to generate a gradient or local accumulation, and perception or response to affect development (reviewed in Grunewald and Friml., 2010; Robert and Friml., 2009).

Auxin biosynthesis

Auxin, in its predominant endogenous form indole-3-acetic acid (IAA), is primarily synthesized in shoot apical meristems (SAMs), young leaves and developing fruits and seeds (Ljung *et al*., 2001, 2005). Furthermore, recent findings have indicated that auxin is produced not only in the biosynthetic organs, such as young leaves (Ljung *et al*., 2005), but also in very distinct cell types, such a the quiescent center (QC), root provasculature of meristematic regions and vasculature of hypocotyls and apical hooks (Stepanova *et al*., 2008). Auxin can be synthesized by tryptophane (Trp)-independent as well as Trp-dependent pathways (Woodward and Bartel., 2005; Ljung *et al*., 2002). Several studies have led to the discovery of genes involved in Trp-dependent auxin biosynthesis. Some Trp biosynthesis genes, such as *ASA1/WEI2* and *ASB1/WEI7* encoding anthranilate synthase, were isolated from a screen for weak ethylene-resistant mutants (Stepanova *et al*., 2005). Hydroxylation of tryptamine, an important step in the Trp-dependent auxin biosynthesis pathway, was found to be catalyzed

by YUCCA genes, which encode a flavin monooxygenase-like protein (Zhao *et al*., 2001). Overexpression of *YUC1* and *YUC6* (Zhao *et al*., 2001; Kim *et al*., 2007) leads to phenotypes characteristic for auxin overproducing mutants, and increased IAA levels in the tissues where the gene is expressed (Zhao *et al*., 2001; Cheng *et al*., 2006, 2007). Further characterization of the *Arabidopsis wei8* mutant revealed that *WEI8* encodes a long-anticipated tryptophan aminotransferase, so-called TAA1, involved in the indole-3-pyruvic acid (IPA) branch of the auxin biosynthetic pathway. This IPA route of auxin production is key to the generation of robust auxin gradients in response to environmental and developmental cues (Stepanova *et al*., 2008).

Auxin distribution

After auxin is synthesized in the source tissue, it is transported throughout the plant via the vascular system towards the root (Chandler, 2009). Besides the long-range auxin transport, slower carrier-dependent transport mediates short-range auxin movement in different tissues. This transport takes place in a strictly regulated and directional manner, which contributes to the generation of a graded distribution of auxin within plant tissues (Robert and Friml, 2009). This is a unique feature that sets auxin apart from other plant signaling molecules (Vanneste and Friml., 2009). Based on a series of classical physiological experiments, the chemiosmotic polar diffusion model was proposed as the mechanism underlying this directional cell-to-cell transport (Rubery and Sheldrake, 1974; Raven, 1975; Goldsmith, 1977) (Figure 1). Inside the cell the environment is less acidic (pH=7) than outside (apoplast pH=5), where some IAA molecules remain undissociated. Those auxin molecules that are neutrally charged can enter the cells by passive diffusion, although those that remains negatively charged would need an active transporter system to get into the cell. Once auxin molecules enter the cell through the membrane, they are exposed to a more basic pH, and they dissociate to the anionic form (IAA^-), which is unable to cross the plasma membrane again. Because the anionic IAA^- auxin molecules get trapped inside the cell, transporters are required for their exit from cell. Thus, according to the model the short distance transport relies on specific auxin influx and efflux carriers and passive diffusion, which mediates the transport of auxin (IAA) across the plasma membrane.

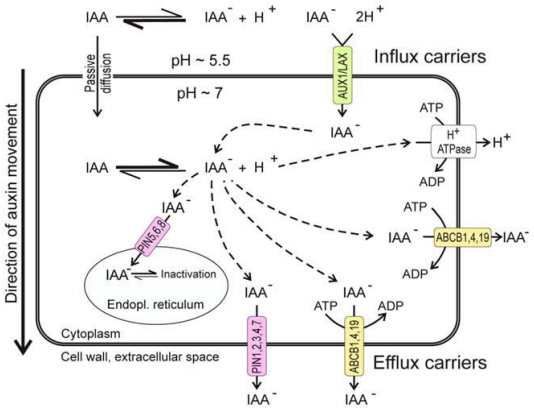

Figure 1. Updated model of intercellular auxin transport. Undissociated IAA molecules enter cells by passive diffusion, whereas the less lipophilic, and therefore less permeable, dissociated auxin anions (IAA-) are imported via auxin influx 2H+ co-transporters oftheAUX1/LAX family. In the more alkaline intracellular environment, IAA dissociates and requires active transport through the PIN or ABCB efflux transporter proteins to exit the cell. Asymmetric, subcellular localization of PIN proteins determines directionality of auxin flow. Some cytosolic IAA is transported by PIN5 and presumably also PIN6 and PIN8 into the lumen of the endoplasmic reticulum. This compartmentalization serves to regulate auxin metabolism. Whereas PIN transporter activityis supposed to use an H+ gradient that is maintained by the action of the plasma membrane H+-ATPase, ABCB transporters have ATP ase activity. Adapted from (Friml, 2010).

Later by genetic approaches in the model organism *Arabidopsis thaliana* AUX/LAX auxin influx (Bennett *et al*., 1996; Parry *et al*., 2001; Swarup *et al*., 2008) and PIN and ABCB/PGP efflux carriers were identified as major gene families involved in the regulation of cell-to-cell transport (Luschnig *et al*., 1998; Muller *et al*., 1998; Friml *et al*., 2002; Geisler *et al*., 2005; Vieten *et al*., 2007; Friml, 2010; Peer *et al*., 2011).

Biochemical and genetic evidences proved that the AUX1/LAX proteins act as influx carriers and play an important role in tropism, lateral root emergence and root hair development (Bennett *et al*., 1996; Yang *et al*., 2006; Stone *et al*., 2008; Swarup *et al*., 2008; Jones *et al*., 2009). The AUX1/LAX influx transporter protein family was identified through the isolation of the *Arabidopsis auxin1* (*aux1*) mutant. This *aux1* mutant shows roots resistant to 2,4-dichlorophenoxyacetic acid (2,4-D) as well as agravitropic growth, both consequences of a defect in cellular auxin uptake (Pickett *et al*., 1990). *AUX1* encodes an amino acid permease-like protein (Bennett *et al*., 1996) that acts as a H+/IAA− symporter (Yang *et al*., 2006; Bennett *et al*., 1996). AUX1/LAX is localized at the plasma membrane of various cell types. Notably this includes asymmetric localization at the upper side of protophloem cells of the root, where presumably AUX1 unloads auxin from the phloem flow into the short-range transcellular transport pathway in the root meristem (Zhong *et al*., 1999).

Interestingly, it has been recently demonstrated that similarly to AUX1/LAX, nitrate transporter AtNRT1.1 (CHL1) facilitates besides nitrate also cellular auxin uptake. This local,

lateral root-specific control of auxin uptake by NRT1.1 appeared to be critical for the nutrient-regulated lateral root outgrowth (Krouk *et al*., 2011).

Export of auxin out of the cells requires auxin efflux carriers. In *Arabidopsis thaliana*, the PIN gene family was identified as crucial factors mediating auxin export that act as auxin efflux carriers (Galweiler *et al*., 1998; Muller *et al*., 1998; Luschnig *et al*., 1998). The PIN gene family consists of eight homologous genes, expressed in an organ- and tissue-specific manner (Vieten *et al*., 2007; Zazimalova *et al*., 2007). Their identification was based on the auxin transport-related shoot and flower phenotypes of *pin-formed1* (*pin1*) mutants (Gälweiler *et al*., 1998; Okada *et al*., 1991) and the agravitropic root growth of *pin2* mutants (Muller *et al*., 1998). Five of the PIN transporters show an asymmetric plasma membrane localization within cells (as was predicted by the chemiosmotic hypothesis for auxin efflux carriers), and are responsible for cell-to-cell auxin transport (reviewed in Feraru and Friml, 2008). In contrast, the PIN5, PIN6 and PIN8 homologues are localized at the membrane of the endoplasmatic reticulum (ER), where they presumably regulate the flow of auxin between cytosol and ER lumen, and thereby contribute to the regulation of the subcellular auxin homeostasis and metabolism (Mravec *et al*, 2009). Thus, differently expressed and localized PIN proteins constitute an auxin distribution network within the plant that mediates the regulation of multiple developmental processes (Grieneisen *et al*., 2007).

Other components of the auxin transport machinery are several phosphoglycoproteins of the ABCB transporter family (ABCB/PGP) (Petrasek *et al*., 2006; Titapiwatanakun et. al., 2009), which have been identified as proteins binding the auxin efflux inhibitor *N*-1-aphthylphthalamic acid (NPA) (Murphy *et al*., 2002; Noh *et al*., 2003). They mediate auxin efflux and show a complex, multilevel functional interaction with the PIN family. Combinations between *pgp* and *pin* mutants indicate that these two types of auxin exporters can act synergistically and antagonistically in different developmental processes (Bandyopadhyay *et al*., 2007; Mravec *et al*., 2008). For instance, at the polar domains where the PGP and PIN proteins co-localize and interact, PGPs can act synergistically with PIN proteins in auxin efflux by stabilizing PIN protein localization at the plasma membrane (Titapiwatanakun et. al., 2009). But PGPs can also act at the non-polar domains independently of the PIN proteins; in such a situation, PGPs would mediate apolar auxin efflux, and thereby control the amount of auxin available in *PIN* expressing cells for the directional PIN driven transport (Mravec *et al*., 2008). As a result, PINs and PGPs mediate directional auxin fluxes

and ensure the fine regulation of the auxin distribution in many developmental processes (Mravec *et al*., 2008).

This tightly controlled distribution of auxin through the plant body, mediated by a polar auxin transport (PAT) machinery composed of auxin influx and efflux carriers is developmentally extremely important. PAT-dependent local auxin accumulation and graded auxin distribution triggers formation of new organs, determines proper tissue patterning and organ formation and thus shapes plant architecture (Reinhardt *et al*,.2003; Benkova *et al*., 2003; Dubrovsky *et al*.,2008; Blilou *et al*.,2005; Friml *et al*., 2003) (Figure 2).

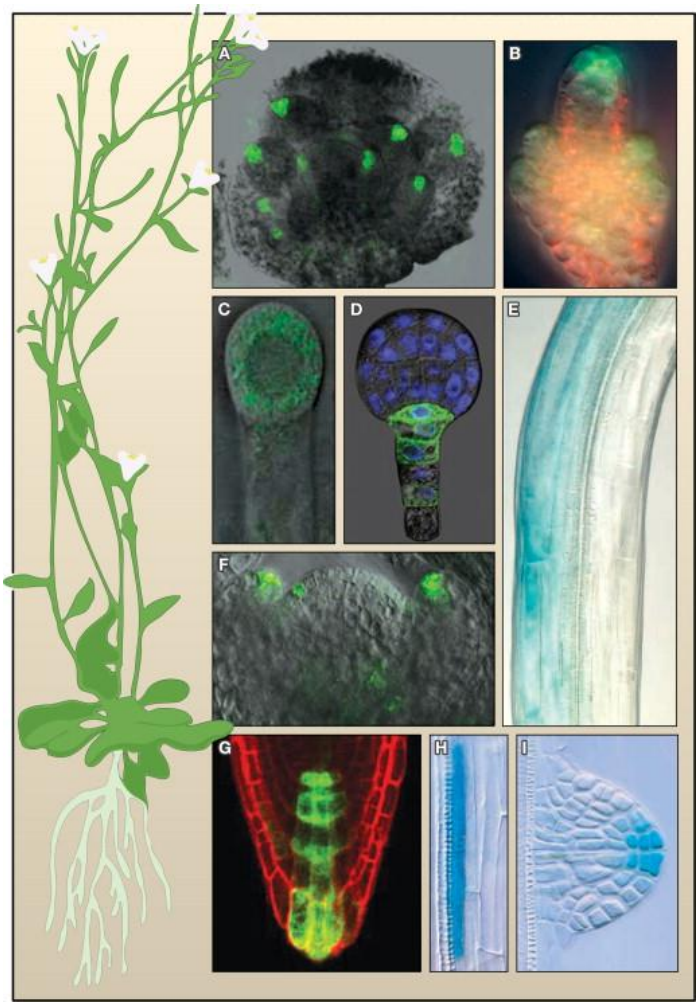

Figure 2. Patterns of Auxin Distribution during *Arabidopsis* DevelopmentDistribution of auxin activity as visualized by the activity of auxin response reporters expressing green fluorescent protein (*DR5rev::GFP)* (A, B, C, D, F, and G) and *DR5::GUS* (E, H, and I). The following are locations of auxin activity: (A) tips of floral organ primordia within developing flowers, (B) the tip of the ovule primordium, (C) the apical cell of a divided zygote, (D) a root pole of the globular stage embryo, (E) the shade side of the photostimulated hypocotyls, (F) the position of incipient organ initiation and tips of flower primordia at the shoot meristem, (G) the root apical meristem, (H) lateral root initiation site, (I) and the tip of the emerging lateral root primordium. Adapted from (Vanneste and Friml, 2009).

Auxin perception and signal transduction

To execute its developmental function auxin signals must be perceived and transduced into the appropriate downstream response. The nature of auxin perception and signaling comes from two important observations: 1. a rapid and specific increase in gene expression in response to exogenous auxin and 2. analyses of auxin-resistant mutations (Theologis *et al*., 1985; Leyser *et al*., 1993; Abel and Theologis, 1996; del Pozo *et al*., 1998). These early auxin response genes belong to three families: *SAURs* (Small Auxin-Up RNAs), *GH3s* and *Aux/IAAs*, which are induced within minutes of external auxin application.

Closer functional studies of Aux/IAA revealed their essential role in auxin response. A number of loss-of-function mutations in *Aux/IAA* genes has been characterized (Theologis *et al*., 1985) with weak phenotypes. However, most of the insights into the biological function of Aux/IAA proteins comes from characterization of gain-of-function mutants (Woodward and Bartel, 2005). Aux/IAA gain-of-function mutants exhibiting a reduced auxin sensitivity suggested that Aux/IAA act as repressors and that their degradation is essential for a normal auxin response (Abel and Theologis, 1996; Hagen and Guilfoyle, 2002).

The identification of the auxin response element (AuxRE) in the promoter of several auxin-responsive genes including *Aux/IAA*s led to isolation of the *Arabidopsis AUXIN RESPONSE FACTOR 1 (ARF1)* transcription factor binding the AuxRE motif (Ulmasov *et al*., 1997). Further genetic and molecular studies have identified 23 *ARF* genes in *Arabidopsis* (Liscum *et al*., 2002). Several genetic reports have implicated various *ARF* genes in distinct developmental processes, including embryogenesis (*ARF5/MONOPTEROS* (Berleth and Jurgens 1993; Hardtke and Berleth., 1998) and *ARF17* (Mallory *et al*., 2005)), root development *ARF7*, *ARF19* (Wilmoth *et al*., 2005; Okushima *et al*., 2005), *ARF10* and *ARF16* (Mallory *et al*., 2005; Wang *et al*., 2005), flower development *ARF2* (Schruff *et al*., 2006), *ARF3* (Sessions *et al*., 1997), *ARF6* and *ARF8* (Ellis *et al*., 2005; Nagpal *et al*., 2005), and senescence *ARF1* and *ARF2* (Ellis *et al*., 2005). Due to some functional redundancy, several *arf* mutants lack obvious phenotypes, but exacerbated developmental defects are seen in several double mutant combinations (Okushima *et al*., 2005; Nagpal *et al*., 2005).

Obvious similarities in *arf* loss-of-function and *AUX/IAA* gain-of-function mutants, along with the observation that Aux/IAAs interacts with ARFs supported the model in which Aux/IAAs repress the transcriptional activity of ARFs through direct interaction.

The genetic identification of another auxin-resistant mutant *tir1* has provided breakthrough insights into the molecular mechanisms underlying auxin signaling. *TIR1* encodes the F box

protein and implicates the role of the ubiquitin-dependent proteolytic system SCF^{TIR1} (Skp1/Cullin/F-box protein Transport Inhibitor Response 1) in the perception and transduction of the auxin signal to the transcriptional program (Ruegger *et al*.,1998; Dharmasiri and Estelle, 2004; Tiwari *et al*., 2001; Dharmasiri *et al*., 2005; Leyser, 2006; Vanneste and Friml., 2009). The nuclear localized TIR1 F-box protein was found to act as the auxin receptor in the SCF^{TIR1} complex. At the presence of auxin TIR1 interacts with Aux/IAAs thereby promoting their ubiquitination and directing them for degradation by the proteasome complex. As a consequence ARF transcription factors are released from repression and activate the downstream transcriptional response (Gray *et al*., 2001; Zenser *et al*., 2001; Kepinski and Leyser, 2005).

The large number of *Aux/IAA*s (29 genes) and *ARF*s (23 genes) with a tissue-specific and developmentally specific expression raises the question how in the individual cells and plant organs specific auxin responses might be accomplished (Weijers and Jurgens.,2004). It has been proposed that certain combinations of AUX/IAAs and ARFs determine the response specificity (Hamann *et al*. 2002; Knox *et al*. 2003; Weijers *et al*. 2005). In the case of root development, the specific pair of IAA12/BDL and ARF5/MP was identified to determine the establishment of the root pole in early embryogenesis (Hamann *et al*. 2002). Besides the *BDL-MP* pair, *SOLITARY ROOT (SLR)/IAA14* acting together with *ARF7* and *ARF19* controls the earliest phases of lateral root initiation (LRI) and some other genes of the auxin signaling pathway (*SHY2/IAA3, AXR3/IAA17*, and *AXR2/IAA7*) were shown to be involved in different aspects of root growth (Leyser *et al*. 1996; Nagpal *et al*. 2000; Tian and Reed 1999), although their direct ARF counterparts are still unknown.

PIN auxin efflux carriers determine directionality of the polar auxin transport

To generate local auxin maxima and gradients crucial for organ initiation, organ formation and tropic responses (Vanneste and Friml 2009), the polar auxin transport machinery must, besides overall facilitating auxin flow through the plant body, regulate its directionality. The key components of the polar auxin transport machinery providing directionality of the auxin flow are PIN auxin efflux carriers (Galweiler *et al*., 1998; Chen *et al*., 1998; Luschnig *et al*., 1998; Muller *et al*., 1998; Utsuno *et al*., 1998; Friml *et al*., 2002a, 2002b; Blilou *et al*., 2005; Sauer *et al*., 2006; Scarpella *et al*., 2006; Petrasek *et al*., 2006; Wisniewska *et al*., 2006). The membrane localization of PINs is strictly linked to the direction of the transport meaning that they control the general trend of moving auxin (Muday *et al*., 2001; Wisniewska *et al*., 2006)

(Figure 3). In the root most PIN proteins are localized at the basal side of the vasculature and stele cells, such as PIN1, PIN3, PIN4 and PIN7 (Friml *et al*., 2002). In the root pericycle cells, PIN3 localizes also at the inner lateral side, whereas it has a symmetric localization in columella cells (reviewed in Feraru and Friml, 2008). PIN2 localizes at the apical cell side in root cap and epidermis cells, but it localizes also at the basal cell side of the cortex cell in the root meristem zone (Muller *et al*., 1998). Wisniewska and coworkers (2006) elegantly demonstrated that the PIN polar membrane localization determines the directions of the auxin stream. Using differences in PIN2 and PIN1 polarity as observed in root epidermal cells they demonstrated that the localization of PIN2 proteins at the upper side of epidermal cells correlated with their ability to facilitate upward auxin movement for the root gravitropic response. In contrast, basally localized PIN1 prevented auxin redistribution, which resulted in a root agravitropic behavior (Wisniewska *et al*., 2006).

An important feature of the PIN polar membrane localization is its developmental plasticity. For example, during lateral root organogenesis, when auxin maxima at the primordial tip are established, the polar localization of PIN1 gradually changes from anticlinal membranes towards the cell sides facing the future primordial tip. This rearrangement correlates with and is required for the auxin gradient formation during lateral root organogenesis (Benková *et al*., 2003).

Furthermore, the localization of PIN proteins is sensitive to environmental stimuli in response to which PIN might relocate to a different side of cells (Abas *et al*., 2006; Kleine-Vehn *et al*., 2010). For example, the direction of the light source and gravity triggers phototropic and gravitropic responses (Friml *et al*., 2002b; Abas *et al*., 2006; Kleine-Vehn *et al*., 2010; Ding *et al*., 2011). The PIN3 protein, after the gravi-stimulus, relocates to the lower side in the columela cells (Kleine-Vehn *et al*., 2010), where auxin is further transported, by a concerted action of AUX1 and PIN2, to the elongation zone (Abas *et al*., 2006; Kleine-Vehn *et al*., 2010). As auxin is pumped asymmetrically to the side of the root, it results in asymmetric growth and bending of the root in response to the gravitropic stimulus (Abas *et al*., 2006; Kleine-Vehn *et al*., 2010). Similarly, gravity-induced PIN3 polarization in the hypocotyl diverts the auxin flow to mediate the asymmetric distribution of auxin for gravitropic shoot bending (Rakusova *et al*., 2011). Ding and colleagues (2011) demonstrated that light regulates the subcellular distribution of PIN3, and that light-dependent changes in PIN3-GFP localization (depletion from the outer lateral membrane and accumulation at the inner

membrane, facing the vasculature) may redirect auxin fluxes during the phototropic bending of hypocotyls.

All these examples nicely demonstrate that a polar plasma membrane localization of PIN auxin efflux carriers determines the direction of the auxin flow in plant organs and during responses to environmental stimuli.

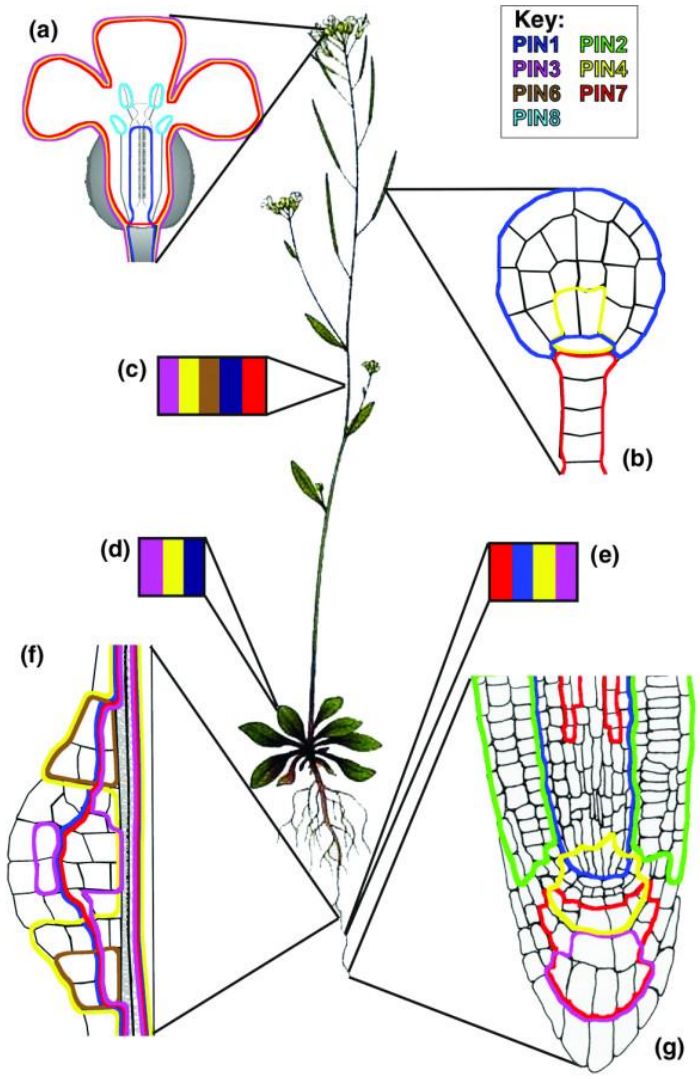

Figure 3. Expression map of *Arabidopsis thaliana PIN* genes compiled from both promoter activity data and protein localization. Each *PIN* gene expression domain is marked out by a colored line (see key in upper right corner. The organs depicted are **(a)** flower; **(b)** embryo (late globular stage); **(c)** stem; **(d)** rosette leaf; **(e)** mature part of the primary root; **(f)** lateral root primordium (stage 5); **(g)** root tip. Adapted from (Krecek *et al*., 2009).

PIN proteins cellular trafficking

PIN proteins are synthesized in the ER and transported through the Golgi apparatus (Mravec *et al*., 2009) and the *trans* Golgi network/early endosomes (TGN/EE) towards the membrane (Dettmer *et al*., 2006). Those newly synthesized PINs secreted from the TGN show a non-

polar plasma membrane localization, indicating that the establishment of PIN polarity requires endocytosis and polar recycling (Dhonukshe *et al*., 2008).

Although membrane located, PIN proteins are subjected to a constitutive recycling mechanism mediated through clathrin-dependent endocytosis (Dhonukshe *et al*., 2007; Kitakura *et al*., 2011) and ARF-GEF-dependent exocytosis (Geldner *et al*., 2001, 2003). Some important insights into the regulation of this trafficking were obtained by using different chemicals that affect protein recycling within the cell. For instance, in response to treatment of *Arabidopsis* roots with the fungal toxic brefeldin A (BFA), PIN1 proteins are reduced from the plasma membrane, creating clusters of endosomes – so-called BFA compartments (Geldner *et al*., 2001). The primary cellular target of BFA in terms of its effect on PIN1 localization is GNOM, an ARF guanine-nucleotide exchange factor (ARF-GEF) that regulates the formation of vesicles in membrane trafficking (Geldner *et al*., 2003). GNOM regulates PIN recycling to the basal side of cells, while apical targeting is GNOM-independent (Kleine-Vehn *et al*., 2008; Tanaka *et al*., 2009).

The functional role of the constitutive recycling mechanism of PINs remains still unclear (Kleine-Vehn *et al*., 2010). This constitutive trafficking might regulate the cellular auxin transport rates (Geldner *et al*., 2001), enable the rapid PIN polarity changes and allow the auxin flow to be quickly redirected in response to various developmental and environmental signals (Kleine-Vehn *et al*., 2008, 2010). For example, it has been shown that PIN3 trafficking events, including clathrin-mediated PIN3 endocytosis, ARF-GEF-mediated recycling and endosome-based translocation, underlie gravitropic root growth in gravity-responding columella cells (Kleine-Vehn *et al*., 2010).

The regulatory mechanisms controling PIN-dependent auxin transport

The regulation of PIN-dependent PAT occurs at multiple levels including control of PIN protein abundance (transcription, translation and silencing), PIN activity (post-translational modifications and degradation) and targeting (PIN abundance at the specific cell side) (reviewed in Vieten *et al*., 2007; Friml *et al*., 2003).

Transcriptional regulation of PIN genes

Members of the PIN protein family are considerably homologous and functionally redundant, as indicated by the increasingly severe phenotypes of the multiple *pin* mutants and the observations that they can complement each other in knockouts (Vienet *et al*., 2005; Blilou *et al*., 2005).

Vieten and coworkers (2005) demonstrated functionally distinct as well as overlapping roles of multiple *PIN* genes in embryonic and root development. During early embryo development PIN1 and PIN7 regulate auxin maxima establishment in the embryo, while PIN3 and PIN4 expressed at the globular and heart stage act together to specify the apical-basal axis of the embryo (Friml *et al*., 2003b). Noteworthy, in the *pin7* embryos, PIN4 was found ectopically expressed in the *PIN7* expression domain as early as the pre-globular stages, revealing that PIN family members might be functionally interchangeable and replace each other (Vieten *et al*.,2005).

A similar situation occurs in the root where *PIN1* became ectopically induced in the *PIN2* expression domain in cortical and epidermis cells of the *pin2* mutant. In case of the *pin1* mutant, *PIN2* and *PIN4* homologues were ectopically expressed in the endodermis and weakly in the stele (Vieten *et al*., 2005). The triple mutant of *pin3pin4pin7* showed an enhanced *PIN1* expression that was detected in the lateral-basal membranes of the endodermis, and ectopic *PIN2* was detected at the basal end of provascular cells that normally express *PIN3* and *PIN7*. Thus, defects in *pin* mutants can be masked by an ectopic activity of the remaining *PIN* genes and ectopic *PIN* expression in *pin* mutants can partially compensate the function of the missing *PIN* protein (Friml *et al*., 2003b; Blilou *et al*.,2005). Additionally, auxin regulates *PIN* gene expression in a tissue-specific manner through the AUX/IAA-dependent signaling pathway (Vieten *et al*., 2005).

Auxin feedback control of the PIN activity

Auxin transport and the directional polarization of auxin flow is under complex feedback control (Sachs., 1991; Tanaka *et al*., 2006). Auxin regulates transcription, turnover, cellular trafficking and localization of PIN proteins (Blilou *et al*., 2005; Leyser, 2005; Paciorek *et al*., 2005; Vieten *et al*., 2005).

The PIN protein recruitment into the clathrin pathway (Kitakura *et al*., 2011) was found to be inhibited by auxin itself, thus providing a mechanism by which auxin might promote its own efflux (Geldner *et al*., 2001; Robert *et al*., 2010).

Rapid auxin-induced inhibition of the clathrin-mediated endocytosis is independent on TIR1-like receptors, but dependent on the auxin binding protein ABP1 (Robert *et al*., 2010; Paciorek *et al*., 2005). The promoting effect of ABP1 on clathrin-dependent endocytosis could be diminished by auxin application, indicating that auxin binding to ABP1 negatively regulates this activity (Robert *et al*., 2010). ABP1 is involved in the regulation of endocytosis, but the underlying mechanism of how extracellular ABP1 could modulate the clathrin machinery remains still unclear.

Moreover, the steady-state levels of PIN auxin efflux carriers were found to be actively regulated by ubiquitin-mediated protein degradation. It has been found that the root gravitropic response is facilitated by differential degradation of PIN2, promoted at the upper side of the gravistimulated roots. On the upper side of the root, PIN2 was found in intracellular compartments as well as on the plasma membrane. Such a internalization of PIN2 appears to be controlled by proteasomic activity, because internalization and degradation of PIN2 was prevented by pretreatment with the proteasome inhibitor MG132 (Abas *et al*., 2006; Kleine-Vehn *et al*., 2008).

Mechanisms controling PIN membrane polarity establishment

Polar membrane localization of PIN proteins was found to be dependent on their phosphorylation status regulated by protein kinases PINOID (PID) (Friml *et al*., 2004) and protein phosphatases (PP2A) (Michniewicz *et al*., 2007). Detail analyses of the *PID* gene reported that *PID* encodes a protein-serine/threonine kinase (Christensen *et al*., 2000; Benjamins *et al*., 2001). The PINOID loss-of-function mutant induces an apical-to-basal shift in PIN1 polar targeting at the inflorescence apex (Friml *et al*., 2004). PID gain-of-function (Friml *et al*., 2004) and reduced PP2A function (Michniewicz *et al*., 2007) lead to an increased PIN phosphorylation and as a consequence a shift of PIN proteins from basal-to-apical localization (Friml *et al*., 2004; Michniewicz *et al*., 2007). Thus, PP2A phosphatase and PID kinase act on the same auxin transport-related developmental processes, by antagonistically regulating PIN polar targeting (Michniewicz *et al*., 2007).

Some of the PID-dependent phosphorylation sites in PIN proteins have been recently identified. The evolutionary conserved TPRXS(S/N) motif found in the cytosolic loop of PIN1 proteins is phosphorylated at its central serine residues by PID (Huang *et al*., 2010). Mutations in the PIN phosphorylation sites in one single serine (Ser231, Ser252 and Ser290)

residue to alanine (Ala) disable the PIN phosphorylation at these residues with resulting in PIN basal targeting, while the substitutions mimicking constitutive phosphorylation induce an apical PIN1 localization (Huang *et al*., 2010; Zhang *et al*., 2010).

Besides the PIN protein phosphorylation status, the PIN apical-basal polar localization was found to be influenced by cytosolic Ca^{2+} levels and by inositol trisphosphate (InsP3). Pharmacological and genetic increases in InsP3 or Ca^{2+} levels lead to PIN1 proteins being targeted to the basal cell side, whereas inhibited cytosolic Ca^{2+} signaling promoted apical PIN targeting (Zhang *et al*., 2011).

The localized cargos distribution is ensured by interactions between cell wall, plasma membrane, plasma membrane localized proteins and components of the cytoskeleton (Heisler *et al*., 2010; Feraru *et al*., 2011). Some experiments suggest that the polar distribution of PIN auxin transporters in plant cells is maintained by connections between the cell wall and the polar domains of the plasma membrane (Feraru *et al*., 2011). In this sense, a role for cellulose has been suggested in regulating cell polarity, as the genetic and pharmacological manipulation of the synthesis of cellulose resulted in PIN1-HA polarity defects (Feraru *et al*., 2011). Furthermore, mechanical stimuli leading to rearrangement of the microtubule cytoskeleton, which correlates with PIN1 membrane reorientation, were proposed to participate in PIN polarity control (Heisler *et al*., 2010).

Hormonal pathways converge on the regulation of polar auxin transport in the root

Plant growth and development is controlled by a complex hormonal regulatory network. Recent studies clearly demonstrated that hormones do not only act in an inert linear pathway, but are interconnected by a complex network of interactions and feedback circuits, which determine the final outcome of the individual hormone actions (Figure 4).

Auxin and its graded distribution have emerged as one of the key factors involved in the regulation of many aspects of plant growth and development. Thus, components regulating polar auxin transport might be an important point of convergence with other hormonal pathways. Indeed, recent research reveals that multiple hormones through modulation of the polar auxin transport activity control different developmental processes.

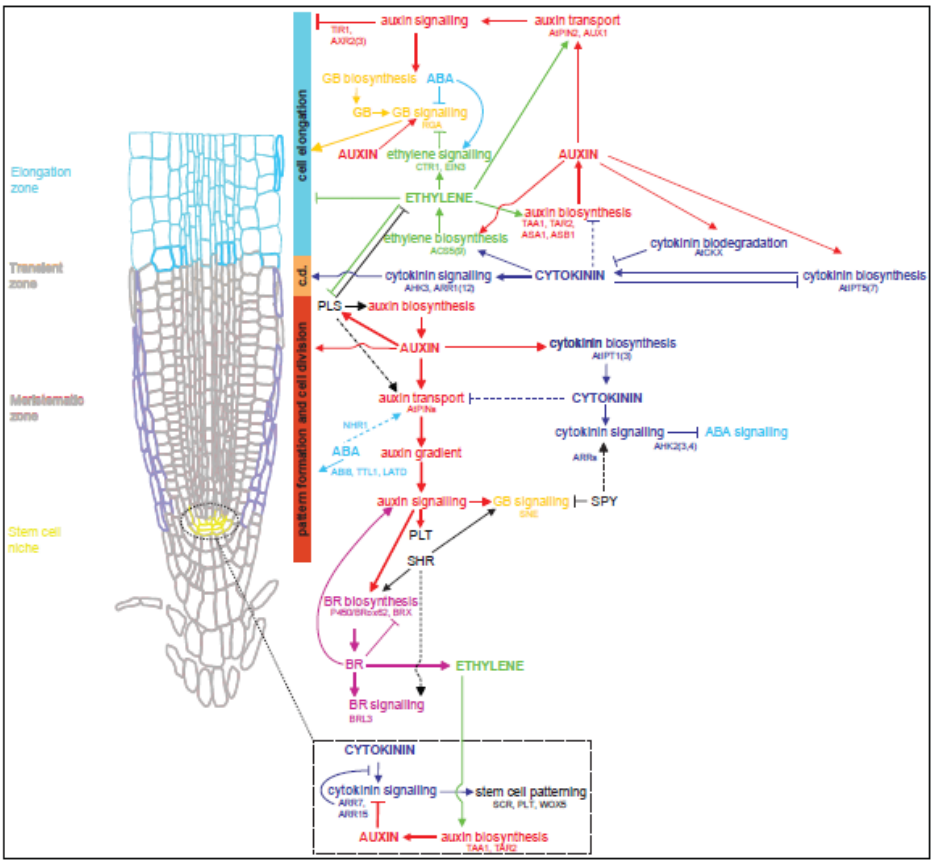

Figure 4. Scheme of the hormonal cross-talk involved in the regulation of the root apical meristem growth and development. Selected regulators of the cross-talk are highlighted. Dashed lines correspond to not completely clear or mostly indirect regulations. c.d. is transition zone where differentiation starts. Adapted from (Benkova and Hejatko 2009).

Cytokinin, gibberellin and brassinosteroids regulate root apical meristem activity through modulation of auxin transport

In the root meristem (RM) of *Arabidopsis thaliana*, two hormones cytokinin (CK) and auxin, are known to act antagonistically in controling meristem activities. Cytokinin 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 maintaining root meristem size and for ensuring root growth (Dello Ioio *et al*., 2007; Ruzicka *et al*., 2009, reviewed in Moubayidin *et al*., 2009). Cytokinin modulates the auxin pathway by affecting the expression of its regulatory component. Genetic and molecular analyses showed that the CK signal, perceived by the AHK3 receptor kinase and transduced by ARR1 and ARR12 response regulators, activates the transcription of auxin repressor *SHY2/IAA3*, resulting in the negative regulation of the transcription of PIN auxin efflux carriers (Dello Ioio *et al*., 2008; Ruzicka *et al*., 2009). Besides repression of *PIN1* transcription, the expression of other PIN family members is also affected by CK. For example, *PIN4* and *PIN3*

are downregulated, while *PIN2* expression is CK insensitive. In contrast, *PIN7* was clearly upregulated after CK treatments (Ruzicka *et al*., 2009).

Besides this transcription based way of altering auxin activity and distribution, CK was found to act in a transcription-independent manner. Cytokinin modulates the endocytic trafficking of PIN1 and redirects this membrane protein for lytic degradation into the vacuoles. In this case, CK activity is transduced through the AHK4 receptor, however the mechanism by which CK interferes with PIN1 endocytotic trafficking remains to be understood (Marhavý *et al*., 2011).

Gibberellins (GAs), in analogy to auxin, positively control root growth (Ubeda-Tomas *et al*., 2008) and meristem size by sustaining cell division (Achard *et al*., 2009; Ubeda-Tomas *et al*., 2008; Moubayidin *et al*., 2010). Moubayidin (2010) described that high levels of GAs in young root meristems antagonize the CK effect on cell differentiation by suppressing the activity of ARR1, what might interfere with the expression of the PIN genes and auxin distribution in the RM. Accordingly, in the inflorescence stems of *ga1*, *gid1ac* and *gai-1* mutants, PIN1 accumulation is reduced when compared with the wild type (Willige *et al*., 2011). However, similarly to CK, GAs also use alternative pathways to modulate auxin distribution. In roots with limited GA signaling, the amount of the membrane PIN proteins was dramatically reduced due to enhanced lytic degradation. Noteworthy, PIN protein sensitivity to GAs does not necessarily correlate with GA-driven transcriptional regulation (Willige *et al.,* 2011), indicating that GAs, similarly to CK, may control *PIN* expression using alternative pathways.

Besides GAs, also brassinosteroids (BRs) provide an important input in the control of the acropetal polar auxin transport (Bao *et al*., 2004), and the expression of auxin efflux carriers, such as *PIN1* and *PIN2* (Li *et al*., 2005). BR and auxin synergistically regulate the expression of several auxin responsive genes (Bao *et al*., 2004; Mouchel *et al*., 2006). Closer insight into the synergistic regulation of those hormones, was provided by analyses of the BREVIX RADIX (BRX) transcription factor (Mouchel *et al*., 2006), involved in the regulation of auxin activity in the RM. BRX is an auxin-responsive target of ARF5(MP), and transiently enhances *PIN3* expression to promote meristem growth in young roots. At later stages, CK induction of SHY2 in the vascular transition zone restricts *BRX* expression to downregulate *PIN3* and thus limit meristem growth (Sacchi *et al*., 2010).

Furthermore, BRs enhance acropetal polar auxin transport (PAT) and the expression of *PIN1* and *PIN2* is upregulated (Bao *et al*., 2004, Li *et al*., 2005). In contrast, short-term treatments with BRs do not influence the response of the *PIN* genes, indicating that modulation of their expression is rather a result of the long-term adaptation to increased levels of the hormone (Hacham *et al*., 2011). Brassinosteroids do not seem to exert a strong impact on the auxin metabolism, but they have an effect in the transcriptional regulation of auxin responsive genes (Nakamura *et al*., 2003; Bao *et al*., 2004; Mouchel *et al*., 2004, 2006).

Jasmonate, abscisic acid and strigolactone converge on auxin in the root apical meristem activity regulation

High concentrations of jasmonate (JA) inhibit primary root growth (Sun *et al*., 2009, Chen *et al*., 2011). Increasing JA activity significantly reduces root meristem size activity Chen *et al*., 2011), indicating that JA antagonizes auxin in the regulation of the root apical meristem (RAM). Recent work bring us insights into the mechanism of JA - auxin crosstalk. Jasmonate, through the CORONATINE INSENSITIVE (COI1) receptor and MYC2 transcription factor mediated signaling (Boter *et al*., 2004; Lorenzo *et al*., 2004, Xie *et al*., 1998; Xu *et al*., 2002), directly represses the expression of the *PLETHORA1 (PLT1)* and *(PLT2)* genes (Aida *et al*., 2004), encoding transcription factors acting downstream of auxin. Thereby JA can interfere with the auxin response and inhibit the root meristem growth (Chen *et al*., 2011). Besides the JA activity on *PLT* transcription, JA, through modulation of *PIN* expression, might interfere with auxin distribution (Chen *et al*., 2011; Sun *et al*., 2009). Jasmonate enhances the expression of *PIN1* and *PIN2* in the wild type, while this induction was reduced in *jdl1/asa1-1* roots (Sun *et al*., 2009). After long-term JA treatment, PIN1 and PIN2 protein levels are reduced and an even stronger reduction effect is seen in the *jdl1/asa1-1* roots (Sun *et al*., 2009). Noteworthy, discrepancies between responses of the individual *PIN*s to JA at the transcript and protein level hint on multiple regulatory pathways underlying JA interaction with auxin distribution (Chen *et al* 2011, Sun *et al* 2009).

Recently the ABI4 ABA-regulated AP2 domain transcription factor was proposed to integrate ABA with CK pathway in regulation of the PAT. Both cytokinin and ABA were found to regulate expression of *ABI4* , which in turn represses *PIN1* expression. Accordingly, the *abi4* mutant and ABI4 overexpressor exhibits enhanced and reduced root acropetal auxin transport, respectively (Shkolnik-Inbar & Bar-Zvi 2011).

Newly identified plant hormone strigolactone (Gomez-Roland *et al*., 2008) play an important role in regulation of the primary root growth. Treatment with synthetic strigolactone analogue (GR24) increases the RM size and overall root growth, but reduces size of the meristematic cell(Ruyter-Spira *et al*., 2011). To which extent this strigolactone effect on the RAM activity involves the observed modulation of the expression of the *PIN* auxin carriers remains to be investigated (Ruyter-Spira *et al* 2011).

Ethylene - Auxin interaction control*s* root cell elongation

In higher plants ethylene represents one of the key regulators of root growth. Ethylene has been shown to mediate its growth inhibitory effects on root elongation and cell expansion in *Arabidopsis* (Cary *et al*., 1995; Stepanova *et al*.,2005; Swarup *et al*., 2007). Ethylene and auxin synergistic effects in the regulation of root growth (Pickett *et al*., 1990; Rahman *et al*., 2001) suggest that these two signaling pathways also interact at the molecular level (Ruzicka *et al*., 2007; Swarup *et al*., 2007).

Ethylene was shown to stimulate auxin biosynthesis and modulate the capacity of auxin transport via the transcriptional regulation of auxin transport components (Ruzicka *et al*., 2007; Swarup *et al*., 2007). In the primary root, auxin efflux carriers such as PIN1, PIN2, PIN4, and the influx carrier AUX1, are transcriptionally upregulated in response to ethylene (Ruzicka *et al*., 2007). This regulatory mechanism is important for the basipetal auxin transport towards the elongation zone, where it activates a local auxin response inhibiting cell elongation (Ruzicka *et al*., 2007; Swarup *et al*., 2007; Lewis *et al*., 2011).

Hormonal regulation of the lateral root organogenesis

The root system in higher plants is a complex organ composed of primary, adventitious and lateral roots (Hochholdinger *et al*., 2004; Osmont *et al*., 2007). It is a developmentally dynamic structure influenced by many environmental inputs, like nutrition availability, soil aeration, humidity, salinity or plant hormones (Vert and Chory, 2012). The root system is crucial for securely anchoring plants into the soil, as well as for the absorption of nutrients and water uptake. In the last decade, lateral roots (LRs) at all their developmental stages (LR initiation, organogenesis, development and emergence) have become an important model system. In *Arabidopsis thaliana* lateral root primordia (LRP) initiation of LR occurs away from the RAM in the differentiation zone of the root called the developmental window (Dubrovsky *et al*., 2006). In this zone the pericycle cells are not actively dividing (Malamy

and Benfey 1997; Beeckman *et al*. 2001; Dubrovsky *et al*., 2011), but some pericycle cells located opposite to the xylem poles in the developmental window acquire the founder cell (FC) status (Dolan *et al*., 1993; Dubrovsky *et al*., 2006; 2008; 2011; 2012; De Smet *et al*., 2006; De Rybel *et al*., 2010). The specification of the FC identity correlates with the elevated auxin response (De Rybel *et al*., 2010). This local auxin responsiveness oscillates with peaks of expression of the *DR5* auxin reporter and correlates with the formation of a consecutive LR (De Smet *et al*., 2007; De Rybel *et al*., 2010; Moreno-Risueno *et al*., 2010). The pericycle cells, once stimulated, divide asymmetrically to form short initial cells bordered by longer cells, that might further divide by several coordinated cell divisions to give rise to a LRP (Malamy and Benfey, 1997; Swarup *et al*., 2008). In the LRP an auxin gradient is gradually established depending on the PIN auxin efflux carriers (Malamy and Benfey 1997, Benkova *et al*., 2003). Changes in PIN1 polar localization, that involve ARF-GEF-GNOM-dependent transcytosis (Kleine-Vehn *et al*., 2008), are required for the establishment of the auxin gradient, which will ensure proper LR development (Benkova *et al*., 2003). Brefeldin A (BFA) inhibition of ARF-GEFs leads to the recruitment of basal PIN cargoes into opposite membranes and, consequently, the loss of auxin maxima and defective LRP organogenesis (Benkova *et al*., 2003; Geldner *et al*., 2004; Kleine-Vehn *et al*., 2008).

Except for auxin, also several other hormones are involved in LRP development and organogenesis (De Smet *et al*., 2006; Mouchel *et al*., 2006, Laplaze *et al*., 2007, Ivanchenko *et al*., 2008, Ruyter-Spira *et al*., 2011). Cytokinins were described to have an antagonistic role to auxin in plant growth and development (Laplaze *et al*., 2007). The exogenous application or elevated endogenous levels of CK via increased expression of *IPT* led to a significant reduction of lateral root initiation (LRI) and decreased root growth (Laplaze *et al*., 2007; Ruzicka *et al*., 2009, Kuderova *et al*., 2008). In contrast, overexpression of cytokinin oxidase (CKX) led to 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, while mutations in type-A ARRs decrease the number of LRs (Mason *et al*., 2005, To *et al*., 2008). Exogenous application of CK also affects the expression of several *PIN* genes (Ruzicka *et al*., 2007). Indeed, 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, that is required for normal LRP patterning (Laplaze *et al*., 2007, Marhavy *et al*., 2011).

A high concentration of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), or an enhanced ethylene synthesis or signaling through the *eto1-1* and *ctr1-1* mutations, strongly inhibits the initiation of LRP (Ivanchenko *et al*., 2008, Negi *et al*. 2008). Contradictory, treatment with higher doses of ACC promotes the emergence of existing LRP, an effect that is also observed in the *eto1* mutant (Ivanchenko *et al*., 2008). On other hand, low doses of ethylene stimulates auxin biosynthesis, which promotes LRI, that involves Aux/IAA and ARF proteins (Ivanchenko *et al*., 2008). Ethylene seems to inhibit LR development by the local modulation of auxin-transport protein abundance needed to form local auxin maxima that drive lateral root formation from prebranch sites as reported (Lewis *et al*., 2011).

Strigolactone application (GR24) suppresses LRP development, and reduces PIN1-GFP in the LRP (Ruyter-Spira *et al*., 2011). This could lead to the disruption of PIN1 polar localization in LRP, which will inhibit the optimal auxin level necessary for creating auxin maxima for proper LRP development (Ruyter-Spira *et al*., 2011). Similarly to CKs, strigolactones are able to modulate local auxin levels, and the net result of strigolactone action is dependent on the auxin status of the plant.

Abscisic acid (ABA) plays a role on the root branching as a negative regulator of LR emergence (reviewed in De Smet *et al*. 2006). Detailed analyses of *abi4* mutants show its involvement in the regulation of LRP development, where the number of LRs is increased at later developmental stages, while the *ABI4*-overexpressing plants exhibit reduced LR formation (Shkolnik-Inbar and Bar-Zvi1, 2011). Furthermore, analyses of *PIN1:PIN1-GFP* protein expression levels in *abi4-1* mutants were higher than in the *ABI4*-overexpressor line. Application of exogenous ABA and CK markedly reduced PIN1 protein levels in the roots of wild-type plants and to a lesser extent in *abi4-1* mutant plants. Alltogether it seems that CK and ABA through *ABI4* repress *PIN1* expression and thereby modulate auxin transport required for proper LR organogenesis (Shkolnik-Inbar and Bar-Zvi, 2011).

Brassinosteroids act synergistically with auxin, and are required for LRP initiation and development (Bao *et al*., 2004). IAA-induced elongation of LRs is suppressed by either BR biosynthesis inhibitor brassinazole (Brz) treatment or a *dwf4* mutation, and this suppression can be rescued by bioactive BR, brassinolide (BL). *DWARF4* (*DWF4*) encodes a C-22 hydroxylase crucial for BR biosynthesis and for the feedback control of endogenous BR

levels. Moreover, PEO-IAA inhibited the IAA-stimulated induction of LR growth, and this inhibition is partially rescued by BL (Yoshimitsu., *et al* 2011). Brassinosteroids promote acropetal auxin transport in the root, what might suggest that BRs promote LRI by increasing acropetal auxin transport (Bao *et al*., 2004).

It has been shown that JA together with auxin modulates the LR development in *Arabidopsis thaliana* (Grunewald *et al*., 2009; Sun *et al*., 2009). Exogenous application of JA stimulates lateral root formation in wild type, but inhibits LR formation in *asa1-1* (Sun *et al*., 2009). Based on a genetic screen, JA response mutant for LR formation, *jdl1/asa1-1*, was identified. By using *CYCB1;1:GUS* as a reporter for cell division, it has been shown that while methyl jasmonate (MeJA) promotes LR formation in the wild type, it represses LRP initiation in *jdl1/asa1-1*, meanwhile reducing cell proliferation and cell elongation in primary root (Sun *et al*., 2009, Chen *et al*., 2011).

The role of GAs in LR formation in *Arabidopsis* has not been deeply studied, but Bidadi *et al*., 2009 demonstrate that external application of GA to the shoot has a positive effect on primary root elongation as well as on increasing the number of LRs (Bidadi *et al*., 2010). Furthermore, the importance of GA in the regulation of LR development was demonstrated by transgenic poplar, as this species exhibits increased LR proliferation, an effect that can be reversed by exogenous GA treatment (Gou *et al* ., 2010).

In last decade our knowledge about hormonal interaction during the different developmental processes made strong progress. Current view on the existing hormonal networks indicates that each ensure an important regulatory properties throughout the plant development. Indeed there are common interaction modules that are adopted by the different hormonal networks regardless of the developmental context. For example, polar auxin transport is significantly controlled by all hormones on transcriptional as well as on post-transcriptional level.
Future research on hormonal network models should still be conducted, requiring both the dissection of direct interactions occurring in a cell autonomous manner and those processes involving communication between cells.

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Chapter 2.

Cytokinin modulates endocytic trafficking of PIN1 auxin efflux carrier to control plant organogenesis

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PM and EB initiated the project and designed most of the experiments, PM carried out most of the experiments. AB and AA performed LR- analyses. LA performed the western blot. JD performed qPCR. HK provide seeds. PM and PJ provide Tobacco VBI-0 cells. JF and JKV discussed the experimental procedures. All authors analysed and discussed the data; PM and EB wrote the manuscript and all authors saw and commented on the manuscript.

Cytokinin modulates endocytic trafficking of PIN1 auxin efflux carrier to control plant organogenesis

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Cytokinin is an important regulator of plant growth and development. In *Arabidopsis thaliana*, the two-component phosphorelay mediated through a family of histidine kinases and response regulators is recognized as the principal cytokinin signal transduction mechanism activating the complex transcriptional response to control various developmental processes. Here, we identified an alternative mode of cytokinin action that uses endocytic trafficking as a means to direct plant organogenesis. This activity occurs downstream of known cytokinin receptors, but through a branch of the cytokinin signaling pathway that does not involve transcriptional regulation. We show that cytokinin regulates endocytic recycling of the auxin efflux carrier PINFORMED1 (PIN1) by redirecting it for lytic degradation in vacuoles. Stimulation of the lytic PIN1 degradation is not a default effect for general downregulation of proteins from plasma membranes, but a specific mechanism to rapidly modulate the auxin distribution in cytokinin-mediated developmental processes.

INTRODUCTION

Cytokinin is one of the key plant growth regulators that controls many developmental processes, including branching (Ongaro and Leyser, 2008), root growth (Dello Ioio *et al*., 2008), establishment of root pole during early embryogenesis (Muller and Sheen, 2008), shoot apical meristem maintenance (Zhao *et al*., 2010), and lateral root (LR) organogenesis (Laplaze *et al*., 2007). Over the past decades, molecular components and signal transduction mechanism of the cytokinin pathway have been disclosed. Cytokinin signal transduction is based on the two-component phosphorelay mechanism. In *Arabidopsis*, cytokinin receptors from the histidine kinase family activate the histidine phosphotransfer proteins that transduce signals towards the B-type response regulators in the nucleus. This transcriptional response is responsible for controlling a variety of developmental processes (Hwang and Sheen, 2001).

An important part of the cytokinin-mediated regulation of development involves an interaction with the auxin pathway. A specific developmental output is ensured by the crosstalk between these two signaling pathways. Previous work has revealed that the communication primarily occurs at the transcriptional regulation level (Dello Ioio *et al*., 2008; Muller and Sheen, 2008; Zhao *et al*., 2010). Here, we identify a different mode of cytokinin action that uses endocytic trafficking as a means to modulate the auxin activity and to direct plant organogenesis. This cytokinin activity requires cytokinin receptors but does not involve transcriptional regulation. We show that cytokinin regulates recycling of the auxin efflux carrier PIN1 (Galweiler *et al*., 1998) to the plasma membrane by redirecting it for lytic degradation in vacuoles. This rapid, nontranscriptional, regulation of the PIN1 abundance enables a precise control of auxin fluxes and distribution during LR organogenesis and might also contribute to other cytokinin-mediated developmental regulations, such as root meristem differentiation.

RESULTS

Cytokinin rapidly reduces PIN1 at plasma membranes during LR organogenesis

To follow the development of lateral root primordia (LRP) and to monitor the impact of hormonal and genetic manipulations on the progress of LRP through defined developmental stages, we have established a real-time *in vivo* analysis. Within 8 hours, LRP of untreated seedlings typically underwent several rounds of anticlinal and periclinal divisions, progressing

from the early first-to-second developmental stage (Malamy and Benfey, 1997) (Figure 1A; see Figure S1A). As expected, treatment with cytokinin repressed the LRP development and no additional divisions were observed (Figure 1B; Figure S1B). In contrast, auxin promoted cell divisions that occurred during the LRP organogenesis (Figure S1C) in agreement with its stimulatory function.

LRP organogenesis has been shown to strictly depend on a gradient of auxin distribution that elicits the relevant downstream auxin signaling events which are decisive for LRP organogenesis. Perturbations in either auxin distribution or signaling lead to severe defects in LRP formation (Benkova *et al*., 2003; Dharmasiri *et al*., 2005; Vanneste *et al*., 2005). Previous work has revealed that normal LRP organogenesis correlates with *DR5* auxin reporter expression maxima at the primordia tip (Benkova *et al*., 2003) In LRP treated with cytokinin, the expression pattern of *DR5* was dramatically changed, with no response maximum at the primordia tips (Figure S1D). Real-time monitoring of *DR5::RFP* expression revealed that auxin maxima decreased dramatically within 12 h of treatment (Figure S1E). These results suggested that cytokinin might interfere with auxin-related regulations, such as auxin signaling or distribution.

PIN1 has been identified as one of the principal auxin efflux carriers controlling the auxin distribution during LRP organogenesis. PIN1 loss-of-function results in defective and often arrested LRP (Benkova *et al*., 2003). To investigate the effect of cytokinin on PIN1, PIN1-GFP was monitored in cytokinin-treated LRP. The active cytokinin derivatives N^6-benzyladenine (BA) and zeatin (ZA) rapidly decreased the PIN1-GFP signal on the plasma membrane in a dose-dependent manner (Figure 1C; Figure S1F; Figures S1I and S1J). Also the endogenous PIN1 levels were significantly reduced in protein extracts from cytokinin-treated wild-type (Col-0) roots (Figure 1E; representative Western blot Figure S2C). Within 1.5 h of BA treatment, the membrane PIN1-GFP signal was reduced by 45-50% and almost completely absent within 5 h (Figure 2A). These results indicate that, besides the previously shown transcriptional regulation (Dello Ioio *et al*., 2008; Ruzicka *et al*., 2009), an additional mode of cytokinin action implying a rapid modulation of PIN1 protein levels might be involved in the cytokinin-regulated LRP organogenesis.

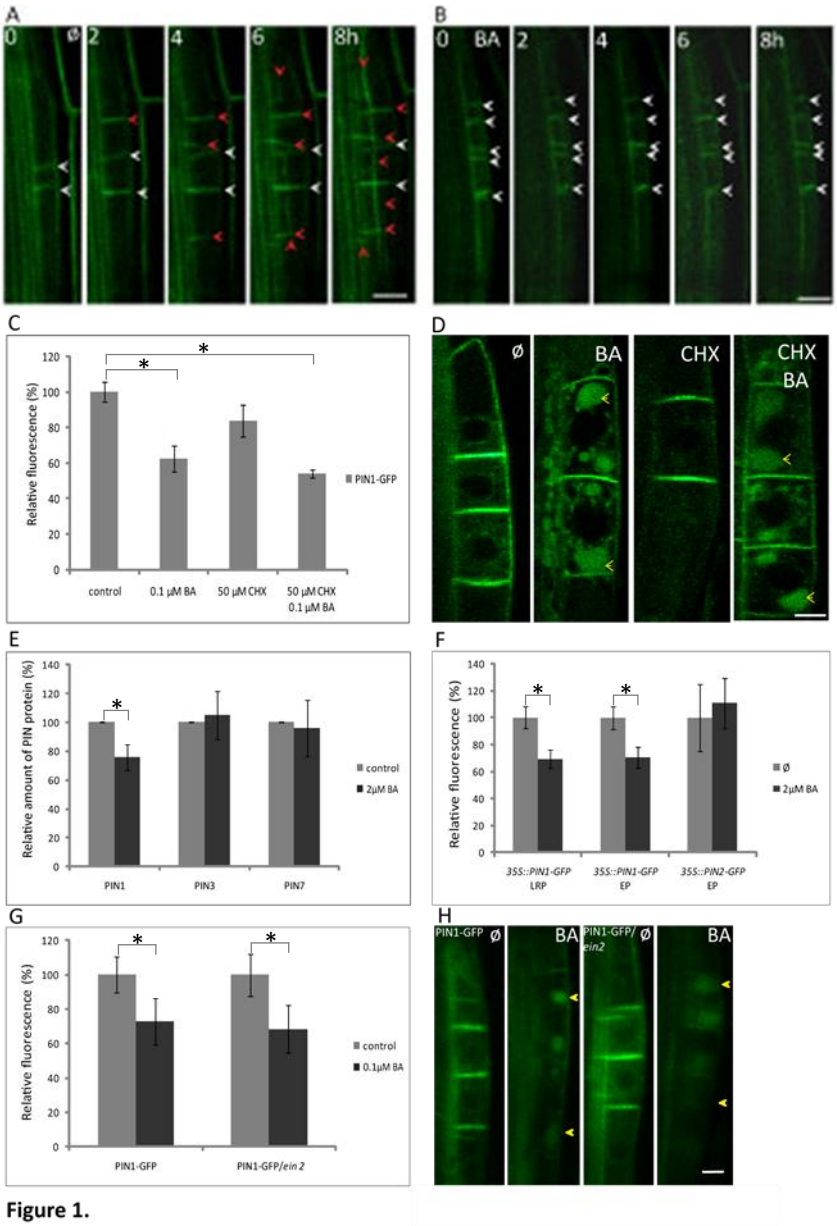

Figure 1.

Figure 1. Cytokinin inhibits LRP development and rapidly depletes PIN1 from the plasma membranes.
(A and B) Real-time monitoring of LRP development on control medium (A), and in the presence of 0.1 µM BA (B). WAVE138-YFP was used to visualize cell membranes (Geldner *et al*., 2009). White arrows indicate anticlinal divisions in the early initiation stage of the LRP at time 0; red arrows mark new cell divisions. Scale bar, 20 µm. (C and D) Membrane PIN1-GFP signals decrease after BA or simultaneous BA and CHX treatment, but not with CHX alone (*$p<0.05$, n=10 LRP). Yellow arrows indicate vacuoles with GFP accumulation. Scale bar, 5 µm. (E) PIN1, but not PIN3 and PIN7 decreases in protein extract from BA-treated wild-type roots (* $p<0.01$, n=7). (F) PIN1-GFP, but not PIN2-GFP, expressed under the control of the *35S* promoter is down-regulated after BA treatment in both LRP and root epidermal cells (*$p<0.05$, n=10). (G,H) BA stimulates the PIN1-GFP degradation in the *ein2* mutant (*$p<0.05$, n=10 LRP). The PIN-GFP membrane signal was measured in stage-I LRP (C,F,G) and root epidermal cell (EP) 1.5 h after BA treatment (F). Error bars mark standard error of the mean. See also Figure S1.

Cytokinin downregulates PIN1 levels at the plasma membrane independently of transcription

To determine whether the PIN1 decrease was either due to transcriptional or to posttranscriptional regulation, we uncoupled *PIN1* from its natural transcriptional control by using the *35S* promoter. In the *35S::PIN1-GFP* line the cytokinin had the same effect on PIN1-GFP in LRP as that observed with the endogenous *PIN1* promoter in *PIN1::PIN1-GFP* (Figure 1F). In contrast, *35S* promoter-driven expression of *PIN2* was not affected by the cytokinin treatment, demonstrating that cytokinin did not interfere with the *35S* promoter activity itself (Figure 1F). Furthermore, the PIN1-GFP membrane signal was monitored in the presence of cycloheximide (CHX), an inhibitor of protein biosynthesis. Previously, incubation of roots in 50 µM CHX had been shown to reduce the ^{35}S-labeled methionine incorporation into proteins to below 10% of the control value (Geldner *et al*., 2001). Whereas CHX led to decrease of the PIN1-GFP membrane signal by approximately 15% in 1.5 h, the cytokinin treatment resulted in a more than 40% reduction during the same time (Figures 1C and 1D). Simultaneous application of cytokinin and CHX did not interfere with the decrease in PIN1-GFP (Figures 1C and 1D). Next we tested inhibitor of transcription cordycepin (COR)(Holtorf *et al*., 1999). Pre-treatment with 400 □M COR for 30 min fully prevented the BA-induced upregulation of the *ARABIDOPSIS RESPONSE REGULATOR15* (*ARR15*) expression and reduced the expression of *ARR3* and *ARR5* by 75% (Figure S1G). Under these conditions of strongly diminished transcription, PIN1-GFP plasma membrane signal dropped by approximately 15% in 1.5 h after COR treatment alone, but the cytokinin-mediated downregulation of PIN1 was not affected in presence of COR (Figure S1H). These findings suggest that the cytokinin effect on PIN1 abundance does not depend on transcription and new protein biosynthesis.

The plant hormone ethylene has been shown to accumulate in response to cytokinin and to execute some of the cytokinin functions in plant development (Cary *et al*., 1995). Modulation of the ethylene level or response by using either the ethylene precursor 1-aminocyclopropane-1-carboxylate (ACC), an ethylene biosynthesis inhibitor 2-aminoethoxyvinylglycin (AVG), or the *ein2* (*ethylene insensitive2*) mutant defective in ethylene signaling (Roman *et al*., 1995), did not affect the cytokinin-induced PIN1 down-regulation (Figures 1G and 1H; Figures S1I and S1J). Thus, the posttranscriptional regulation of PIN1 by cytokinin does not involve ethylene biosynthesis or signaling. This observation is also consistent with cytokinin regulation being independent of ethylene for LR organogenesis (Laplaze *et al*., 2007).

Altogether, our results reveal a mode of cytokinin activity that, independently of transcription, regulates PIN1 levels at the plasma membrane, presumably by stimulation of PIN1 degradation. This direct method of control might modulate the PIN1 abundance at the plasma membrane more quickly than the transcriptional regulation and, consequently, rapidly influence the auxin distribution, critical for the LRP organogenesis.

Cytokinin affects stability of membrane proteins in protein specific manner

To analyze whether cytokinin has a general effect on plasma membrane protein turnover, we examined the cytokinin sensitivity of several membrane proteins in LRP cells. Neither PIN7, another member of the PIN auxin efflux carrier family (Friml *et al*., 2003), nor AUX1, an auxin influx carrier (Bennett *et al*., 1996) were influenced by cytokinin. PIN7-GFP typically remained unchanged for the first 2 hours after cytokinin application and, from 3 hours on, the signal gradually increased (Figure 2A), most likely following the onset of the cytokinin-mediated transcriptional stimulation, as previously described for *PIN7* (Ruzicka *et al*., 2009). Although the PIN3-GFP plasma membrane signal was reduced upon cytokinin treatments, the kinetics of the signal decrease were slower than those of PIN1 (Figure 2A). Western blot analysis of endogenous PIN proteins in membrane protein extracts from wild-type roots confirmed that PIN proteins differed in their sensitivity to cytokinin and that cytokinin strongly enhanced primarily the PIN1 depletion (Figure 1E; and representative Western blot Figure S2C). Similarly, immunolocalization of PIN1 and PIN2 in the root meristem treated for 1.5 h with cytokinin revealed a strong reduction in membrane PIN1 in the endodermis, whereas PIN2 in epidermis remained unaffected (Figures S2A and S2B). As *PIN1* and *PIN2* are not normally expressed in the same cells, we compared the cytokinin effects on PIN1 and PIN2 proteins by expressing them both under the *PIN2* promoter. When expressed in root epidermal cells, PIN1-GFP was downregulated, in contrast to PIN2-GFP that was cytokinin insensitive (Figures 2B and 2C). To further test whether differential control of PIN degradation is determined by cell type, PIN cytokinin sensitivity in cultured cells of tobacco (*Nicotiana tabacum* Virginia Bright Italia [VBI-0]) was examined. Accordingly, in the stably-transformed tobacco cell line VBI-0, PIN1-GFP, but not PIN7-GFP, was reduced after cytokinin treatment (Figures S2D-S2F). Our data demonstrate that cytokinin effect on the stability of membrane proteins has a pronounced protein specificity and that it is not a default mechanism for general depletion of proteins from membranes. Furthermore, we show that

PIN1 is responsive to cytokinin treatments in different cell types, including LRP, root stele/epidermal cells and suspension culture cells.

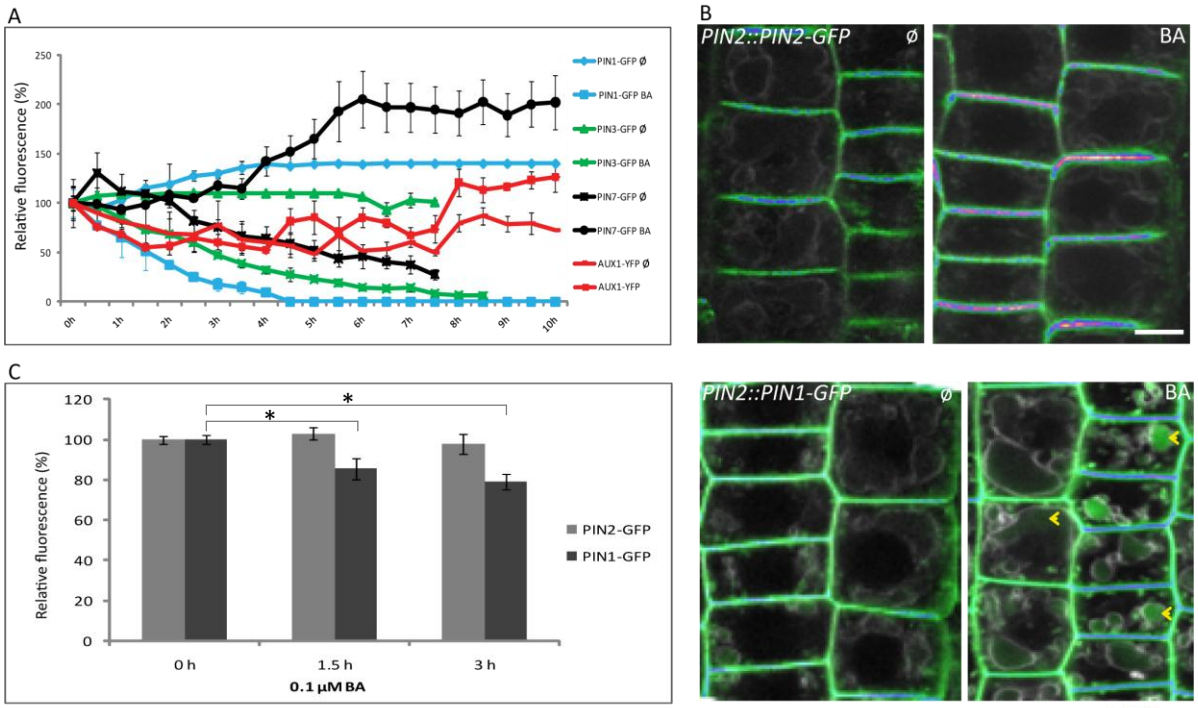

Figure 2.

Figure 2. PIN1 exhibits high sensitivity to cytokinin.
(A) Real-time monitoring of membrane proteins response to BA treatment. PIN-GFP and AUX1-YFP plasma membrane signals were measured in stage-I LRP 1.5 h after 0.1 µM BA treatment (n=10 LRP). (B and C) PIN1-GFP, but not PIN2-GFP, decreases after 0.1 µM BA treatment in root epidermal cells (*p<0.05, n=10 roots, five cells per root). Yellow arrows indicate vacuoles with GFP accumulation. FM4-64 used to visualize vacuoles. Scale bar, 10 µm. Error bars mark standard error of the mean. See also Figure S2.

Cytokinin directs PIN1 to lytic vacuoles for degradation

Next, we assessed the cellular mechanism by which cytokinin regulates PIN1 levels at the plasma membrane. A strongly increased GFP vacuolar signal that coincided with the membrane PIN1-GFP depletion (Figures 1D and 2B; Figure S2E) suggested that cytokinin regulates the vacuolar targeting or sorting of PIN1 for lytic degradation. Therefore, we tested the cellular processes that are required for PIN vacuolar trafficking. PIN1 undergoes complex subcellular dynamics. It constitutively recycles between the plasma membrane and endosomal compartments (Geldner *et al*., 2001) or alternatively, it might be targeted to vacuoles (Abas *et al*., 2006). The trafficking occurs along actin filaments and requires the brefeldin A (BFA)-sensitive ARF-GEF activity (Kleine-Vehn *et al*., 2008). Indeed, depolymerization of actin filaments by treatment with latrunculin B (LatB) (Figure 3A; Figure S3A) prevented PIN1 trafficking into the vacuoles in response to cytokinin, while depolymerization of microtubules with oryzalin (Oryz) did not (Figure 3A; Figure S3A). As expected, the treatment of roots or VBI-0 tobacco cells with BFA reduced the PIN1-GFP signal at the plasma membrane and

enhanced its intracellular accumulation (Figure 3A; Figures S3A-S3C). BFA prevented the PIN1 targeting to the vacuoles in response to cytokinin without additional decrease of the membrane PIN1-GFP signal (Figures S3B and S3C). The PIN1 membrane signal fully recovered after the removal of BFA. This recovery was diminished by cytokinin indicating that PIN1 recycling is affected by cytokinin (Figures S3B and S3C). Thus, the cytokinin effect on the PIN1 plasma membrane levels involves both actin and ARF-GEF activity.

Next we interfered with the vacuolar targeting by wortmannin (Wm), an inhibitor of phosphatidylinositol-3-kinase (PI3K) and phosphatidylinositol-4-kinase (PI4K) that affects recycling of vacuolar sorting receptors between the prevacuolar compartments/multivesicular bodies and the trans-Golgi network, thus interfering with the targeting of proteins to the lytic vacuoles (Kleine-Vehn *et al*., 2008). In the presence of Wm, cytokinin could not mediate any decrease in the PIN1-GFP signal at the plasma membrane nor any increase in the vacuoles (Figure 3A). This pharmacological approach showed that perturbations of cellular processes that are required for protein trafficking into lytic vacuoles interfere with the cytokinin effect on PIN1 degradation. This strongly supports hypothesis that cytokinin might regulate constitutive cycling of PIN1 by its alternative sorting to lytic vacuoles and degradation.

Cytokinin requires functional endocytic trafficking to regulate PIN1degradation

To further dissect the role of endocytic trafficking in the cytokinin-controlled PIN1 degradation, we analyzed mutants affected in PIN1 endocytosis. Previously, *ben1* (*BFA-visualized endocytic trafficking defective1*) and *ben2* were identified in a screen for mutants defective in PIN1 endocytosis. *BEN1* encodes an ARF-GEF regulator of PIN1 endocytosis, while the identity of *BEN2* is unknown (Tanaka *et al*., 2009). Both *ben1* and *ben2* mutants exhibited a strong resistance to the cytokinin-stimulated PIN1 targeting to vacuoles and PIN1-GFP membrane signal did not decrease after cytokinin treatments (Figure 3B). Thus, genetic perturbations of the PIN1 endocytic trafficking had a severe impact on the targeting of PIN1 to vacuoles by cytokinin treatment.

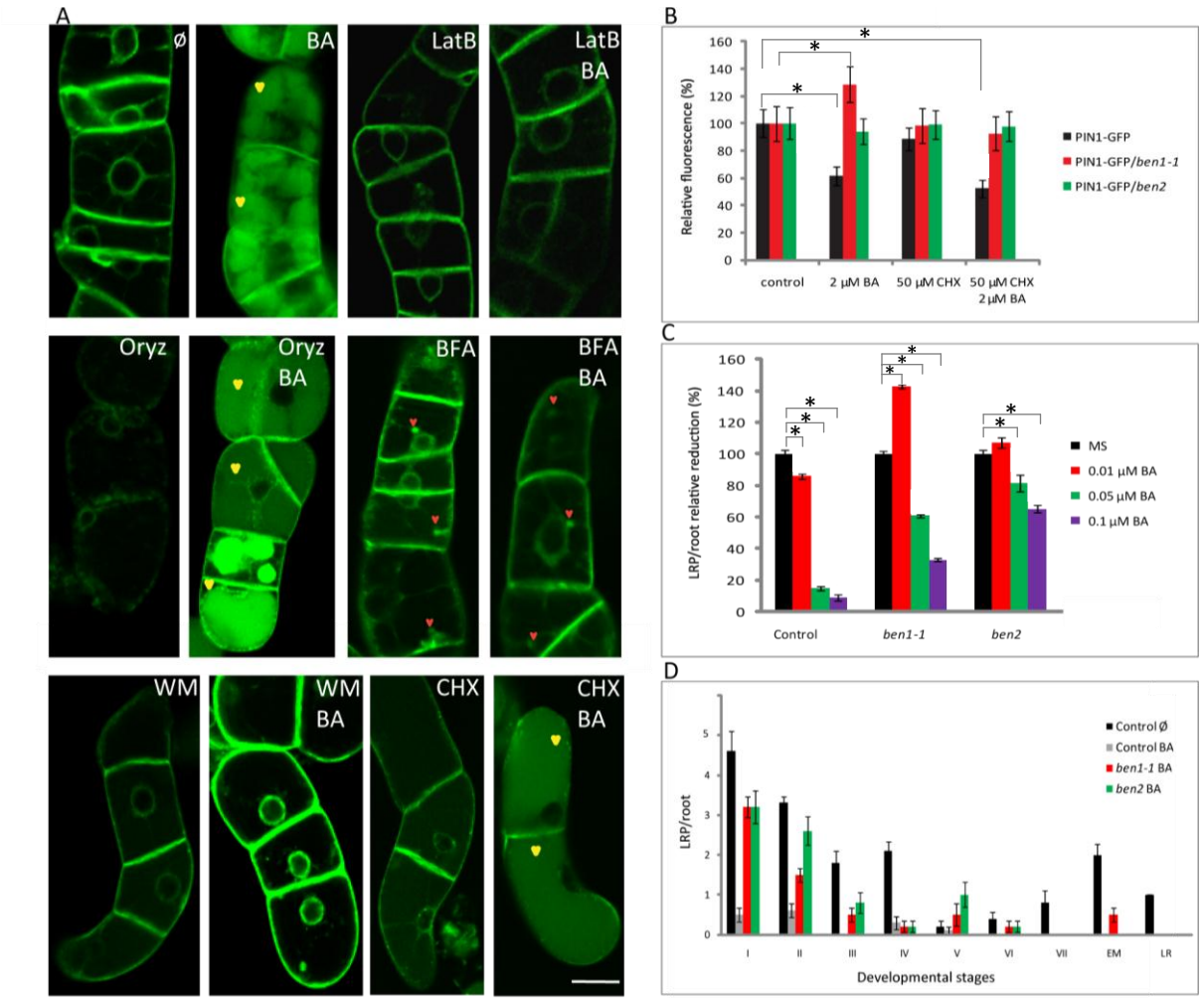

Figure 3.

Figure 3. Cytokinin activity depends on the functional endocytic trafficking.
(A) In VBI-0 tobacco suspension cells, depolymerisation of actin by LatB, but not of microtubules by Oryz, interferes with PIN1-GFP trafficking to vacuoles in response to BA. Inhibition of exocytosis/vacuolar trafficking by BFA and vacuolar trafficking by Wm prevent PIN1-GFP accumulation in vacuoles. CHX treatment does not affect PIN1-GFP accumulation in vacuoles. Yellow and red arrows indicate PIN1-GFP accumulation in vacuoles and BFA bodies, respectively. Scale bar, 20 µm. (B) The membrane PIN1-GFP is insensitive to BA in *ben1* and *ben2* mutants (*p<0.05, n=10 LRP). The PIN1-GFP membrane signal was measured in stage-I LRP. (C and D) *ben1* and *ben2* exhibit cytokinin-insensitive LR initiation (C) and LRP development (D) when grown on BA containing media for 8 days (*p<0.05, n= 14 roots). EM emerged LRP. Error bars mark standard error of the mean. See also Figure S3.

To examine the developmental consequences of the modified cytokinin sensitivity towards the regulation of the PIN1 degradation, the effect of cytokinin on LR organogenesis was studied in mutants defective in endocytosis. In both *ben1* and *ben2*, LRP initiation and development were cytokinin resistant (Figures 3C and 3D). These results reveal a correlation between cytokinin-mediated PIN1 lytic degradation and LR organogenesis.

To corroborate on the notion that cytokinin regulates plant growth and development at least in part through its effect on PIN degradation, we assessed the cytokinin effect on the primary root meristem. Root meristem differentiation had been previously shown to be strongly enhanced by cytokinin, supposedly by crosstalk with auxin signaling at the transcriptional

level (Dello Ioio *et al*., 2008). Consistently with the results of LRP, the meristem differentiation (as inferred from meristem size and onset of cell differentiation) in *ben1* and *ben2* primary roots was cytokinin insensitive (Figures S3G and S3F). In contrast, the overall root elongation, which is primarily under the control of ethylene overproduced in response to cytokinin (Cary *et al*., 1995), remained unaffected in *ben1* and *ben2* mutants, displaying sensitivity as in control seedlings (Figures S3D and S3E).

These results show that cytokinin requires a functional endocytic trafficking to efficiently modulate the amount of membrane-located PIN1. The data indicate that cytokinin-mediated regulation of PIN1 degradation underlies the cytokinin effect on different developmental processes, such as LRP organogenesis and root meristem differentiation. Altogether, our experiments revealed a role for endocytic trafficking in cytokinin-controlled plant development.

AHK based cytokinin perception is required for cytokinin-mediated PIN1 lytic degradation

Cytokinin is perceived by cytokinin receptors belonging to a family of histidine kinase receptors. Three of these, *AHK2* (*ARABIDOPSIS HISTIDINE KINASE2*), *AHK3* and *AHK4*, have been confirmed to act in cytokinin perception (Higuchi *et al*., 2004). To test whether known cytokinin perception mechanisms are required for the PIN1 lytic degradation in response to cytokinin, we examined the *ahk2*, *ahk3* and *cre1*/*ahk4* single and multiple loss-of-function mutants. The cytokinin effect on PIN1 degradation did not change significantly in either single *ahk2* and *ahk3* or *ahk2 ahk3* double mutant backgrounds (Figures 4A and 4B; Figures S4A and S4B). In contrast, the PIN1-GFP degradation in response to cytokinin was dramatically reduced in *cre1/ahk4* (Figures 4A and 4B). Likewise, in the multiple loss-of-function mutant combinations *ahk2 cre1/ahk4* and *ahk3 cre1/ahk4*, cytokinin was ineffective in targeting PIN1 to lytic vacuoles (Figures S4A and S4B). As the expression of all three cytokinin receptors overlapped in the stage-I LRP (Figure S4C), the phenotypic differences are unlikely to be the consequence of tissue-specific receptor expression. The impact of the PIN1 cytokinin insensitivity due to the lack of AHK4 activity on the LR organogenesis was investigated. In both single and multiple *cre1/ahk4* mutants, in which the PIN1 degradation was resistant to cytokinin, LRP initiation and development were as well. In contrast, the *ahk2* and *ahk3* single and *ahk2 ahk3* double mutants showed only minor differences in the cytokinin effect on LR organogenesis (Figures S4D and S4E).

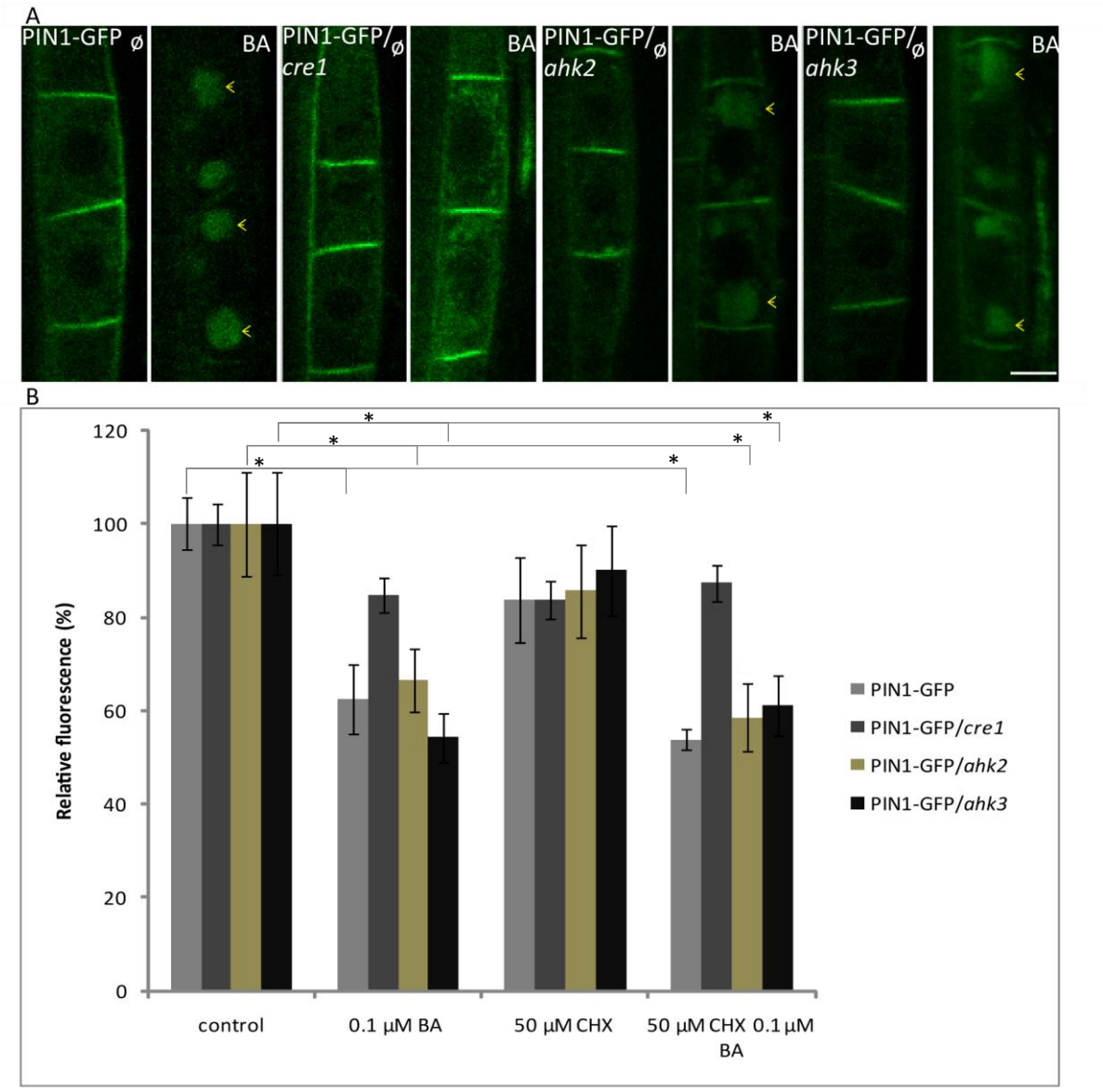

Figure 4.

Figure 4. Cytokinin induced PIN1 degradation requires a functional cytokinin perception.
(A and B) PIN1-GFP is degraded upon BA and simultaneous BA and CHX treatments in the *ahk2*, *ahk3*, but not in the *cre1*/*ahk4* mutant (*p<0.05, n= 10 LRP). The PIN1-GFP membrane signal was measured in stage-I LRP 1.5 h after BA treatment. Yellow arrows indicate vacuoles with GFP accumulation. Scale bar, 8 □m. Error bars mark standard error of the mean. See also Figure S4.

To analyze the role of downstream components of the signaling pathway, we examined the cytokinin sensitivity of PIN1 in the root meristem of several B-type *ARR* loss- of-function mutants. The PIN1 membrane signal decreased in *arr1-2* and *arr10-1* comparably to that of the cytokinin-treated root meristem of control roots, but PIN1 was not diminished in *arr2* and *arr12-1* mutants (Figure S4F and S4G).

Altogether, these findings show that functional cytokinin perception is required to mediate the PIN1 lytic degradation in response to cytokinin and imply a specific role for the AHK4 receptor-mediated branch of the pathway, including some B-type ARR components (ARR2

and ARR12) in the transduction of this cytokinin activity. Moreover, cytokinin-dependent regulation of the vacuolar PIN1 trafficking appears to be functionally important for regulation of the LRP organogenesis and meristem control.

DISCUSSION

Establishment and maintenance of shoot and root apical meristems (Dello Ioio *et al*., 2008; Zhao *et al*., 2010), shoot branching (Ongaro and Leyser, 2008), and LR organogenesis (Laplaze *et al*., 2007) are developmental processes controlled by antagonistic activities of auxin and cytokinin. Thus, an accurate balance between opposing auxin and cytokinin effects is crucial for proper developmental output. The auxin-controlling activity of the cytokinin signaling pathway has been shown to be mediated by the transcriptional modulation of its signal transduction. Conversely, cytokinin has been shown to feedback on the auxin activity through modification of the expression of the *Aux/IAA* genes that suppress the auxin signaling pathway. Consequently, cytokinin directly impacts on the auxin distribution mediated through the auxin efflux carriers of the PIN family that are under transcriptional control of the auxin signaling pathway (Dello Ioio *et al*., 2008; Ruzicka *et al*., 2009). Thus far, all disclosed mechanisms of the auxin-cytokinin communication act through mutual modulation of their transcriptional responses.

Here, we reveal another mechanism underlying the cytokinin control of plant development and crosstalk with the auxin pathway. We show that cytokinin, independently of transcription, affects the PIN1 trafficking and redirects it for lytic degradation in vacuoles. At early stages of LR organogenesis, cytokinin depletes in 90 min approximately 40% of the membrane-localized PIN1. Such a rapid posttranscriptional regulation of the PIN1 abundance provides for very efficient and precise mechanisms to control auxin fluxes and distribution during cytokinin-mediated developmental regulations, including LRP organogenesis and root meristem differentiation. The results imply that the endocytic trafficking plays a role in cytokinin-controlled development and that cytokinin activity downstream of the cytokinin perception is not restricted to transcriptional regulation. However, unraveling exactly how both transcriptional and transcription-independent effects are mediated by the cytokinin pathway is a challenge for future investigations. Our data reveal that the functional AHK4 receptor and several B-type ARR regulators are required for cytokinin-stimulated PIN1 degradation. Although underlying mechanisms are still elusive, we hypothesize that cytokinin

perception might either target the endocytotic pathways, or affect the PIN1 protein itself, thus preventing PIN1 recycling and promoting its vacuolar targeting.

EXPERIMENTAL PROCEDURES

Plant material

The transgenic *Arabidopsis thaliana* (L.) Heynh. lines have been described elsewhere: *PIN1::PIN1-GFP, DR5::GUS* (Benkova *et al*., 2003); *PIN2:PIN2-GFP* (Xu and Scheres, 2005); *PIN7::PIN7-GFP* (Blilou *et al*., 2005); *PIN3::PIN3-GFP* (Zadnikova *et al*., 2010); *PIN2::PIN1-GFP* (Wisniewska *et al*., 2006); *AUX1*::*AUX1-YFP* (Swarup *et al*., 2004); *35S::PIN1-GFP* (Ruzicka *et al*., 2007); *WAVE138::YFP* (Geldner *et al*., 2009); *AHK2::GUS, AHK3::GUS ,CRE/AHK4::GUS, cre1-12, ahk2-2, ahk3-3, cre1-12 ahk2-2, ahk2-2 ahk3-3, cre1-12 ahk3-3* (Higuchi *et al*., 2004); *ben1-1* and *ben2* (Tanaka *et al*., 2009); *pin2 pin3 pin7* (Friml *et al*., 2003) and *ein2(Roman et al., 1995). arr1-2* (N6368), *arr10-1* (N6369) and *arr12-1* (N6978), (Mason *et al*., 2005), *arr2* (SALK_043107C) were obtained from the European Arabidopsis Stock Centre (NASC). Q RT-PCR analysis confirmed that *arr2* is null mutant allele (primers TTATTAAATGCCAGTGGCAGC and CGACAAGAACTCGAAGATTCG). The tobacco cell line VBI-0 (*Nicotiana tabacum* L. cv. Virginia Bright Italia) (Opatrný and Opatrná, 1976)was used as suspension-cultured cells.

Growth conditions

Seeds of *Arabidopsis* (ecotype Columbia-0) were plated on 0.5 MS medium (Duchefa) with 1% sucrose, 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. Tobacco VBI-0 cells were grown in liquid media and stably transformed with *Arabidopsis PIN1::PIN1-GFP* (Benkova *et al*., 2003) and *PIN7::PIN7-GFP* (Blilou *et al*., 2005). For transformation details see Supplementary materials.

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 22°C. Drugs and hormones used were: CHX (50 μM), COR (400 μM), BFA (50 μM), LatB (20 μM), Wm (30 μM), Oryz (20 μM), BA (0.1 μM and 2 μM), NAA (10 μM), ZA (2 μM), AVG (0.2 μM), ACC (0.04 μM

and 5 μM). For double treatments, a 30-min pretreatment with CHX, BFA, COR, LatB, Wm or Oryz was done prior to the BA application. Seven-day-old tobacco VBI-0 cells were incubated in liquid medium supplemented with CHX (50 μM), BFA (20 μM), LatB (20 μM), Wm (30 μM), Oryz (15 μM), BA (0.1 μM) for 1.5 to 2 h in the dark at 22°C. Vacuoles visualized by FM4-64 (4 μM) as described (Kleine-Vehn *et al*., 2008).

Real-time analyses of membrane protein dynamics and GFP signal quantification.

The membrane GFP signal was quantified on scans of stage-I LRP, root epidermal cells and tobacco cells. Pictures were taken by a FV10 ASW confocal microscope (Olympus) with a 20 or 60 (water immersion) objective. Criteria for quantifications of membrane signals in particular tissues and microscope settings are specified in Supplementary materials. The fluorescence intensity of the membrane PIN-GFP signal was quantified with ImageJ (NIH; http://rsb.info.nih.gov/ij) as described (Zadnikova *et al*., 2010). The statistical significance was evaluated with Student's *t*-test.

Analysis of primary root and LRP organogenesis

For real-time analysis of the LRP development, 6-day-old seedlings were placed on chambered cover glass (Nunc Lab-Tek) and covered with 0.2-mm thin square blocks of solid MS media with or without the indicated chemicals and hormones. LRPs were scanned in 3- or 5-min time intervals for 8-12 hours by the FV10 ASW confocal microscope (Olympus).

For phenotypic analyses of root growth, LR initiation and development, at least 20 seedlings were processed. The LRP density was analyzed in 8-day-old seedlings as described (Malamy and Benfey, 1997). Root growth parameters (root length and root meristem) were analyzed with the ImageJ software (NIH; http://rsb.info.nih.gov/ij) as described (Růžička *et al*., 2009).

Gene expression analysis

GUS activity was detected as described (Benkova *et al*., 2003). For quantitative RT-PCR RNA was extracted with the RNeasy kit (Qiagen) from 5-day-old roots of *Arabidopsis*. Expression levels were normalized to *UBQ10*. For details see Supplementary materials.

Western Blot Analysis and whole-mount protein localization

Fourteen-day-old Col O seedlings were sprayed with a known amount of BA or DMSO. Roots were harvested after 2-3 hours. Isolation of membrane proteins, Western blotting and quantification were done as described (Abas and Luschnig, 2010). Affinity-purified antibodies against PIN1 (Paciorek *et al*., 2005), PIN3 (provided by C. Luschnig), and PIN7 (Friml, 2003) were used. The statistical significance was evaluated with Student's *t*-test (paired, 2-tailed, n=7 independent biological repeats). For details see Supplementary materials.

In situ whole mount localization of PIN1 and PIN2 was done on 6-day-old roots as described (Sauer *et al*., 2006).

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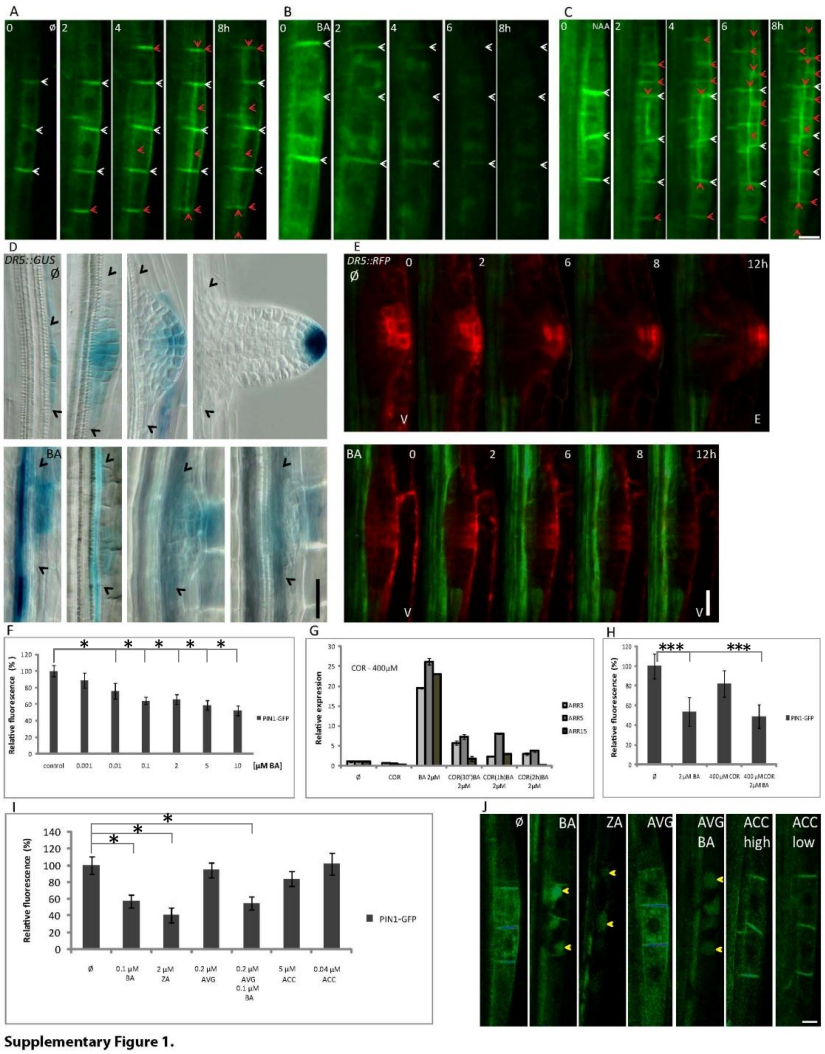
SUPPLEMENTARY FIGURES



Supplementary Figure 1.

Figure S1. Cytokinin inhibits LRP development and depletes PIN1 from the plasma membrane (related to Figure 1)

(A-C) Real time monitoring of PIN1-GFP during LRP development in control (A) seedlings and treated with BA (B) and auxin (NAA) (C). (D) The *DR5::GUS* response in LRP treated with BA (down) compared to the untreated (top). (E) Real time monitoring of *DR5::RFP* expression in BA treated (down) compared to untreated (top). (F) Dose-dependent decrease of the membrane PIN1-GFP in response to BA (*p<0.05, n=10 LRP). (G) Pretreatment with 400 □M COR for 30 min fully inhibits *ARR15* and by 75% expression of *ARR5* and *ARR15* induced by 2 □M BA as detected using q-RT PCR analysis (*p<0.05, n=3) (H) Inhibition of transcription by COR does not interfere with the BA effect on the PIN1-GFP membrane signal (*p<0.05, n=10 LRP). (I and J) BA (N^6-benzyladenine) and ZA (zeatin) induce the PIN1-GFP accumulation in vacuoles and decrease the PIN1-GFP signal at the plasma membrane. Neither AVG nor ACC interfere with the PIN1-GFP membrane localization (*p<0.05, n=10 LRP). Yellow arrows indicate vacuoles. The PIN1-GFP membrane signal was measured in stage-I LRP after 30' min pre-treatment with COR and 1.5 h simultaneous BA treatment (H) or 1.5 h after BA treatment (F,I). White and red arrows indicate anticlinal divisions at the early initiation stage of LRP at time 0 and newly formed cell divisions, respectively (A-C), black arrows position of LRP (D), yellow arrows vacuoles (J). Scale bars: 9 □m (A-C), 70 □m (D), 25 □m (E), 5 µm (J). Error bars mark standard error of the mean.

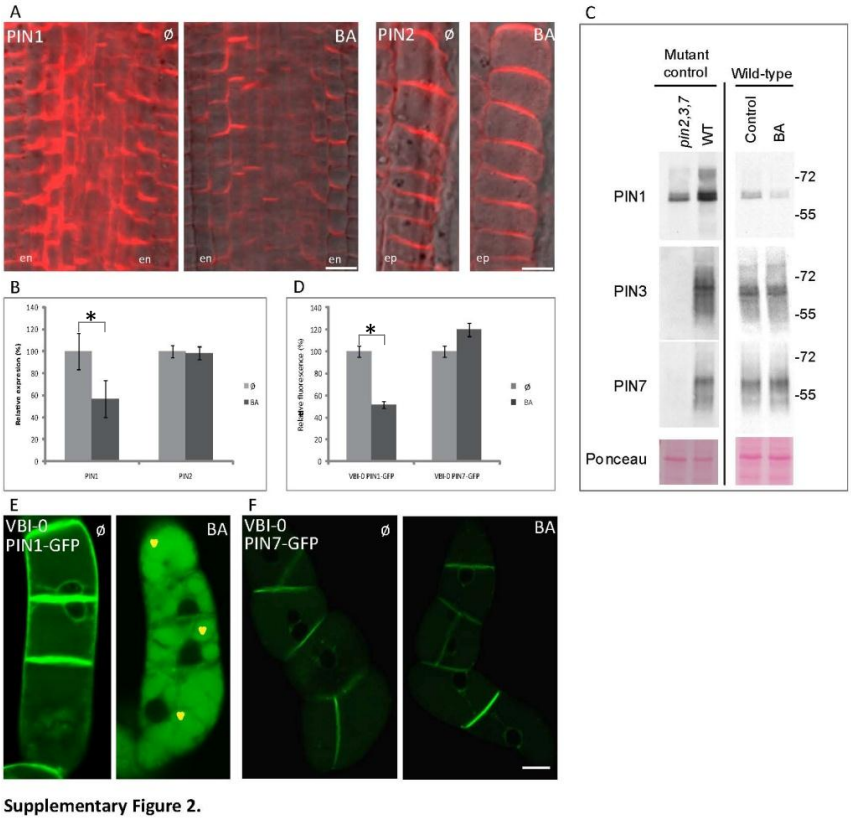
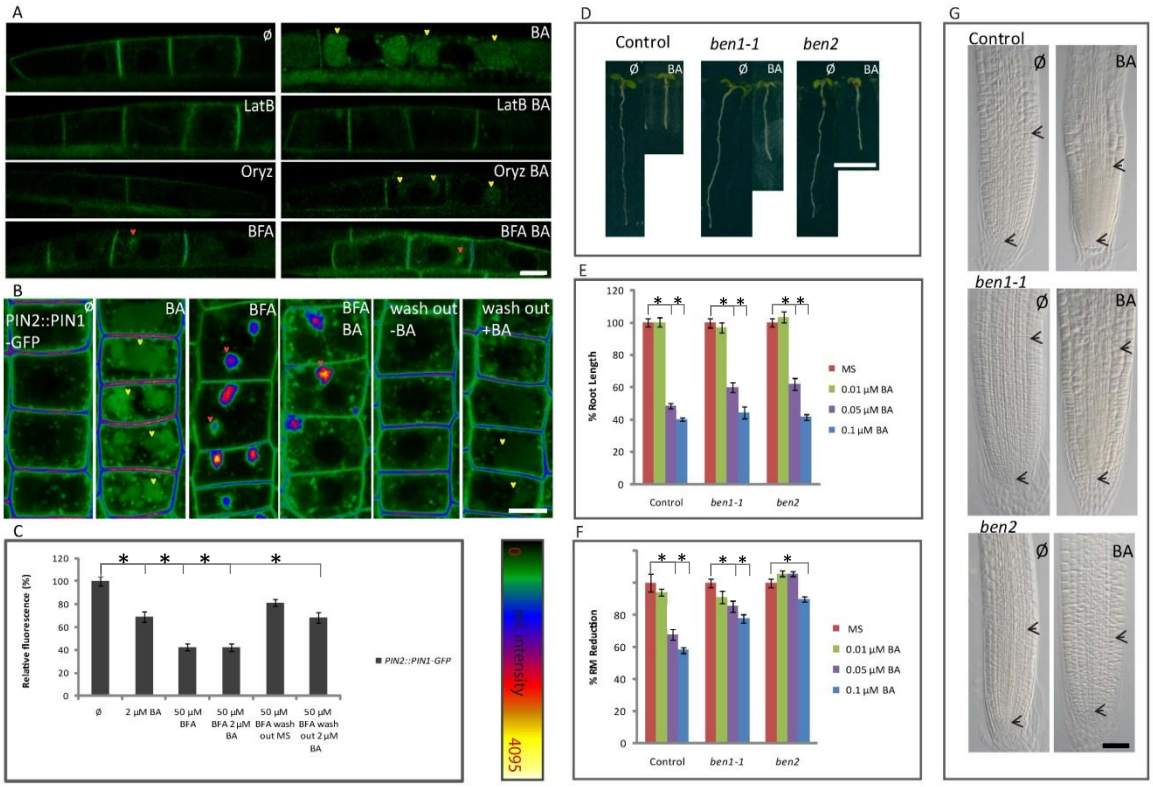
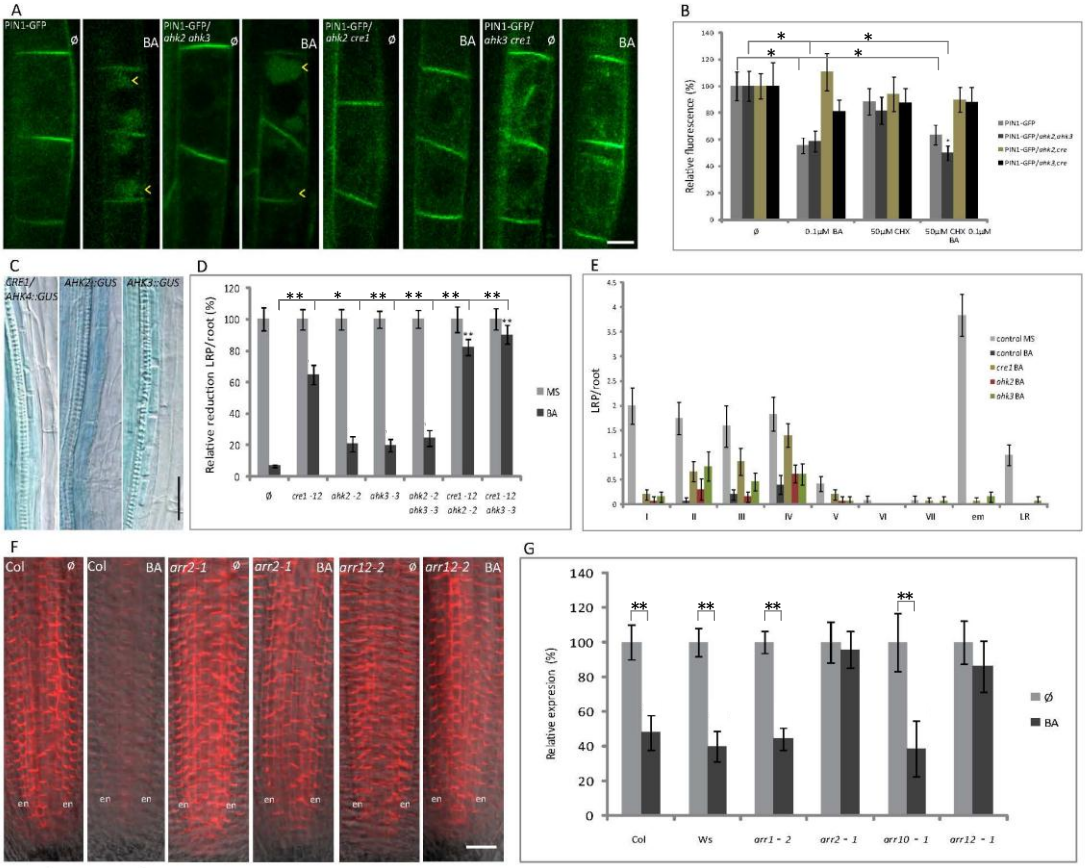


Figure S2. Cytokinin rapidly decreases PIN1-GFP plasma membrane signal (related to Figure 2)
(A and B) Immunolocalization of PIN1 and PIN2 in root meristem. PIN1 decreases in root endodermal cells after BA treatment, in contrast to unchanged PIN2 in epidermis (*$p<0.05$, n=10 roots, ten cells per root). (C) Western blot analysis of endogenous PIN proteins in extracts from untreated and BA-treated wild-type (Col-0) roots. No PIN3 or PIN7 signal was detected in the *pin2 pin3 pin7* triple mutant. (D-F). In response to BA treatment, PIN1-GFP, but not PIN7-GFP, decreases at the plasma membranes of individual tobacco cells (*$p<0.05$, n=30 individual cells). The PIN-GFP signal was measured 1.5 h after the BA treatment. Scale bars: 25 µm (A), 20 µm (E). Error bars mark standard error of the mean.



Supplementary Figure 3.

Figure S3. Cytokinin activity depends on the functional endocytic trafficking (related to Figure 3)
(A) In the stage-I LRP, treatment with LatB, but not with Oryz interferes with the PIN1-GFP trafficking to vacuoles in response to BA. BFA prevents accumulation of PIN1-GFP in vacuoles.
(B and C**)** BFA induced accumulation of PIN1-GFP in BFA bodies (red arrowheads) and prevents cytokinin-induced PIN1-GFP targeting to vacuoles (yellow arrows). The PIN1 plasma membrane signal recovers after BFA had been washed out with control, but not with BA-containing media (*p<0.05, n=10 roots, 5 cells per root). The PIN1-GFP plasma membrane signal was measured in root epidermal cells (C) 1.5 h after BA treatment. (D and E) *ben1* and *ben2* exhibit cytokinin-sensitive root growth (*p<0.05, n=14). (F and G**)** Cytokinin affects meristem size in control (Col-0) root meristems, but not in *ben1* and *ben2* (*p<0.05, n=14). Arrows indicate the distance between the quiescent centre (QC) and the cortex transition zone. Seedlings grown on 0.1 µM BA for 6 days. Scale bars: 10 µm (A), 7 µm (B), 1 cm (D), 200 µm (G). Error bars mark standard error of the mean.



Supplementary figure 4.

Figure S4. Cytokinin-induced PIN1 degradation requires a functional cytokinin perception (related to Figure 4)

(A and B) PIN1-GFP is degraded upon BA or simultaneous BA and CHX treatment in *ahk2 ahk3*, but not in *ahk2 cre1*, and *ahk3 cre1* mutant ($p<0.05$, n=10 LRP). The PIN1-GFP membrane signal was measured in stage-I LRP 1.5 h after BA treatment. Yellow arrows indicate vacuoles. (C) *AHK2*, *AHK3* and *AHK4/CRE1* expression in stage-I LRP. (D and E) LR initiation (D) and LR development (E) in the *cre1* and *ahk2 cre1* and *ahk3 cre1* mutants when grown on 0.1 µM BA for 8 days (*$p<0.05$,**$p<0.01$, n=15). (F and G). PIN1 membrane signal after cytokinin treatment in *arr1-2* and *arr10-1*, *arr2-1* and *arr12-1* (**$p<0.01$, n=10 roots, ten cells per root). PIN1 was visualized by immunolocalization in control and with 0.1 µM BA for 1,5 h treated root meristems and membrane signal in endodermal cells quantified. WS (Wassilewskija), Col (Columbia) ecotype used as control for *arr1-2*, *arr10-1* and *arr2-1*, *arr12-1*, respectively. Scale bar: 8 µm (A), 20 µm (C), 30 µm (F). Error bars mark standard error of the mean.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Plant material

Growth conditions and gene transformation

Tobacco VBI-0 cells were grown in liquid media as described (Petrášek *et al*., 2002) and stably transformed with *Arabidopsis PIN1::PIN1-GFP* (Benkova *et al*., 2003) and *PIN7::PIN7-GFP* (Blilou *et al*., 2005) as described for BY-2 cells (Petrášek *et al*., 2003). One-week-old cells were co-incubated with the *Agrobacterium tumefaciens* strain GV2260 carrying the corresponding genes in the pBINPLUS binary vector. After regeneration and selection of fluorescent cells, PIN1-GFP and PIN7-GFP cells were maintained in culture media containing 100 μg ml^{-1} kanamycin and 100 μg ml^{-1}cefotaximum.

Real-time analyses of membrane protein dynamics and GFP signal quantification.

The membrane GFP signal was quantified on scans of stage-I LRP, root epidermal cells and tobacco cells. Pictures were taken by a FV10 ASW confocal microscope (Olympus) with a 20 or 60 (water immersion) objective. Fluorescence signals were detected for GFP (excitation 488 nm, emission 505–550 nm), FM 4-64 (excitation 561 nm, emission >575 nm), and RFP (543 nm excitation, 560-615 nm emission). Sequential scanning was used to avoid any cross-talk between fluorescence channels. For all the experiments, the scans were done with identical microscope and laser settings. For quantifications of membrane signals in particular tissues following criteria were applied: (i) minimum 10 LRP in stage-I positioned in the plane with two xylem strands were selected, and 2 anticlinal plasma membranes per LRP were measured. (ii) Files of five root atrichoblast epidermal cells below the transition zone were selected, 10 roots with five cells per root were measured. (iii) Confocal sections through tobacco BY-2 cell in the plane with nucleus were used for analysis. Minimum 30 tobacco cells per treatment were measured. The fluorescence intensity of the membrane PIN-GFP signal was quantified with ImageJ (NIH; http://rsb.info.nih.gov/ij) as described (Zadnikova *et al*., 2010). The statistical significance was evaluated with Student's *t*-test. Images were processed in Adobe Illustrator.

Quantitative RT-PCR.

For quantitative RT-PCR RNA was extracted with the RNeasy kit (Qiagen) from roots of 5-day-old *Arabidopsis* seedlings. A DNase treatment with the RNase-free DNase Set (Qiagen) was carried out for 15 min at 25°C. Poly(dT) cDNA was prepared from 1 µg total RNA with iScript™cDNA Synthesis Kit (Bio-Rad) and quantified with a LightCycler 480 (Roche) and SYBR GREEN I Master (Roche) according to the manufacturer's instructions. PCR was carried out in 384-well optical reaction plates heated for 10 min to 95°C to activate hot start Taq DNA polymerase, followed by 40 cycles of denaturation for 60 sec at 95°C and annealing/extension for 60 s at 58°C. Targets were quantified with specific primer pairs designed with the Beacon Designer 4.0 (Premier Biosoft International). Expression levels were normalized to *UBQ10* expression levels. All RT-PCR experiments were done at least in triplicate. Following primers were used: *UBQ10* (CACACTCCACTTGGTCTTGCGT and TGGTCTTTCCGGTGAGAGTCTTCA), *ARR3* (CCGTTGATGACAGCCTAGTTGA and CGTGACTTTGCAGGATGTGATT), *ARR5* (ACACTTCTTCATTAGCATCACCG and CTCCTTCTTCAAGACATCTATCGA), and *ARR15* (CTTCAGCACTCAGAGAAATCC and GTCTCTAGATTAACCCCTAGACTCT). Efficiency factor (ef) for tested genes corresponded to: *UBQ* 1.82 (ef) 0.0582 (standard error (std)); *ARR3* 1.81 (ef) 0.0383 (std); *ARR5* 1.79 (ef), 0.0422 (std); *ARR7* 1.83 (ef), 0.0437 (std); and *ARR15* 1.84 (ef), 0.0562 (std).

Western Blot Analysis

Fourteen-day-old Col O seedlings grown on vertical plates were sprayed with a known amount of BA or DMSO (final concentrations of hormones after absorption into the solid medium were approximately 2 µM). Plates were maintained vertically throughout the treatment; both dark and light conditions were tested. Roots were harvested after 2-3 hours. Isolation of membrane proteins, Western blotting and quantification were done as described (Abas *et al*., 2010). Affinity-purified antibodies against PIN1(Paciorek *et al*., 2005), PIN3 (mouse antibodies raised against amino acids 334-483, kindly provided by C. Luschnig), and PIN7 (Friml *et al*., 2003) were used. The statistical significance was evaluated with Student's *t*-test. (paired, 2-tailed, n=7 independent biological repeats).

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Chapter 3.

Cytokinin promotes PIN1 polarity establishment

Adapted from

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PM and EB initiated the project and designed most of the experiments, PM carried out most of the experiments. DJ performed LR- analyses. OR provide seeds. FE and FJ discussed the experimental setup and procedures. All authors analysed and discussed the data; PM and EB wrote the manuscript and all authors saw and commented on the manuscript..

Cytokinin promotes PIN1 polarity establishment

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Abstract

The plant hormones auxin and cytokinin jointly control a variety of plant developmental processes. To ensure a proper developmental output both hormones mutually coordinate their activities, and an intense cross-talk between auxin and cytokinin occurs at multiple levels encompassing a reciprocal regulation of the hormone metabolism, distribution, signaling and downstream responses. Among them, cytokinin-mediated modulation of auxin transport through transcriptional and post-transcriptional regulation of the PIN-formed (PIN) auxin efflux carriers provides an efficient means to rapidly control auxin availability in plant tissues and organs. Here, we reveal that a recently described cytokinin stimulatory effect on lytic degradation of PIN1 not only allows overall regulation of auxin transport efficiency, but might be part of a mechanism determining directionality of the auxin stream. We demonstrate that cytokinin interferes selectively with a trafficking pathway targeting PIN1 towards the basal membrane. Developmental and genetic modulations, increasing the proportion of apically localized PIN1 due to its increased phosphorylation, dramatically reduce cytokinin sensitivity. We demonstrate that during developmental processes, such as lateral root organogenesis requiring redirection of auxin flow, cytokinin might contribute to PIN polarity re-establishment by targeting a specific subset of PIN1 membrane proteins for lytic degradation.

INTRODUCTION

Plant hormones cytokinin (CK) and auxin have already been recognized for a long time as crucial signaling molecules controlling plant growth and development. Recent studies using *Arabidopsis thaliana* provide more insights about the role of CK and auxin in plants (Bishopp *et al*., 2009). In the root of *Arabidopsis thaliana*, these two hormones (CK and auxin) are known to act antagonistically in controling root meristem activities and branching. Auxin sustains root meristem activity and branching by promoting cell division (Ongaro *et al*. 2008) whereas cytokinin promotes cell differentiation and inhibits root branching by repressing both auxin signaling and transport (Dello Ioio *et al*., 2008; Ruzicka *et al*., 2009). Their coordinated action is essential for maintaining root meristem size and for ensuring root growth and branching (Dello Ioio *et al*., 2007; Ruzicka *et al*., 2009, reviewed in Moubayidin *et al*., 2009). Cytokinin signal, perceived by the AHK3 receptor kinase and transduced by ARR1 and ARR12 response regulators, activates the transcription of auxin repressor *SHY2/IAA3*, resulting in the negative regulation of the transcription of PIN auxin efflux carriers (Dello Ioio *et al*., 2008; Ruzicka *et al*., 2009). Specifically, the expression of *PIN1, PIN4* and *PIN3* transcripts is downregulated, while *PIN2* expression is CK insensitive. In contrast, *PIN7* is clearly upregulated after CK treatments (Ruzicka *et al*., 2009). Besides the transcriptional regulation of auxin activity and distribution, CK also acts in a transcription-independent manner. Cytokinin is perceived by the AHK4 receptor kinase and modulates the endocytic trafficking of PIN1, redirecting this membrane protein for lytic degradation into the vacuoles (Marhavý *et al.*, 2011).

Polar membrane localization of the PIN-formed (PIN) auxin efflux carriers is one of the key determinants decisive for the direction of auxin movement through the plant (Wiśniewska *et al*., 2006; Petrášek *et al*., 2006). Thus, disclosure of the mechanisms controling PIN subcellular trafficking and polarity establishment is crucial for our understanding of auxin-regulated plant development. Phosphorylation of PIN proteins controlled by a set of kinases and phosphatases (Friml *et al*., 2004; Michniewicz *et al*., 2007; Zhang *et al*., 2010; Huang *et al*., 2010), Ca^{2+} signaling (Zhang *et al*., 2011), or mechanical signals orienting the plant microtubule network (Heisler *et al*., 2010) were found to contribute to the modulation of subcellular trafficking of PIN proteins and determine their polarity establishment.

Here we show, that the CK stimulatory effect on the lytic degradation of PIN1 not only allows overall regulation of auxin transport efficiency, but might be part of the mechanism determining directionality of the auxin stream. We identify that CK interferes preferentially with trafficking pathways directing PIN proteins from the basal membranes into the vacuole, which leads to a significant extinction of the PIN proteins from basal, but not apical, membranes.

RESULTS

Previously we demonstrated that to control root growth and development cytokinin might, besides other mechanisms (Hwang and Sheen 2001; Muller and Sheen 2007; Dello Ioio *et al*., 2008; Zhao *et al*., 2010), act through modulation of the endocytotic trafficking of PIN1 (Yoshida *et al*., 2011; Marhavý *et al*., 2011). In the root, cytokinin was found to redirect PIN1 for lytic degradation to vacuoles, thereby providing an alternative mechanism for the rapid control of auxin availability in root tissues (Marhavý *et al*., 2011). Notably, PIN1, when ectopically expressed in the root epidermal cells, was targeted by cytokinin in contrast to PIN2, an innate epidermal PIN homolog (Marhavý *et al*., 2011; Figure 1a,e compared to b,f). Thus, although they are members of the same gene family, PIN homologs differ in their response to cytokinin, which indicated the existence of specific cues determining the sensitivity of PIN proteins to cytokinin. In the root epidermal cells PIN2 and PIN1 are recruited by distinct trafficking pathways to either the apical (shootward) or basal (rootward) membranes, respectively (Wiśniewska *et al*., 2006; Kleine-Vehn *et al*., 2008). To test whether cytokinin targets the PIN1 protein with different efficiency according to its apical or basal membrane location, we compared the cytokinin sensitivity of basal PIN1-GFP2 with PIN1-GFP3 and PIN2-GFP that both localize similarly at the apical membranes of the root epidermal cells (Wiśniewska *et al*., 2006). Typically 1.5 h exposure to cytokinin resulted in the 30% ± 9 (n=10 roots) reduction of the PIN1-GFP2 signal from the basal membrane (Figure 1a,e). In contrast, in roots expressing the PIN1-GFP3 allele the GFP membrane signal did not diminish (Figure 1c,g) and thus strongly resembled cytokinin insensitive PIN2 (Figure 1b,f).

To further examine whether PIN basal and apical trafficking pathways differ in cytokinin sensitivity, we analyzed the cytokinin effect on the root cortex cells where all PIN1-GFP2 and PIN1-GFP3 protein fusions as well as PIN2 are targeted to the basal membranes (Wiśniewska *et al*., 2006). As expected, the PIN1-GFP2 membrane signal reduced in response to cytokinin

by 30% ± 9 (Figure 1a,b). Importantly, differently from the epidermis, in the cortex cells the membrane signal of both PIN2-GFP and PIN1-GFP3 proteins was significantly lower (30% ± 8 and 32% ± 13, respectively) (Figure 1c,d and Figure 1e,f).

Altogether, these data indicate that cytokinin interferes preferentially with trafficking pathways directing PIN to the basal membranes, which leads to a significant extinction of the PIN proteins from basal, but not apical membranes.

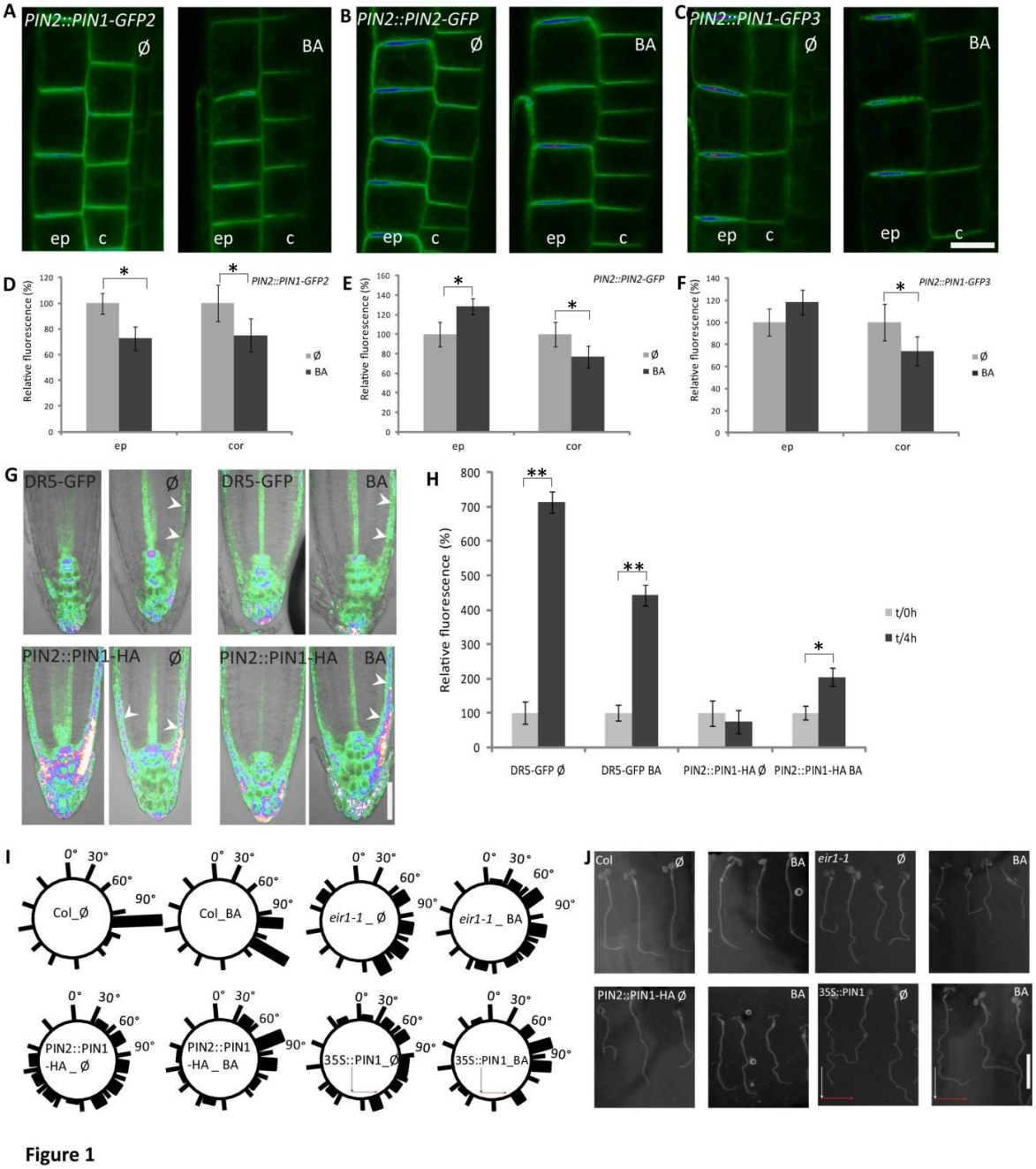

Figure 1

Figure 1. Cytokinin depletes basally localized PINs from the plasma membranes.
(A - F) Cytokinin derivate N^6-benzyladenine (BA) depletes basally localized PIN1-GFP2 in cortex and epidermal cells, PIN2-GFP and PIN1-GFP3 in cortex cells, but not apically localized PIN2-GFP and PIN1-GFP3 in root epidermal cells. The PIN-GFP membrane signal was measured in root cortex (c) and epidermal cells (ep) 1.5 h after BA treatment. **(D - F)** Scale bars: 15 µm. Error bars mark the standard error of the mean (*p<0.05,

n=10 roots, ten cells per root). **(G and H)** Quantification evaluation of DR5rev::GFP fluorescence intensity ration (upper/lower) side of root in the wild type and *PIN2::PIN1-HA,pin2/eir1* roots after (0h and 4h) of gravistimulation. Enhanced DR5rev::GFP response at the gravistimulated side of control, but not *PIN2::PIN1-HA,pin2/eir1* roots. BA treatment partially rescues asymmetric auxin response in *PIN2::PIN1-HA;pin2/eir2* roots. Values are the average of three biological replicates (n = 20 per time point on each replicate). Error bars mark standard error of the mean (*p<0.05, **p<0.001, n=20 roots). Scale bars: 30 μm. **(I and J)** Gravitropic response is rescued by BA treatment in *PIN2::PIN1-HA;pin2* and *35S::PIN1-GFP* roots, but not in the *eir1/pin2* mutant. Seven days old roots were exposed to a gravistimulus for 24 h. Scale bars: 0.5 cm.

Cytokinin restores agravitropic root growth caused by non-polar PIN1 localization in the root epidermis

Polar localization of PIN2 at the apical membranes of the root epidermal cells directs the basipetal auxin stream after gravistimulation and thus determines proper root gravitropic response (Muller *et al*., 1998). Ectopic expression of *PIN1* (when driven by *PIN2* or *35S* promoters) in the root epidermal cells fails to rescue the agravitropic root phenotype of the *pin2/eir1-1* loss-of-function mutant due to predominantly non-polar and basal localization of PIN1 in these cells (Wiśniewska *et al*., 2006; Feraru *et al*., 2011). We hypothesized that cytokinin through elimination of PIN1 at the basal, but not apical membranes of the epidermal cells, might restore basipetal auxin flux in the root epidermal cells and consequently root gravitropic response. Using immunolocalization we confirmed that cytokinin treatment reduced the amount of the PIN1-HA proteins at the basal side, but not the apical side of the epidermal root cells (Supplementary Figure 1a and b). Scoring epidermal cells with non-polar, basal, or apical PIN1 polarity showed that in cytokinin-treated roots the proportion of the epidermal cells with apically localized PIN1 increased (76.5% ± 8; n=209) when compared to untreated roots where most of the cells exhibited non-polar PIN1 localization (52% ± 11, n=113) or basal localization (24% ±7, n=25)(Supplementary Figure 1b). To examine the cytokinin effect on the auxin redistribution in roots expressing PIN1-HA driven by the PIN2 promoter in *eir1-1* mutants, we monitored DR5::GFP auxin reporter expression. As expected four hours of gravistimulation led to a significant increase of auxin response at the stimulated side of control roots (Figure 1g and h). Examination of the DR5 response in PIN2::PIN1-HA/*eir1-1* gravistimulated roots revealed that despite a generally stronger DR5 response in the epidermal cells, there was no statistically significant auxin response asymmetry between both sides of the roots (Figure 1g and h). However, when grown on cytokinin, gravistimulus promoted an asymmetric DR5 response, indicating partial restoration of auxin basipetal transport (Figure 1g and h).

Next we tested whether cytokinin restoration of the auxin redistribution in gravistimulated PIN2::PIN1-GFP2/*eir1-1* roots correlates with an improvement of the gravitropic response. We observed that cytokinin treatment caused a slight delay in the gravitropic response of wild-type roots and did not restore the agravitropic phenotype of the *eir1-1*/*pin2* loss-of-function mutant (Figure 1i and j). In contrast, roots of *PIN2::PIN1-HA* as well as *35S::PIN1* lines exhibiting a strong agravitropic phenotype were able to react to gravitropic stimulation when treated with cytokinin (Figure 1i and j).

In summary, the analysis of the auxin redistribution and root response to the gravistimulus further supported our hypothesis that cytokinin promotes the degradation of PIN1 selectively from the basal membranes. This local, membrane-specific decrease of the auxin carrier at the basal side of the root epidermal cells restores the basipetal auxin transport stream and consequently the gravitropic response.

Loss- and gain-of-phosphorylation mutations of PIN1 modulate cytokinin sensitivity.

The PIN phosphorylation state was found to be an important determinant of the PIN polarity establishment. Ser residues in the conserved TPRXS(N/S) motifs within the PIN1 hydrophilic loop were recognized as important targets that are phosphorylated by PINOID kinase in vitro (Huang *et al*., 2010; Zhang *et al*., 2010). In planta analyses of loss-of-phosphorylation and phosphomimic PIN1:GFP mutants proved that reversible phosphorylation of these motifs is required and sufficient for proper PIN1 polar localization and auxin-regulated plant development (Zhang *et al*., 2010; Huang *et al*., 2010). The loss-of-phosphorylation PIN1 mutations PIN1-GFP-(Ala) and PIN1:GFPS1,3A correlated with basal, while phosphomimic mutations PIN1-GFP-(Asp) and PIN1:GFPS1,3E with apical membrane localization (Zhang *et al*., 2010; Huang *et al*., 2010). Basally, but not apically localized PIN1 was found to be sensitive to cytokinin treatment, thus we examined whether loss-of-phosphorylation and phosphomimic PIN1-GFP mutated versions differ in their cytokinin sensitivity, accordingly. Measurements of the GFP signal at the plasma membrane in the lateral root primordia at stage I (Malamy and Benfey, 1997) revealed that loss-of-phosphorylation PIN1-GFP-(Ala) and PIN1:GFPS1,3A mutants were sensitive to cytokinin, while phosphomimic PIN1-GFP-(Asp) and PIN1:GFPS1,3E versions were cytokinin insensitive (Figure 2a-c). Previously we showed that a rapid decrease of membrane PIN1 correlates with an arrest in the lateral root primordia development, and accordingly in mutants defective in cytokinin perception (*cre1/ahk4*) or endocytosis (*ben2*), PIN1 cytokinin insensitivity is accompanied with cytokinin resistant lateral root primordia (LRP) development (Marhavy *et al*., 2011). To examine whether the

phosphorylation state of PIN1 impacts on the LRP development sensitivity to cytokinin we performed real time analysis of LRP development during 16 h. Typically, under control conditions, 87% of the LRP progressed from stage I/II to stage III/IV (n=23; Table 1). After cytokinin treatment, 85% of the LRP were arrested and did not progress to the next developmental stage (n=27) (Table 1; Supplementary Figure 2a). Lateral root primordia development in PIN1-GFPS1,3(A) and PIN1-GFPS1,3E lines was mildly affected and developmental arrest was detected in 36% (n=19) and 33% (n=30) of examined primordia, respectively, caused probably by deficiency in proper PIN1 polarity establishment. However, while cytokinin treatment significantly increased the proportion of the arrested LRP to 83% (n=12), in loss-of-phosphorylation PIN1-GFPS1,3A mutants only 40% (n=20) of the primordia expressing PIN1-GFPS1,3E were arrested (Table 1; Supplementary Figure 2). Consistently, lateral root initiation was resistant to the inhibitory cytokinin effect in PIN1-GFP-(Asp) and PIN1-GFPS1,3E, but not in PIN1-GFP-(Ala) and PIN1-GFPS1,3A lines (Figure 2e,f).

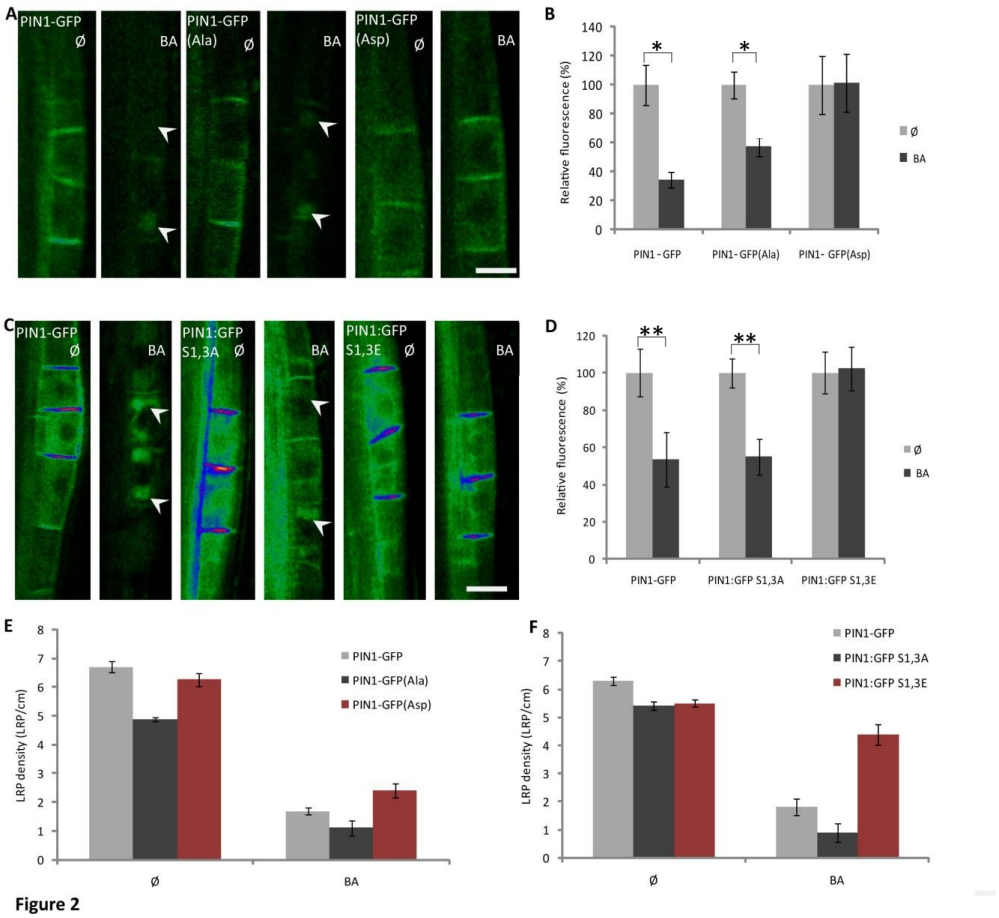

Figure 2. Loss- and gain-of-phosphorylation mutations of PIN1 modulate its cytokinin sensitivity.
(A - D) Gain-of-phosphorylation PIN1-GFP(Asp) and PIN1:GFPS1,3E, but not loss-of-phosphorylation PIN1-GFP-(Ala) and PIN1-GFPS1,3A alleles are BA insensitive (*p<0.05, **p<0.001, n=10 LRP). White arrows indicate vacuoles with GFP accumulation. The PIN1-GFP membrane signal was measured in stage-I LRP. **(E - F)** PIN1-GFP-(Asp) and PIN1:GFPS1,3E exhibit cytokinin-insensitive LR initiation (E) when grown on BA

containing media for seven days (*p<0.05, n= 15 roots). Scale bars: 20 µm. Error bars mark the standard error of the mean. BA - cytokinin derivate N^6-benzyladenine.

A well-established developmental function of cytokinin is to maintain the root meristem size by balancing the cell differentiation and division activities. Enhancement of the cytokinin signaling results in the reduced root apical meristem size (Dello Ioio *et al*., 2008). Analysis of the root meristem size in lines expressing either loss-of-phosphorylation PIN1-GFPS1,3A or phosphorylated PIN1-GFPS1,3E alleles revealed that roots expressing phosphomimic PIN1-GFP variants exhibit a dramatically reduced sensitivity to cytokinin. While cytokinin reduced the root meristem size in all wild-type, PIN1-GFP-(Ala) and PIN1-GFPS1,3A lines by 26% ± 8, 22% ± 3 and 22% ± 4 (n=15), respectively, in phosphomimic PIN1-GFP-(Asp) and PIN1-GFPS1,3E lines the root meristem size was not affected (Supplementary Figure 3a and b).

The data reveal that genetic modulations mimicking PIN1 phosphorylation reduce its sensitivity to cytokinin and weaken the cytokinin inhibitory effect on lateral root organogenesis and root meristem size. Thus we conclude that the degree of PIN1 protein phosphorylation might significantly affect its sensitivity to cytokinin.

Modulations in PINOID kinase (PID) and PP2A activity affect the cytokinin response.

Protein phosphatase PP2A and PINOID (PID) kinase act antagonistically on the phosphorylation of PIN proteins and directly impact on the apical-basal localization of the PIN auxin efflux carriers (Friml *et al*., 2004; Michniewicz *et al*., 2007). A PIN polarity shift towards the apical membrane was shown to be facilitated in *pp2A* loss-of-function mutants or by *PID* overexpression in *35S::PID* plants. Conversely, *pid* loss-of-function induces an apical-to-basal PIN1 polar targeting (Friml *et al*., 2004). Therefore, to further dissect the role of phosphorylation in cytokinin-mediated PIN1 degradation we analyzed mutants defective in PP2A and PID function. The cytokinin sensitivity of PIN1 in the endodermal cells of the root meristem of *pp2A* mutants (*pp2aa1, pp2aa1 pp2aa2, pp2aa1pp2aa3*) (Michniewicz *et al*., 2007) was examined using immunolocalization. Cytokinin treatment reduced the PIN1 membrane signal by 37% ± 12 (n=10) in wild-type roots and similarly a decrease of PIN1 was detected in the *pp2aa1* mutant background (30% ± 11; n=10) indicating that a lack of the RCN1 activity does not significantly interfere with cytokinin sensitivity. However, in the loss-of-function mutants *pp2aa2* and *pp2aa3*, lacking additionally the activity of one or two more

subunits of the PP2A complex, respectively, the cytokinin sensitivity was reduced and PIN1 lowered by 21% ±11 (n=10) and 19% ± 12 (n=10), respectively (Figure 3a and b).
To further test whether the changed PIN1 cytokinin sensitivity due to malfunction of the PP2A activity impacts the cytokinin-regulated root development, the root meristem size and lateral root organogenesis in the presence of cytokinin were analyzed. Cytokinin strongly inhibits lateral root initiation in wild-type roots (6,9 ± 0,2 LRP/cm versus 4,2 ± 0,1 LRP/cm, n=10 in cytokinin-treated roots) and reduces root meristem size by 24% ± 3 (n=15) (Figure 3c and Supplementary Figure 3a and b). While the *pp2aa1* loss-of-function mutation does not change root cytokinin sensitivity, roots of the *pp2aa2* and *pp2aa3* mutants exhibited a strong cytokinin resistance in both analyzed root parameters - lateral root initiation and root meristem size (Figure 3c and Supplementary Figure 3a and b).

PP2A phosphatase activity might be antagonized by PID kinase (Michniewicz *et al*., 2007). Therefore, we next examined whether enhancement or loss of PID activity, respectively, affects PIN1 cytokinin sensitivity and cytokinin-regulated root development. In roots overexpressing PID under control of the *35S* promoter, the PIN1 sensitivity to cytokinin was dramatically decreased when compared to cytokinin-treated control roots, shown by the 39% ± 14 reduced PIN1 membrane signal (Figure 3d and e). Accordingly, the root meristem size of the *35S::PID1* was not affected by cytokinin treatment (Supplementary Figure 4c and d).

Surprisingly, PIN1 cytokinin sensitivity was strongly reduced also in the *pid* loss-of-function mutant (Figure 5a and b). PID kinase belongs to the family of AGC kinases, and WAG1 and WAG2 homologs were identified recently as another PIN1 polarity-mediating kinases. However, similarly to the *pid* loss-of-function mutant, PIN1 cytokinin sensitivity was dramatically reduced in the *pid,wag1,wag2* tripple mutant background (Supplementary Figure 5c and d). These results indicated that the reduced PIN1 cytokinin sensitivity is most probably not due to the upregulation of *WAG1* or *WAG2* homologs in the *pid1* mutant. The reduced PIN1 cytokinin sensitivity in both *pid* and *pid,wag1,wag2* mutant backgrounds correlated with a cytokinin insensitive root development, including both lateral root initiation and root meristem size (Supplementary Figure 5c-f).
Altogether, these results show that modulations in kinase and phosphates activities impact the cytokinin-stimulated PIN1 targeting to lytic vacuoles and modulate root cytokinin sensitivity.

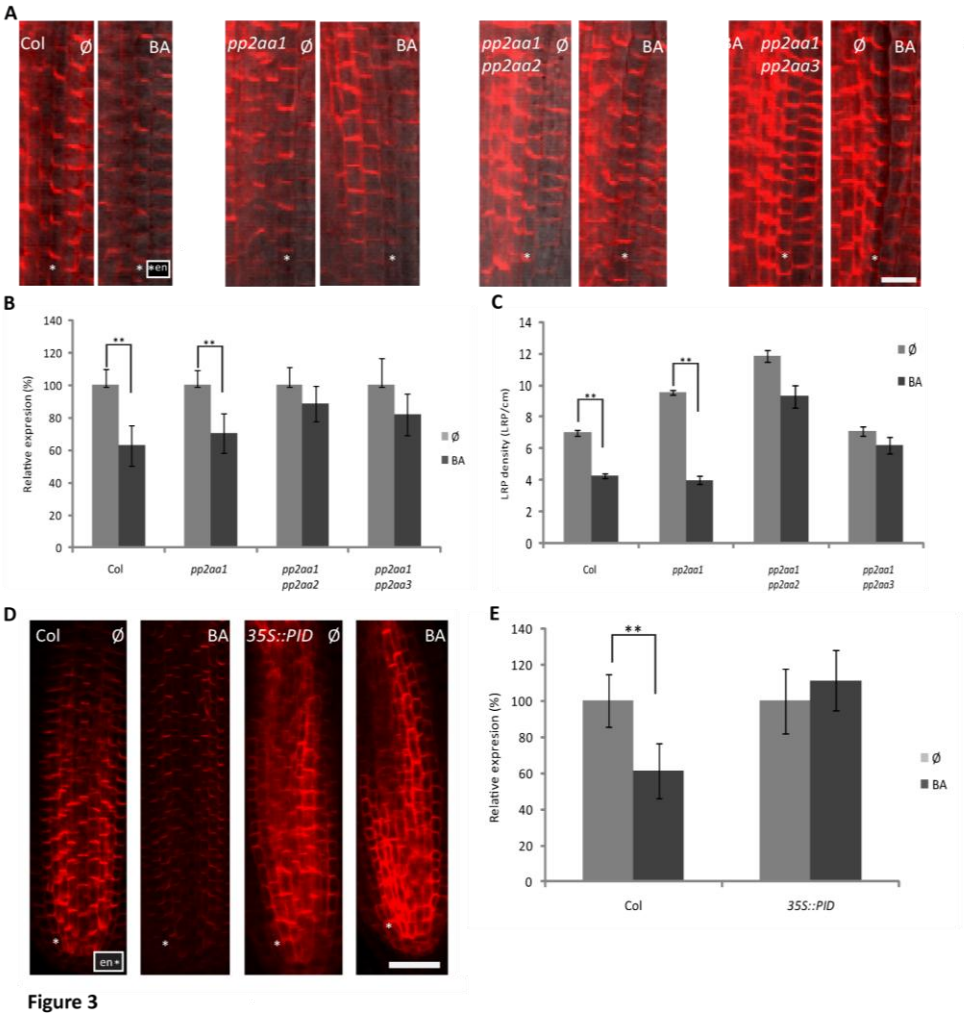

Figure 3

Figure 3. Reduced PP22 phosphatase or enhanced PID kinase activity affect PIN1 cytokinin sensitivity
(A, B and D, E) PIN1 is cytokinin insensitive in the endodermal cells (en) of *pp2aa1 pp2aa2* and *pp2aa1pp2aa3* (Scale bars: 30 µm) and *35S::PID* roots (Scale bars: 40 µm). (**C**) *pp2aa1 pp2aa2* and *pp2aa3 pp2aa1* exhibit cytokinin-insensitive LR initiation (*p<0.05, **p<0.001, n=10 roots, ten cells per root). The PIN1 signal is detected by immunolocalization in endodermal cells of the root meristem. White asterisks indicate the endodermal root cell files (en). BA - cytokinin derivate N^6-benzyladenine.

Cytokinin contribution to PIN1 polarity re-establishment during lateral root primordia organogenesis.

Proper LRP development depends on the auxin maxima formation coordinated by dynamic PIN polarity rearrangements. The switch from an anticlinal to transversal PIN1 polarity underlies redirecting of an auxin flux and promotes the establishment of the local auxin gradient with maxima at the lateral root primordia tip (Benková *et al*., 2003). Previously, we showed that cytokinin stimulates the lytic degradation of PIN1 from the anticlinal membrane at the first stage of lateral root primordia consisting of a few short initial cells (Marhavy *et al*., 2011). Apparent differences in PIN1 cytokinin sensitivity dependent on its phosphorylation state and polar membrane localization prompted us to investigate cytokinin effects in more developed LRP, where a distinct polarity of PIN1 at either the anticlinal or transversal

membranes are set up (Figure 4a). To evaluate cytokinin sensitivity of PIN1 respective to its polarity, LRP at stage III were treated with cytokinin and the PIN1 membrane signal at the anticlinal and transversal membranes were monitored in time (Figure 4a and b). Cytokinin led to a rapid decrease of PIN1 from the anticlinal membrane and the membrane signal was lowered by 30% ± 9 and by 60% ± 17 in three and seven hours of the treatment, respectively, when compared to untreated primordia. In contrast, PIN1 at the transversal membranes was reduced significantly less: about 10% ± 10 and 17% ± 7 in three and seven hours of treatment, respectively, (Figure 4c compared to d).

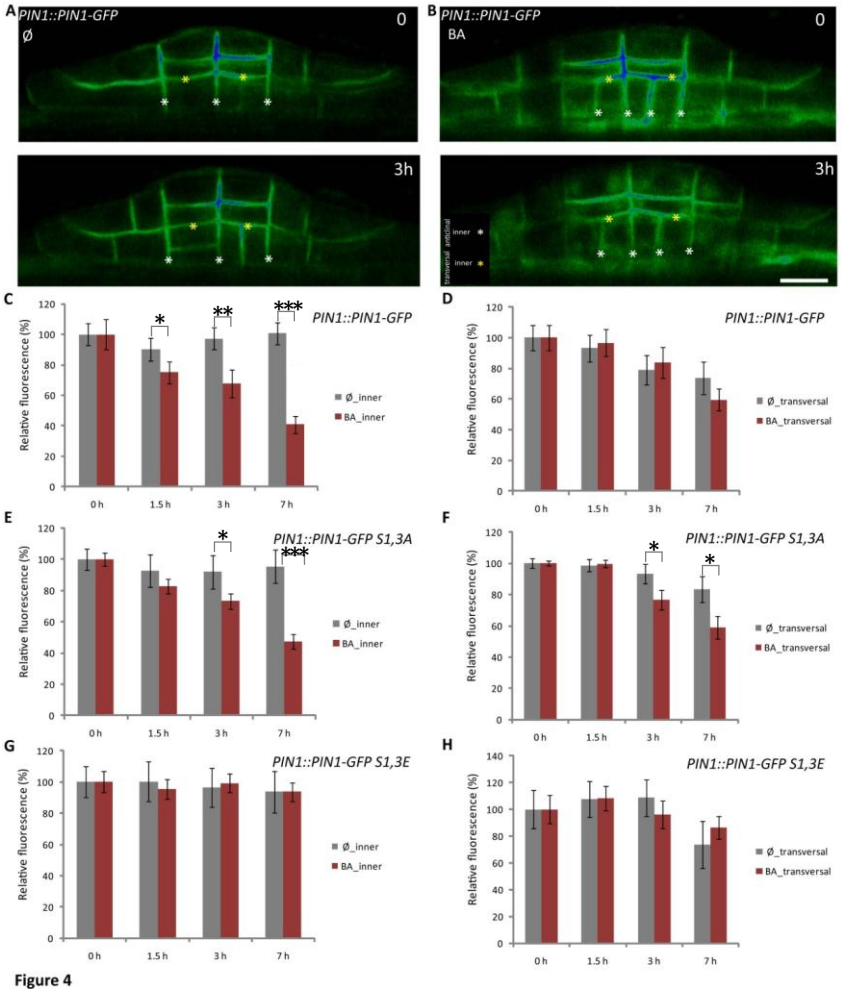

Figure 4. Cytokinin depletes PIN1 from anticlinal but not transversal membranes in lateral root primordia.
(A, B) Real time monitoring of PIN1-GFP in control **(A)** and with BA-treated **(B)** lateral root primordia. **(A – D)** PIN1-GFP localized on anticlinal membranes is more sensitive to BA compared to transversal membranes in wild type. **(E and F)** Loss-of-phosphorylation PIN1:GFPS1,3A at both anticlinal and transversal membranes is BA sensitive **(G and H)**, in contrast to the BA insensitive PIN1:GFPS1,3E allele. (*p<0.05, **p<0.001, n=5 LRP). White asterisks indicate the measured anticlinal membranes, yellow asterisks indicate transversal membranes. Roots were treated for 1.5 h, 3h and 7h with 0.1 µM BA. Scale bars: 40 µm. Error bars mark the standard error of the mean. BA - cytokinin derivate N^6-benzyladenine.

PIN1:GFPS1,3A and PIN1:GFPS1,3E loss- or gain-of-phosphorylation alleles, respectively, exhibited different sensitivities to cytokinin corresponding to their phosphorylation degree. While cytokinin-treated primordia of PIN1:GFPS1,3A roots dramatically decreased from both anticlinal and transversal membranes (20% ± 5, 53% ± 5; 22 ± 7.3, 28 ± 7.3) (Figure 4e and f), PIN1:GFPS1.E reacted differently, and the membrane signal on both transversal and anticlinal membranes was insensitive to cytokinin treatment (Figure 4g and h).

These results demonstrate, that cytokinin targets PIN1 with different efficiency when located at anticlinal or transversal membranes of lateral root primordia. An overall reduction in the PIN1 phosphorylation state sensitizes PIN1 sensitivity to cytokinin on both anticlinal and transversal membranes, while phosphomimic mutation reduces PIN1 sensitivity. Based on our results we hypothesize, that during lateral root organogenesis when PIN1 polarity from anticlinal to transversal membranes must be re-established, cytokinin might promote the elimination of a subset of PIN proteins from anticlinal membranes and thus reinforce the new PIN1 polarity arrangement in the developing organ.

DISCUSSION

Directional auxin transport is instructive for various developmental processes including organ formation or tropic responses (Reinhardt *et al*., 2003; Friml *et al*., 2002; Benkova *et al*., 2003). The direction of the auxin flow is largely defined by the membrane polarity of auxin efflux carriers (Wiśniewska *et al*., 2006; Petrasek *et al*., 2006). As shown by a number of studies, PIN polarity is highly dynamic and can change in response to different endogenous or environmental inputs such as induction of new organogenesis or tropic responses (Benkova *et al*., 2003; Heisler *et al*., 2010; Reinhardt *et al*., 2003; Friml et el.,2002; Kleine-Vehn *et al*., 2010). Different mechanisms were found to contribute to PIN polarity establishment including PIN protein phosphorylation (Huang *et al*., 2010; Zhang *et al*., 2010) controlled by antagonistic activities of PP2A phosphatase and PINOID/AGC kinases (Michniewicz, *et al*.,2007; Friml *et al*.,2004; Donukshe *et al*., 2010), Ca^{2+} signaling (Zhang *et al*.,2010) or mechanical stimuli affecting microtubules reorientation (Heisler *et al*., 2010).

Recent studies demonstrated that the plant hormone cytokinin is an effective modulator of polar auxin transport (Dello Ioio *et al*., 2008; Ruzicka *et al*., 2009; Pernisova *et al*., 2009). Cytokinin not only significantly impacts the transcription of several PIN genes, but was also found to directly interfere with endocytotic trafficking of PIN1 and to target it for lytic degradation into vacuoles. Detailed studies of cytokinin interference with PIN1 trafficking

pathways reveal that cytokinin selectively targets PIN1, recruited by the GNOM-ARF-GEF dependent pathway, towards basal membranes. In contrast, apically targeted PIN1 is largely insensitive to cytokinin treatment. Genetic manipulations increasing the proportion of phosphorylated PIN1 and promoting its apical localization, such as restriction of PP2A or enhancement of PID activities, respectively, or phosphomimic mutations of the PIN1 sequence, dramatically reduced PIN1 cytokinin sensitivity and led to cytokinin insensitive root development. Surprisingly, the reduction of PID kinase activity also resulted in a reduced PIN1 cytokinin sensitivity. Analysis of mutants lacking functions of two other PID homologs, WAG1 and WAG2, which were recently found to participate in PIN phosphorylation (Donukshe *et al*., 2010), limits the possibility that another known homolog from the same family might simply substitute the lack of PID activity at the presence of cytokinin. This result hints on the existence of an additional feedback mechanism linked to the PID pathway, which might contribute to the modulation of PIN1 sensitivity to cytokinin.

Selective interaction of cytokinin with a subset of PIN proteins might provide a mechanism for the rapid, membranę-specific elimination of auxin efflux carriers, which could be employed in developmental processes requiring PIN polarity re-establishment and redirection of the auxin flow. We demonstrate that during lateral root organogenesis, where a switch in PIN polarity from anticlinal towards transversal membranes is crucial for the formation of the auxin maxima and proper primordia development (Benkova *et al*, 2003), cytokinin application reduces PIN1 from anticlinal membranes with a significantly higher efficiency compared to transversal membranes. Altogether these data support the cytokinin role in mechanisms regulating PIN polarity re-establishment in developmental processes when auxin flow redirection is required.

Table 1 Real primordia

PIN1-GFP				PIN1:GFP S1,3A				PIN1:GFP S1,3E			
Ø		2µM BA		Ø		2µM BA		Ø		2µM BA	
from I to I	n=1	from I to I	n=10	from I to I	n=2	from I to I	n=3	from I to I	n=7	from I to I	n=4
from I to II	n=5	from I to III	n=1	from I to II	n=3	from I to II	n=1	from I to II	n=9	from I to II	n=6
from I to III	n=5	from II to II	n=13	from I to III	n=2	from II to II	n=7	from I to III	n=6	from I to III	n=1
from I to IV	n=1	from II to III	n=3	from I to IV	n=1	from II to III	n=1	from II to II	n=3	from II to II	n=4
from I to V	n=1			from II to II	n=5			from II to III	n=4	from II to III	n=2
from II to II	n=2			from II to IV	n=2			from II to IV	n=1	from II to IV	n=2
from II to III	n=5			from II to V	n=1					from II to V	n=1
from II to IV	n=2			from II to VI	n=1						
from II to V	n=1										
total	n=23	total	n=27	total	n=17	total	n=12	total	n=30	total	n=20
fully arested	n=3	fully arested	n=23	fully arested	n=7	fully arested	n=10	fully arested	n=10	fully arested	n=8
fully arested %	n=13.04%	fully arested %	n=85.18%	fully arested %	n=41.17%	fully arested %	n=83.33%	fully arested %	n=33.33%	fully arested %	n=40%
Em - emergence				red - primordia arrested in development				MS - Murashige Skoog medium			

EXPERIMENTAL PROCEDURES

Plant material

The transgenic *Arabidopsis thaliana* (L.) Heynh. lines have been described elsewhere: *PIN1::PIN1-GFP, DR5::GUS,DR5rev::GFP* (Benková *et al*., 2003); *PIN2::PIN2-GFP* (Xu and Scheres, 2005); *PIN2::PIN1-HA;eir1-1/pin2*, *PIN2::PIN1-GFP* (Wiśniewska *et al*., 2006); *35S::PIN1-GFP* (Ruzicka *et al*., 2007); *35::PID* (Benjamins *et al*., 2001); *pid14* (Huang *et al*., 2010), *wag1wag2, wag1wag2 pid* (Zhang *et al*., 2010); *rcn1, rcn1/rcnl(1-2-3), rcn1/L2-2* (Michniewicz *et al*.,2007); *PIN1::PIN1-GFP(Asp), PIN1::PIN1-GFP(Ala)* (Zhang *et al*., 2010); *PIN1::PIN1-GFPS1.3A, PIN1::PIN1-GFPS1.3E* (Huang *et al*.,); *cre1-12,* (Higuchi *et al*., 2004).

Growth conditions

Seeds of *Arabidopsis* (ecotype Columbia-0) were plated on 0.5 MS medium (Duchefa) with 1% sucrose, 0.8% or 1.2% agar (pH 5.7), and 0.5 MS medium (Duchefa) with BA (0.1 μM, 0.6 μM and 0.7 μM) 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 or on continues light at 21°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 22°C. Drugs and hormones used were: PI (10 μM), BA (0.1 μM and 2 μM).

Real-time analyses of membrane protein dynamics and GFP signal quantification.

The membrane GFP signal was quantified on scans of stage-I and stage-III LRP, root epidermal and cortex cells. Pictures were taken by a FV10 ASW confocal microscope (Olympus) with a 20 or 60 (water immersion) objective. For all the experiments, the scans were done with identical microscope and laser settings. At minimum 10 LRPs (2 anticlinal PMs in stage-I LRP) for stage-III 5 LRPs (2 PM from each layer were analyzed), 10 roots with 10 cells per root. Criteria for quantifications of membrane signals in particular tissues and microscope settings are described in (Marhavy *et al*., 2011). The fluorescence intensity of the membrane PIN-GFP signal was quantified with ImageJ (NIH; http://rsb.info.nih.gov/ij) as

described (Zadnikova *et al*., 2010). The statistical significance was evaluated with Student's *t*-test. Images were processed in Adobe Illustrator.

Analysis of primary root and LRP organogenesis

For real-time analysis of the LRP initiation and development was performed as described in (Marhavý *et al*., 2011). For phenotypic analyses of root growth, LR initiation and development, at least 20 seedlings were processed. The LRP density was analyzed in 8-day-old seedlings as described (Malamy and Benfey, 1997; Benková *et al*., 2003). Root growth parameters (root length and root meristem) were analyzed with the ImageJ software (NIH; http://rsb.info.nih.gov/ij) as described (Ruzicka *et al*., 2009).

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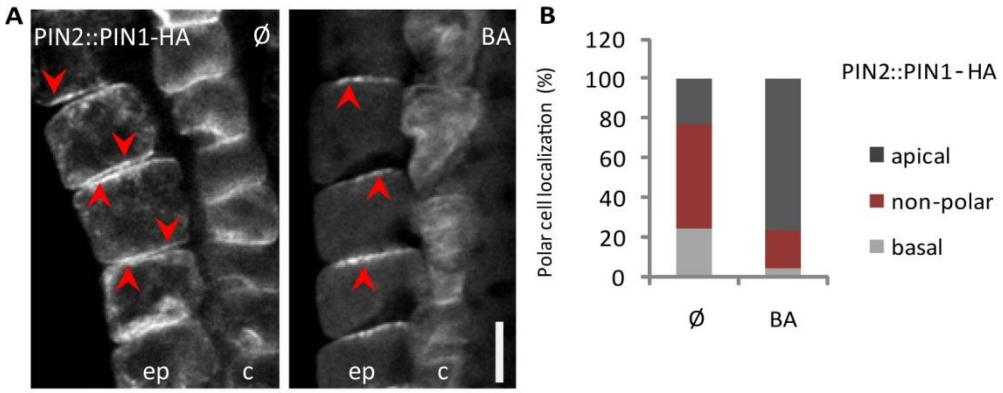
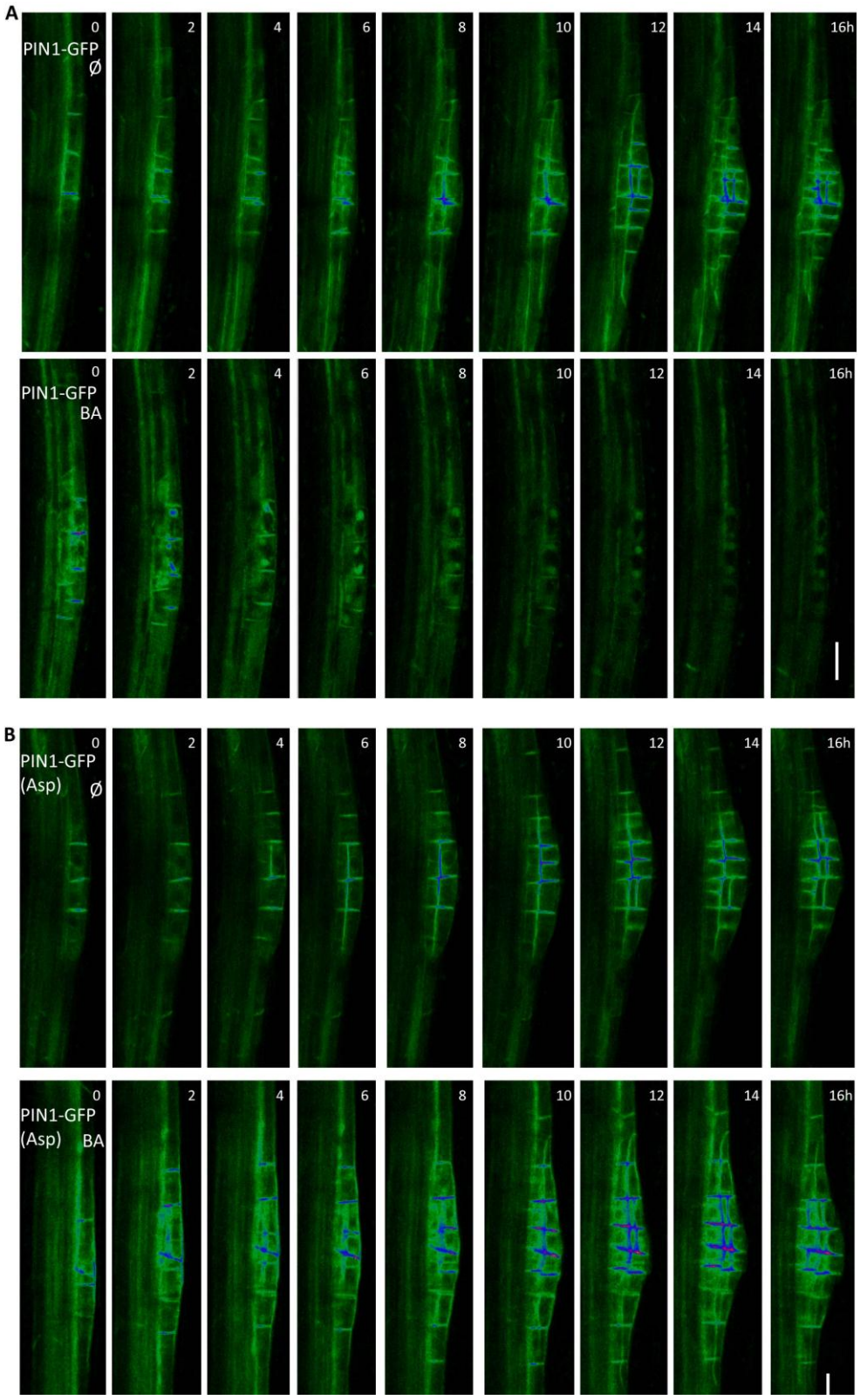
SUPPLEMENTARY FIGURES

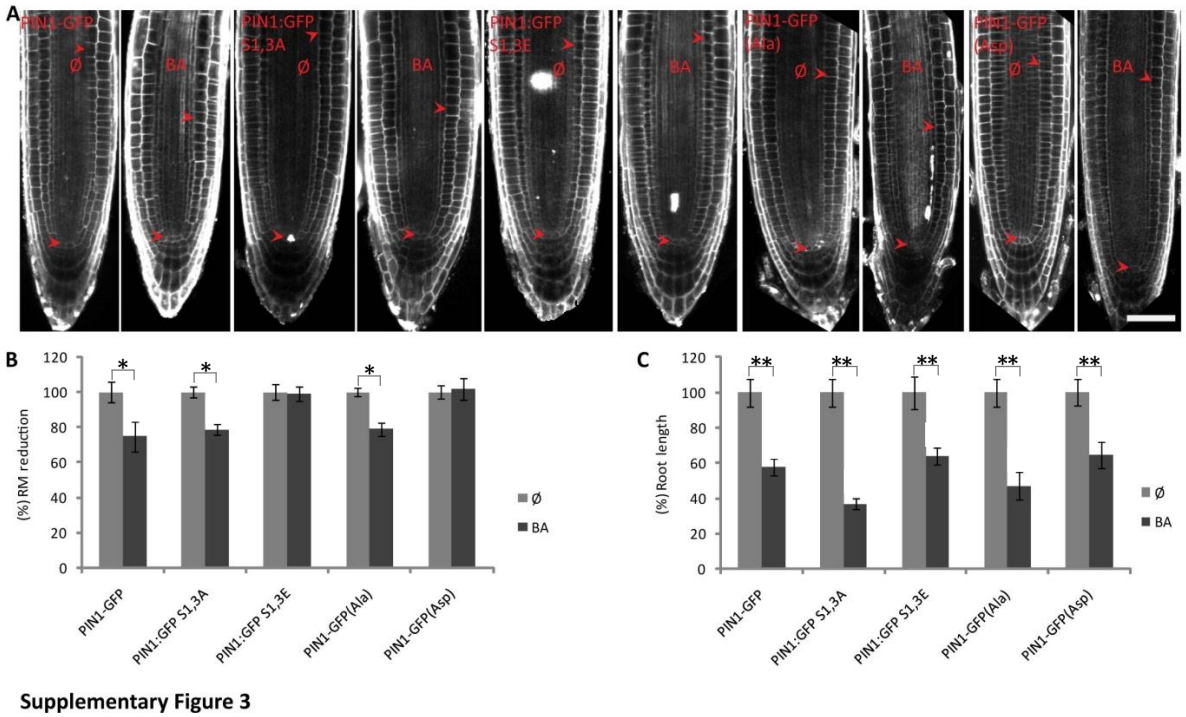


Supplementary Figure 1

Supplementary Figure 1
(A, B) PIN1-HA localization in epidermal (ep) and cortex (co) cells, as shown by immunolocalization of PIN1. *PIN2::PIN1-HA;pin2* shows predominantly basal and nonpolar PIN1-HA localization. Cytokinin depletes PIN1-HA from basal membranes of root epidermal cells of and increases the proportion of cells with apically localized PIN1-HA. Seedlings were grown 6 day on 0.6 µM BA. Scale bars: 15 µm.



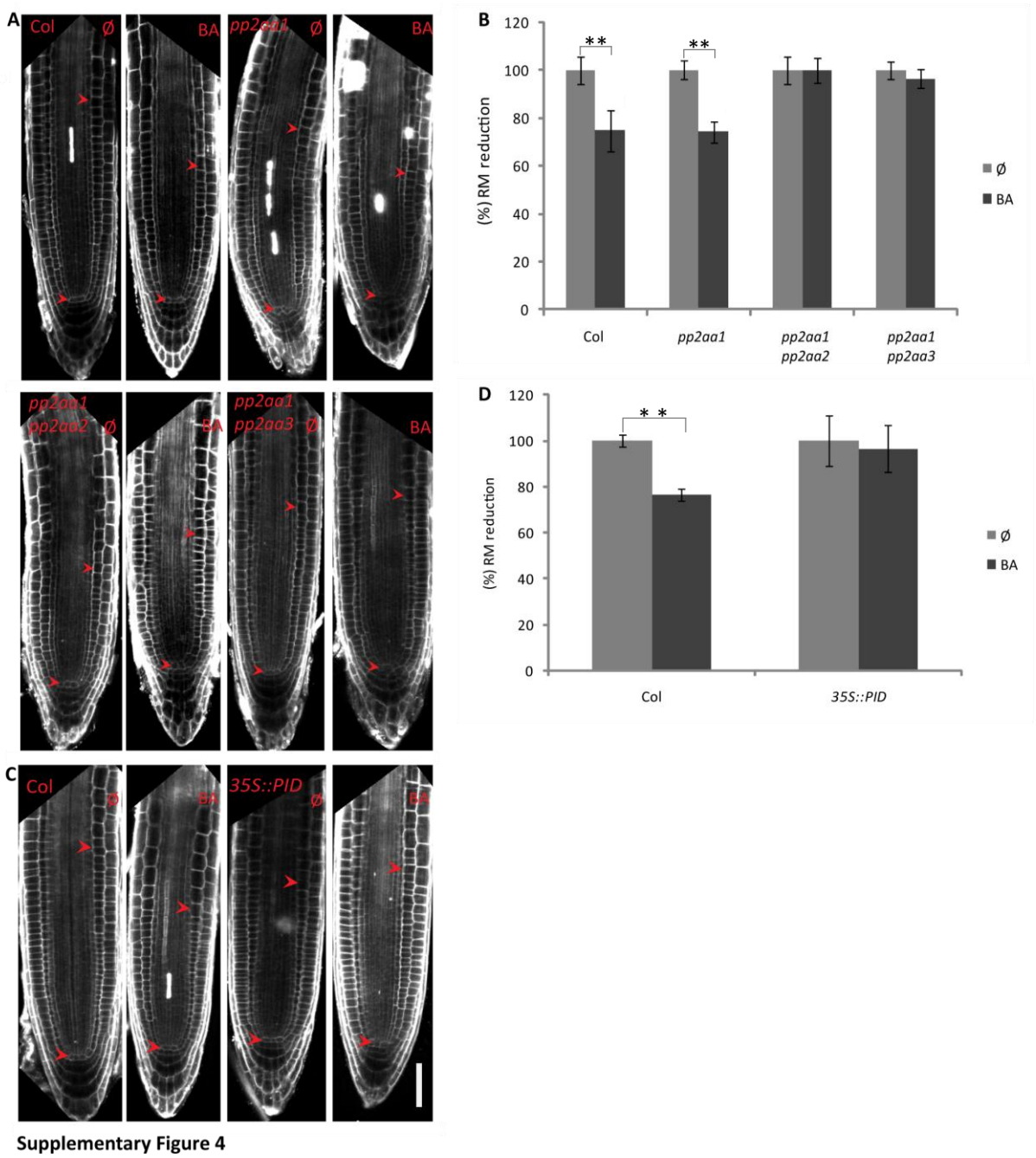
Supplementary Figure 2
(**A, B)** Cytokinin treatment stimulates PIN1-GFP degradation and inhibits LRP development **(A)**, in contrast to cytokinin insensitive PIN1-GFP-(Asp) correlating with cytokinin insensitive LRP development **(B)**. Presented are representative real time analysis of lateral root primordia development on control medium (Ø) and in the presence of cytokinin (0.1 µM BA) (n=10 primordia per line and treatment). Scale bars: 20 µm. BA - cytokinin derivate N^6-benzyladenine.



Supplementary Figure 3

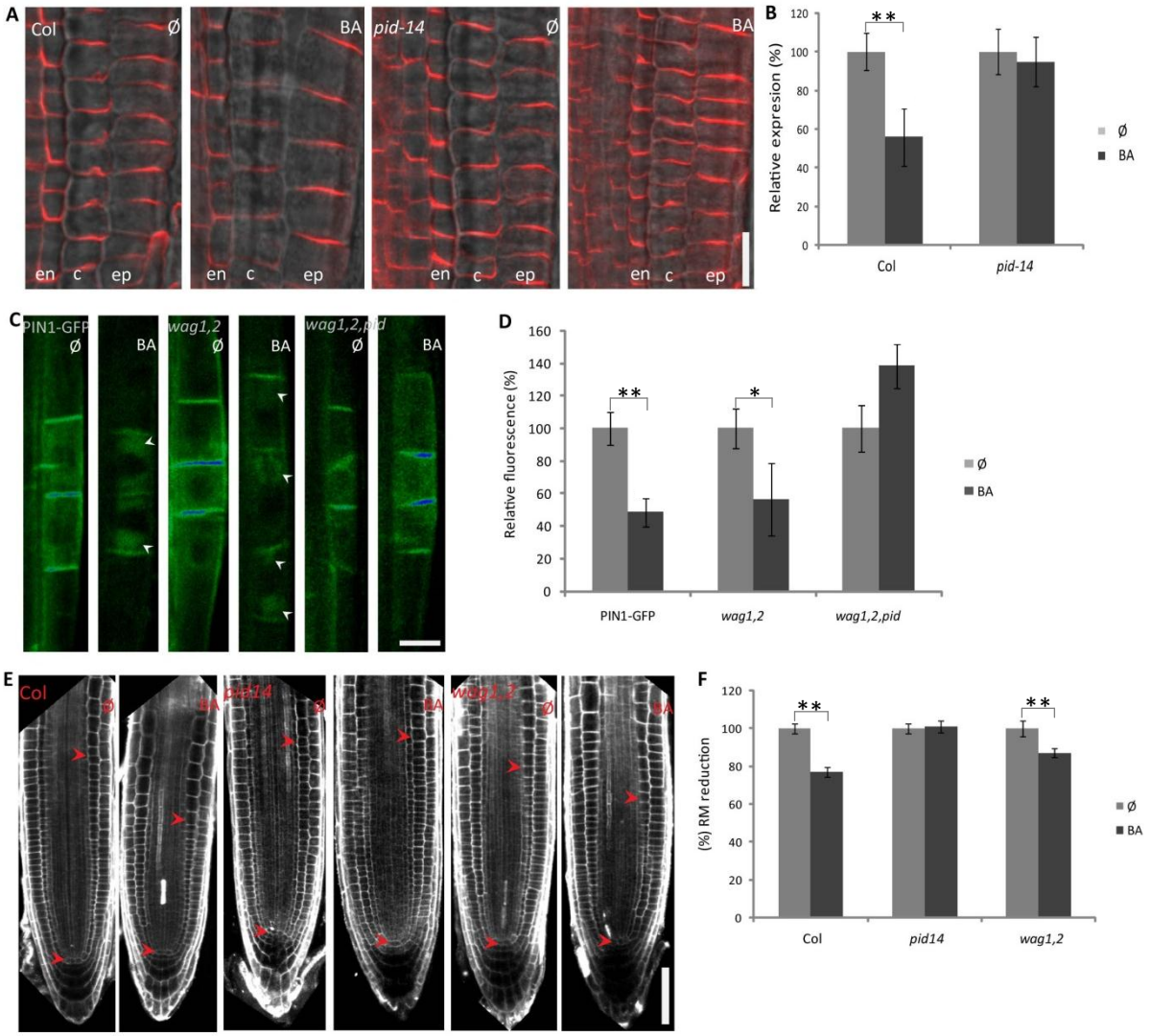
Supplementary Figure 3

(A - C) Root meristems expressing PIN1-GFPS1,3E and PIN1-GFP-(Asp) gain-of-phosphorylation alleles are cytokinin resistant, in contrast to roots expressing loss-of-phosphorylation PIN1-GFPS1,3A and PIN1-GFP-(Ala). Error bars mark the standard error of the mean (*p<0.05, **p<0.001, n=10 roots). Scale bars: 50 µm. Seedlings grown on 0.1 µM BA for 6 days. Scale bars: 50 µm. BA - cytokinin derivate N^6-benzyladenine.



Supplementary Figure 4

Supplementary Figure 4
Root meristems of *pp2aa1 pp2aa2*, *pp2aa1 pp2aa3* (**A, B**) and 35S::PID (**C, D**) lines are cytokinin insensitive (*p<0.05, **p<0.001, n=10 roots). Arrows indicate the distance between the quiescent centre (QC) and the cortex transition zone. Seedlings grown on 0.1 µM BA for 6 days. Scale bars: 50 µm. Error bars mark the standard error of the mean. BA - cytokinin derivate N^6-benzyladenine.



Supplementary Figure 5

Supplementary Figure 5
(**A and B**) PIN1 is insensitive to cytokinin-stimulated degradation in the *pid 14* loss-of-function mutant (**p<0.01, n=10 roots, ten cells per root). PIN1 was visualized by immunolocalization in control root meristems and in root meristems treated with 0.1 µM BA for 1.5 h. The membrane signal in endodermal cells (en) was quantified. Scale bars: 15 µm. (**C and D**) PIN1-GFP is BA sensitive in roots of the control (Col-0) and *wag1,wag2* lines, but not of the *wag1,wag2,pid* mutant. The PIN1-GFP membrane signal was measured in stage-I LRP 1.5 h after BA treatment. White arrows indicate vacuoles. (*p<0.05, **p<0.001, n=10 LRP). Scale bars: 20 µm. (**E and F**) Root meristem size of *pid14* and *wag1,wag2,pid* mutants are BA insensitive in contrast to Col-0 and *wag1,wag2* (**p<0.001, n=10). Red arrows indicate the distance between the quiescent centre (QC) and the cortex transition zone. BA - cytokinin derivate N^6-benzyladenine.

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Chapter 4.

Auxin Re-flux between Endodermis and Pericycle Controls Lateral Root Initiation

Adapted from

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Auxin Re-flux between Endodermis and Pericycle Controls Lateral Root Initiation (manuscript in preparation).

PM, MV and EB initiated the project and designed most of the experiments, PM and MV carried out most of the experiment. BDR contributed to the construction of the plasmids and assisted in critical revising of the manuscript. MB and TB assisted in critical revising of the manuscript. MV, PM and EB wrote the manuscript, all authors saw and commented on the manuscript.

Auxin Re-flux between Endodermis and Pericycle Controls Lateral Root Initiation

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Lateral root formation is initiated when a pericycle cell accumulates auxin, thereby acquires the founder cell status and divides asymmetrically. How this auxin maximum in the pericycle cells builds up and remains focused, is not understood. Here, we reveal that the endodermis plays an active role in the regulation of auxin accumulation in founder cells and that this function is ultimate for the progress from the founder cell into the lateral root initiation phase. The developmentally specific auxin moves from endodermal to founder cells, so called auxin re-flux, and mediated through PIN3 (PIN-formed) auxin efflux carrier.

Lack of this re-flux causes dramatic defects in the progress of founder cells towards the next initiation phase. Our data identify an unexpected regulatory function for the endodermis in lateral root initiation as part of the fine-tuning mechanism which might act as one of the check-points in LR organogenesis after FCs are specified.

INTRODUCTION

Lateral roots originate from the pericycle cells, that are embedded as a single tissue layer within the Arabidopsis root (Beeckman *et al*., 2001; Dubrovsky *et al*., 2000). This new developmental program starts when a restricted number of pericycle cells acquire the founder cell (FC) identity and divide asymmetrically. Accumulation of the plant hormone auxin is one of the earliest events taking place during FC specification tightly preceding lateral root initiation (LRI) (Dubrovsky *et al*., 2008). Interference with this auxin increase by chemical or genetic inhibition of auxin transport or the downstream auxin response, leads to severe defects in FC cell establishment and LRI (Benková *et al*., 2003; Casimiro *et al*., 2001; Dharmasiri *et al*., 2005; Fukaki *et al*., 2002; Vanneste *et al*., 2005). Despite the advanced insights into mechanisms of auxin transport in roots, it remains unclear how the auxin threshold is reached in selected pericycle cells for lateral roots to initiate although several hypothesis where recently put forward to address this question (De Smet *et al*., 2007; Laskowski *et al*., 2008; Lucas *et al*., 2008; Moreno-Risueno *et al*., 2010). In *Arabidopsis*, the PIN family of auxin efflux carrier proteins is part of the polar auxin transport machinery determining the direction of the auxin flow and thus, auxin levels within plant tissues (Wiśniewska *et al*., 2006). Among them, PIN3 was shown to play an important role during LRI (Benková *et al*., 2003; Laskowski *et al*., 2008). Here, we provide insight into the mechanism of FC transition to LRI and show that PIN3 defines a developmentally specific auxin re-flux that enables FCs to reach auxin threshold levels to initiate lateral roots.

RESULTS

PIN3 is Transiently Induced in the Endodermis during Lateral Root Initiation

To get insight into how PIN3 contributes to LRI, we thoroughly examined *PIN3* expression in the root. As expected, we found *PIN3::PIN3-GFP* (Žádníková *et al*., 2010) expression in the stele including the pericycle cells (Benková *et al*., 2003) (Figure 1A; Figure S1). Remarkably, the PIN3-GFP signal was also detected in the endodermal cell overlaying the FCs and initiating LR primordia at stage I (Malamy *et al*., 1997), while the neighboring endodermal cells were lacking the signal (Figure 1A; Figure S1C). Detailed monitoring of PIN3-GFP in the root endodermis revealed that PIN3 expression varied along the longitudinal axis (Dolan *et al*., 1993). Starting from the most distal end, through the root meristem (RM) and the elongation zone (EZ), a continuous PIN3 signal was detected (Figure S1A). Unlike in the

differentiation zone (DZ), where cells obtain signs of terminal maturation encompassing xylem strand differentiation (Dolan *et al*., 1993), this signal disappeared and PIN3 was exclusively detectable in endodermal cells overlaying LRI sites (Figures S1A and S1C).

To obtain a spatio-temporal view on the dynamics of *PIN3* expression during pre-initiation and LRI phases, real time analysis of LRI were performed using the previously established assay for induction of LRI through mechanical root bending (Laskowski *et al*., 2008; Marhavý *et al*., 2011) (for details see Experimental Procedures). Local enhancement of auxin activity in the pericycle cells monitored by the *DR5pro::N7:Venus* (Heisler *et al*., 2005) nuclear auxin sensitive reporter was considered as the earliest sign of the FC specification (Dubrovsky *et al*., 2008). Directly after bending, *PIN3-GFP* expression was exclusively observed in the vascular tissues (Figure 1G). At 90 min, an enhanced nuclear signal (Figure 1G; red arrowhead) in a restricted pericycle cell indicated FC establishment (Figure 1G; yellow arrowheads). Shortly thereafter (at 120 min), the PIN3-GFP signal was induced in the adjacent endodermal cells (Figure 1G; white arrowheads) and remained there during the entire LRI phase. No *PIN3-GFP* expression in the endodermis could be detected prior to this DR5 activation in the pericycle cells. As LR primordia progressed to the next developmental phase around 900 min, PIN3-GFP in the endodermis gradually disappeared and was absent from the endodermis once the developing primordium obtained three cell layers around 1020 min (Figure 1G). From this developmental stage on, cortex *PIN3-GFP* expression could be observed (Figure S1C). Thus, the transient *PIN3* expression in the endodermis takes place in a narrow window encompassing FC establishment and LRI.

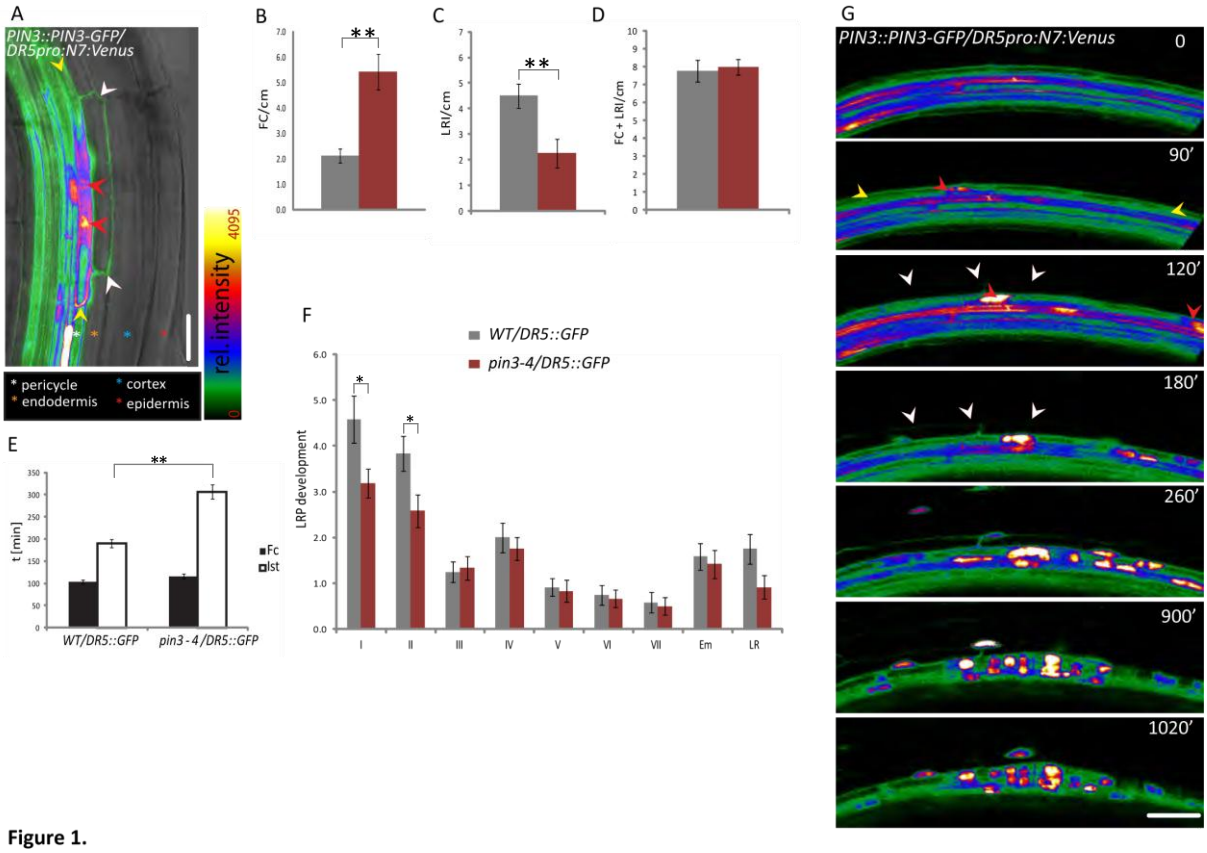

Figure 1.

Figure 1. The *pin3* Mutation Causes a Delay in the Transition from the FC Stage to LRI

(A) *PIN3::PIN3-GFP* expression during LRI. PIN3-GFP was detected in FC pericycle cells (yellow arrowheads), and the overlaying endodermis cell (white arrowheads). Note that the neighbouring endodermal cells are not labelled with the GFP signal. Red arrowheads indicate the migrated nuclei with an enhanced *DR5pro::N7:Venus* (Heisler *et al*., 2005) auxin response. A semiquantitive color-coded heat-map of *the GFP fluorescence intensity* is provided. Color-coded asterisks indicate the different root cell files. (B) FC density is increased in *pin3* mutants compared to WT. (C and D) LRI density is decreased in *pin3* mutants (C), whereas the total of FCs+LRI densities is similar to WT (D).

(E) Timing of FC establishment and LRI after root bending, indicates a delay between FC establishment and LRI in *pin3* mutants compared to WT. (F) Stage distribution of LRP shows a decrease in stage I and stage II LRP in *pin3* mutants. (G) Real time analysis of *PIN3::PIN3-GFP* expression in the endodermis (white arrowheads) relative to FC establishment and LRI. FC establishment and LRI was followed by the accumulation of the nuclear *DR5pro::N7:Venus* (Heisler *et al*., 2005) signal (red arrowheads) in the pericycle FCs (yellow arrowheads) and the division of these nuclei. Time series, depicted is a representative example from at least 10 observations, is relative to root bending (E and G). Error bars represent standard error (SE), (n = 10-20). P values are *<0.05, **P<0.01; Student's t-test. Scale bars represent 25 µm (A) and 20 µm (G). See also Figures S1-S3.

PIN3 Controls the Transition from Founder Cells to the Lateral Root Initiation Phase

To dissect the developmental role of this spatially and timely confined expression of *PIN3* in the endodermis, we re-examined the LRI defect in the *pin3* mutant and focused specifically on the earliest stages of LR formation. The *DR5rev::GFP* (Friml *et al*., 2003) auxin reporter expressed from the FC stage onwards (Dubrovsky *et al*., 2008), was used to analyze densities of FCs (pericycle cells exhibiting *DR5* expression, but no division scored) and LRI events in 5 day old *pin3* mutant and WT roots. As expected, *pin3* mutants displayed a significant decrease in LRI density (Benková *et al*., 2003; Laskowski *et al*., 2008) (4,5 ± 0,5 in WT; 2,3

± 0,6 in *pin3*; n = 10) (Figure 1C). Surprisingly, FC density was dramatically increased in the *pin3* background (2,1 ± 0,3 in WT; 5,4 ± 0,6 in *pin3*; n = 10) (Figure 1B), whereas the total FC plus LRI density remained equal in both WT and the *pin3* mutant (Figure 1D). Scoring of FC and LRI densities in 7 day old WT and *pin3* seedlings confirmed the results obtained in 5 day old roots (Figures S2C-S2F), thus indicating the age independent character of this phenotype. In addition, monitoring of FC and lateral root primordia (LRP) along the acropetal root growth axis revealed that the accumulation of FCs mainly occurred at the distal root end (Figures S2A and S2B), in accordance with the overall acropetal development of lateral root formation described in *Arabidopsis* (Dubrovsky *et al*., 2006).

The increased accumulation of FCs alongside with the decrease in LRI events in the *pin3* mutants, hints on defects in the transition between the FC and LRI. To corroborate this hypothesis, LRI was followed in real time. Typically, in control roots, *DR5* expression in individual pericycle cells, indicative for FC establishment, was induced around the same time after bending in both WT and the *pin3* background (Figure 1E; Figures S2H and S2I). However, while the transition from FC to LRI required on average 100 min (103 ± 10, n = 10) in WT roots, in *pin3* mutants the time was approximately twice as long (206’ ± 16, n = 10) (Figure 1E; Figures S2H and S2I). Hence, *pin3* mutants are delayed in the progression from FC establishment to LRI. To assess the impact of *pin3* loss of function on the subsequent developmental phases of the lateral root organogenesis, we examined the number of the individual stages of lateral root development (Malamy *et al*., 1997) in 7 day old roots. Compared to WT, differences were mostly pronounced in the earliest developmental stages (Figure 1F; Figure S2G). Notably, we observed a decrease in the number of stage I (4,6 ± 0,5 in WT; 3,2 ± 0,3 in *pin3;* n = 20), whereas the following developmental stages were not significantly affected.

Altogether, these data demonstrate that PIN3 activity is required for the transition from the FC stage to LRI.

PIN7 Controls Lateral Root Initiation Differently from PIN3

PIN3 is most closely related to the *PIN7* homolog of the PIN family of auxin efflux carriers (Paponov *et al*., 2005). Both genes share common expression domains and have been shown to act redundantly in numerous plant developmental processes (Benková *et al*., 2003; Blilou *et al*., 2005a; Devlin *et al*., 2003; Friml *et al*., 2003; Paponov *et al*., 2005). To explore the functional redundancy of PIN7 and PIN3 in the early phases of LRI, we examined the expression of *pPIN7::PIN7-GFP* (Blilou *et al*., 2005a) in the root (Figure S3A). As expected,

PIN7-GFP was detected in the central root cap and the stele, similar to PIN3-GFP (Blilou *et al*., 2005b) (Figure S1A compared to S3A). However, *PIN7* expression was not detected in the neighboring endodermal cells in the RM and EZ. The root was subsequently scanned for LRI events. No PIN7-GFP signal was observed in the overlaying endodermal cells (Figure S3A). Hence, expression in the endodermis during LRI is specific to *PIN3*. It has been reported previously that the *pin7* mutation affects the total number of initiated LRP (Benková *et al*., 2003). Although the density of FCs in the *pin7* mutant was significantly increased (2,9 ± 0,2 in WT; 4,6 ± 0,4 in *pin7;* n = 10) (Figure S3C), real time monitoring of the LRI showed that timing of FC specification (140’ ± 11’ in WT; 174’ ± 30’ in *pin7;* n = 10) and the transition from FC to LRI (118,7’ ± 15,4’ in WT; 138,3’ ± 17,7’ in *pin7;* n = 10) was not affected (Figures S3B and S3E). This indicates that lack of PIN7 activity interferes with mechanisms underlying the frequency of FC specification, but not with the FC-LRI transition. The developmental consequences of the *pin7* mutation were manifested by an accumulation of primordia in stage I (3,9 ± 0,3 in WT; 4,82 ± 0,5 in *pin7;* n = 20), raising the density of LRI events (Figure S3F). Thus, both the endodermal expression accompanying LRI and the control over FC-LRI transition are specific to *PIN3.*

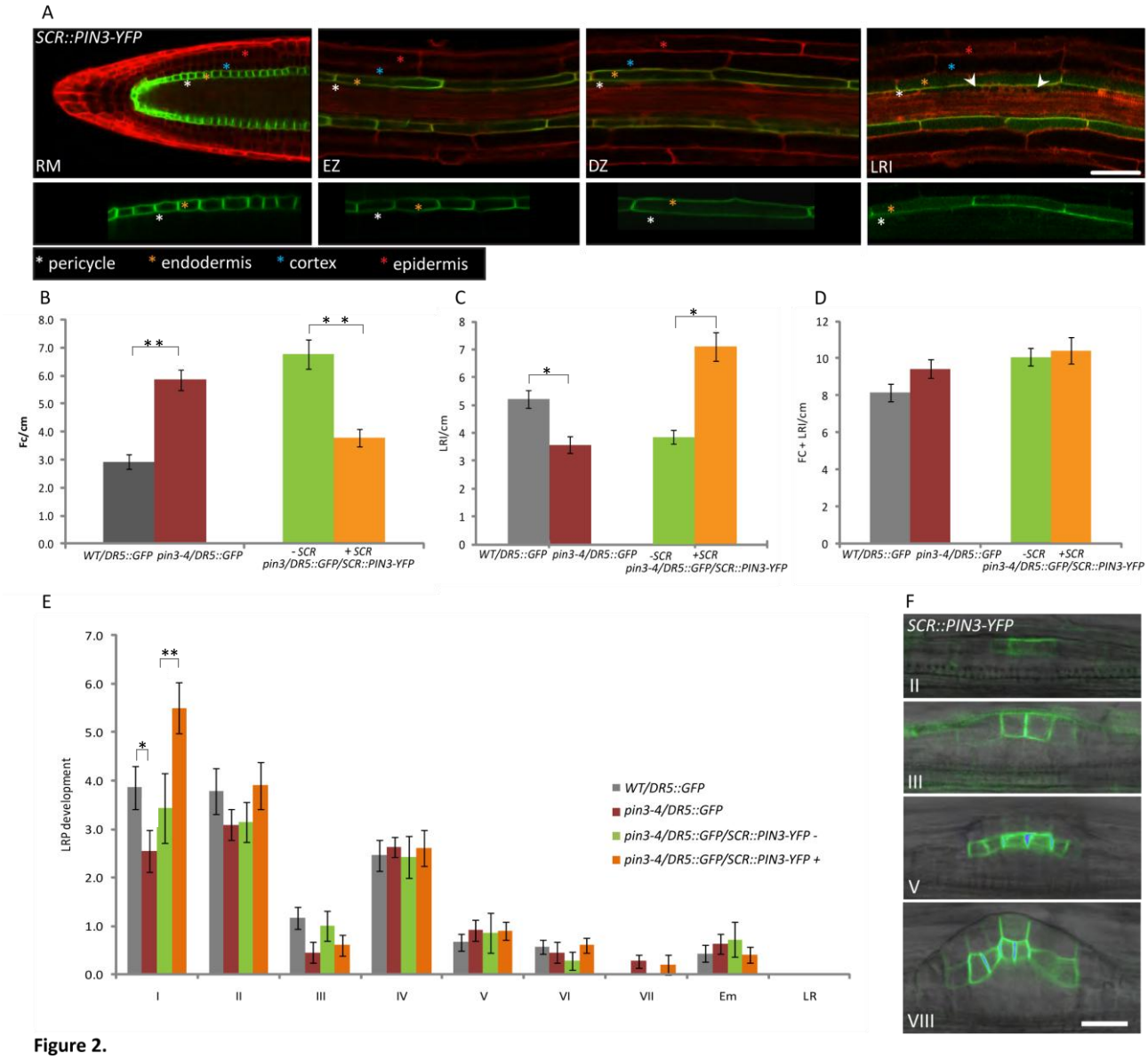

Figure 2.

Figure 2. PIN3 Activity in the Endodermis is Required for FC Progression

(A) Monitoring of *SCR*::*PIN3-YFP* expression (green) in the root meristem (RM), elongation zone (EZ), differentiation zone (DZ), and during LRI (white arrowhead). Propidium iodide (PI) counterstain (red) is shown in the upper panel. The lower panel reveals PIN3 polarity in the endodermal cells of the depicted root zones. Color-coded asterisks indicate the different root cell files.(B and C) FC (B) and LRI (C) densities are rescued in the *pin3/DR5::GFP* roots expressing the *SCR::PIN3-YFP* in the endodermis. (D) The total number of FCs+LRI densities is similar to WT in all genetic backgrounds. (E) Stage distribution of LRP indicates that the decrease in stage I in *pin3/DR5::GFP* mutants is restored in the fluorescent positive *pin3/DR5::GFP/SCR::PIN3-YFP* seedlings compared to *WT/DR5::GFP*. +SCR and –SCR refer to the pooled fluorescent positive and negative seedlings, respectively, from a segregating population in the stable *pin3/DR5::GFP* background (B-E). Error bars represent SE (n = 10-20). P values are *<0.05, **P<0.01; Student's t-test. Scale bars respresent 50 μm (A) and 20 μm (F).

PIN3 Activity in the Endodermis is Required for Founder Cell Progression to Lateral Root Initiation

The complex *PIN3* expression pattern encompassing both the pericycle and the endodermis during LRI, raises the question on the functional contribution of PIN3 in the endodermal cells to the regulation of LRI, notably the transition between the FC to LRI stage. To discern whether PIN3 activity in endodermis cells is required for the proper progression of this developmental event, we set up a tissue-specific complementation assay. The endodermis specific *SCR* promoter was selected to drive a *PIN3-YFP* fusion (Žádníková *et al*., 2010) in the *pin3/DR5::GFP* background. As expected, the *SCR* promoter restricted *PIN3-YFP* to the endodermal cells of the RM, EZ, and the DZ (Figure 2A). Importantly, the *SCR* promoter was active in those endodermal cells overlaying LRI, but no *PIN3* expression was detected in the dividing pericycle cells in the stage I LRP (Figure 2A). Only later from the developmental stage II on, *SCR::PIN3-YFP* expression could be detected in the outer layers of the LRP (Figure 2F). Expression of *PIN3-YFP* exclusively in the endodermis was sufficient to fully rescue FCs (2,9 ± 0,2 in *WT/DR5::GFP*; 5,8 ± 0,3 in *pin3/DR5::GFP*; 6,8 ± 0,5 and 3,2 ± 0,3 in *pin3/DR5::GFP/SCR::PIN3-YFP* fluoresence – and + respectively*;* n = 10) and LRI density (5,2 ± 0,3 in *WT/DR5::GFP*; 3,6 ± 0,3 in *pin3/DR5::GFP*; 3,8 ± 0,3 and 7,1 ± 0,5 in *pin3/DR5::GFP/SCR::PIN3-YFP* fluoresence – and + respectively*;* n = 10) (Figures 2B and 2C). Accordingly, the decrease in density of stage I LRP was eliminated (3,9 ± 0,4 in *WT/DR5::GFP*; 2,5 ± 0,4 in *pin3/DR5::GFP;* 3,4 ± 0,7 and 5,5 ± 0,5 in *pin3/DR5::GFP/SCR::PIN3-YFP* fluoresence – and + respectively; n = 20) (Figure 2E). To further corroborate our findings, the promoter of *CASP1*, a recently identified endodermis specific gene (Roppolo *et al*., 2011), was used. Unlike the *SCR* promoter, endodermis specific expression of *PIN3-YFP* driven by the *CASP1* promoter was restricted to the differentiating part of the root (Roppolo *et al*., 2011) (Figure S4A). Introduction of *CASP1::PIN3-YFP* in the *pin3/DR5::GFP* background completely restored the FC (3,7± 0,3 in *WT/DR5::GFP*; 7,7 ± 0,2 in *pin3/DR5::GFP*; 8,3 ± 0,4 and 5,5 ± 0,5 in *pin3/DR5::GFP/CASP1::PIN3-YFP* fluoresence – and + respectively*;* n = 10) and LRI density (8,2 ± 0,6 in *WT/DR5::GFP*; 4,5 ± 0,3 in *pin3/DR5::GFP*; 5,3 ± 0,7 and 9,4 ± 0,7 in *pin3/DR5::GFP/CASP1::PIN3-YFP* fluoresence – and + respectively*;* n = 10) (Figures S4B and S4C). Also the decrease in stage I LRP was rescued (5,0 ± 0,4 in WT/*DR5::GFP*; 3,7 ± 0,6 in *pin3/DR5::GFP;* 4,1 ± 0,7 and 6,9 ± 0,4 in *pin3/DR5::GFP/CASP1::PIN3-YFP* fluoresence – and + respectively; n = 20) (Figure S4E). Altogether these data show that PIN3 activity in the endodermis adjacent to the FCs and LRI, is required for FC progression to LRI. Furthermore, the data exclude a role of

endodermal PIN3 in the distal root zones, encompassing the basal meristem, from contribution to this FC-LRI regulatory module.

To determine whether PIN3 activity in the root stele participates in the regulation of this developmental event, the *SHR* promoter that is active in the root stele (Helariutta *et al*., 2000) was used to drive *PIN3-YFP* expression in the *pin3*/*DR5GFP* background. The *SHR* promoter activated *PIN3-YFP* in the stele of the RM, EZ, and DZ, comprising the dividing pericycle cells (Lucas *et al*., 2011) (Figures 3A and 3C). The PIN3-YFP signal in the stele masked any *DR5::GFP* signal in the pericycle, thus hindering the scoring of FCs and LRI events. A distribution of the different stages of lateral root development however clearly showed that expression of *PIN3-YFP* in the stele did not increase the number of stage I primordia in the *pin3* background (5,7 ± 0,4 in WT; 4,8 ± 0,5 in *pin3; 4*,5 ± 0,4 and 4,7 ± 0,4 in *pin3/DR5::GFP/SHR::PIN3-YFP* fluoresence – and + respectively) (Figure 3B). Hence, PIN3 action in the stele, including the pericycle cells, does not significantly contribute to the transition from FC establishment to LRI.

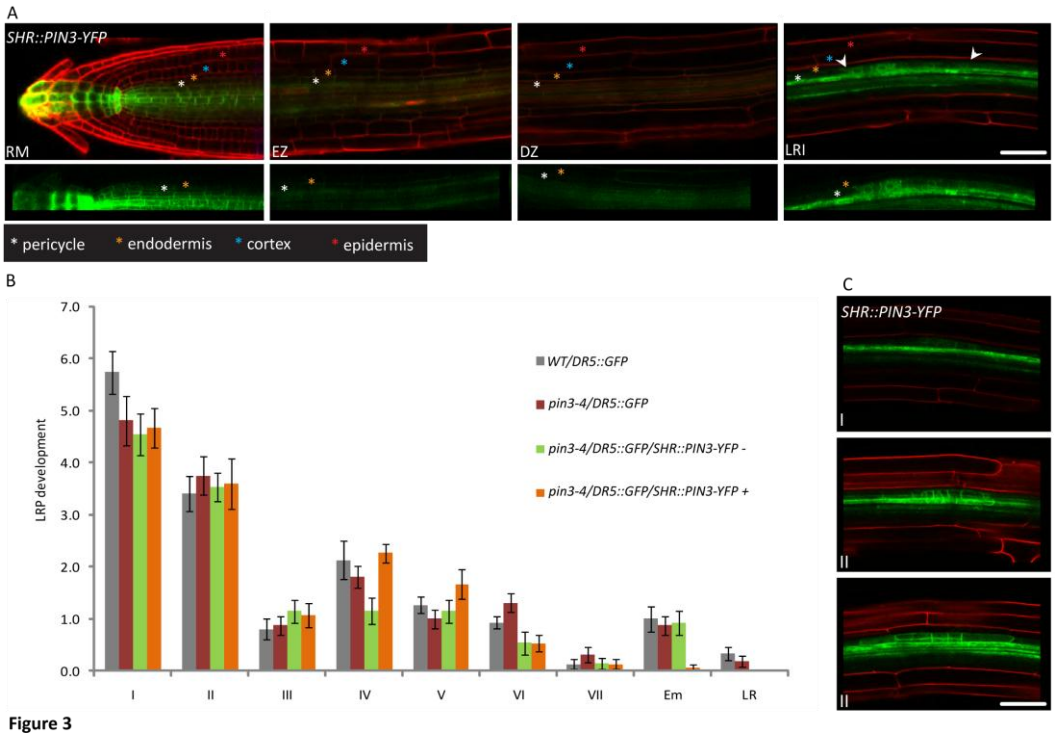

Figure 3. *SHR* driven *PIN3* expression in the stele is not sufficient to rescue the *pin3* LRI phenotype
(A) The *SHR* promoter drives *PIN3-YFP e*xpression (green) in the stele of the root meristem (RM), elongation zone (EZ), differentiation zone (DZ) and during LRI (arrowheads) in the *pin3/DR5::GFP* background, with PI counterstain (red) in the upper panel. Color-coded asterisks indicate the different root cell files. Note that PIN3-YFP is also present in the dividing pericycle cells during LRI. The fluorescent signal in the root cap is derived from the DR5::GFP marker. (B) Stage distribution of LR primordia indicates that the decrease in stage I is not restored in *pin3 m*utants when *PIN3-YFP i*s expressed in the stele. +SHR and –SHR refer to the pooled fluorescent positive and negative seedlings respectively from a segregating population in the stable *pin3/DR5::GFP* background. Error bars represent SE (*n* = 10-20). *P* values are *<0.05, **P<0.01; Student's *t*-

test. (C) The *SHR* promoter drives *PIN3-YFP* expression in de dividing pericycle cells from the first stage of LR development onwards. Corresponding stages of lateral root development are indicated. The PI counterstain is shown in red. Scale bars represent 50 μm.

PIN3 Defines an Auxin Reflux Loop between the Endodermis and Founder Cells

The direction of the auxin flow is largely determined by the polarity of PIN efflux carriers in the cells (Wiśniewska *et al*., 2006). Thus, PIN3 polarity at the plasma membrane is indicative for the direction of PIN3 mediated auxin transport. Optical cross-sections of PIN3-GFP roots at the site of LRI indicated that PIN3 is localised to the inner side of the overlaying endodermal cells whereas no PIN3 signal was observed at the membranes of the remaining endodermal cells (Figures 4A and 4B). *PIN3* promoter activity in the pericycle alongside the endodermal cells limits detailed examination of the PIN3 polarity in either cell type (Figure S1C). Therefore, to study the membrane localization of PIN3 specifically in the endodermal cells, the *SCR::PIN3-YFP* line was analyzed. Differently from the *PIN3* promoter, whose expression is induced only transiently in the endodermis in the DZ during LRI, the *SCR* promoter is active continuously throughout the root zones encompassing RM, EZ, and DZ. Along the root growth axis, PIN3 displayed a variable polarity in endodermal cell file. While PIN3 was basal in the cells of the RM (Figure 2A), this polarity changed to apical in the EZ. In cells which had entered the phase of terminal differentiation, PIN3 was first non-polarly distributed (Figures 2A and 4C). However, more proximal in the root, a dramatic change of PIN3 polarity was again observed where PIN3 was polarized to the inner lateral membrane facing the pericycle cells (Figure 4D). When the root was subsequently searched for LRIs, we found that these always occurred within the region of the DZ where PIN3 was polarised to the inner lateral wall (Figures 2A and 4D). This indicates that when *PIN3* expression is induced in the endodermis during LRI, the PIN3 protein might per default polarize to the inner lateral membrane. Next, we checked whether manipulations that induce LRI, provoke PIN3 lateralization in the region of the DZ where PIN3 is non-polarly distributed along all membranes using the *SCR::PIN3-YFP* line. Bending the root in this region triggered PIN3 lateralisation (Figure 4E). In the course of 4 hours (240 min), the originally non-polar PIN3 membrane distribution had changed and the prevailing part of PIN3 was detected on the inner membrane of the endodermal cells. Consistently, treatment of roots with auxin, a well established enhancer of LRI, induced PIN3 lateralization (Figures 4F-4H). After 2 hr, PIN3 lateralisation was observed, with the strongest significance after 5 hr (Figures 4G and 4H). This auxin-based lateralization of PIN3 was specific to the DZ and was not observed in the

RM (Figures 4F and 4H). Altogether these data indicate that PIN3 induced in the endodermal cell after FC specification might locate towards the inner membrane to direct auxin towards the underlying FC.

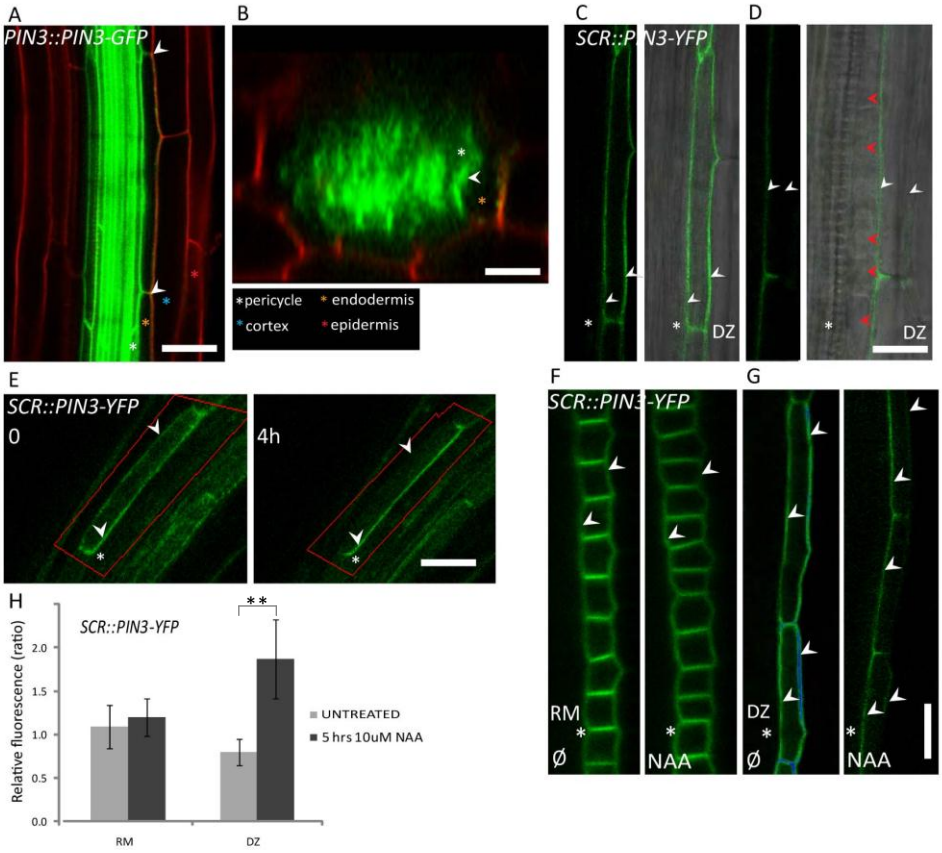

Figure 4.

Figure 4. PIN3 is Polarized to the Inner Lateral Membrane of Endodermal Cells Overlaying LRI Sites
(A and B) PIN3::PIN3-GFP (green) expressing root with PI counterstain in red (A) and the corresponding optical cross-section at the site of LRI (B) shows PIN3-GFP at the inner lateral membrane of the two overlaying endodermal cells (arrowheads), whereas PIN3-GFP is absent from the remaining endodermal cells in the cross-section. (C and D) *SCR* driven *PIN3-YFP* in endodermal cells of differentiation zone (DZ) in close proximity of elongation zone (EZ) (C) and in more proximal region of the differentiation zone (DZ) where the LRIs take place (D). Note non-polar (C) and lateral (D) membrane localization of PIN3-YFP. Fluorescence image (left panel) and overlay with DIC (right panel). (E-H) Manipulations that induce LRI, provoke PIN3 lateralization. Observations were done in the *SCR::PIN3-YFP* line, directly behind the RM, where PIN3 is apolarly distribitued on all membranes. (E) After root bending, *SCR* driven PIN3-YFP in the endodermal cell lateralizes in the course of 4 hr. Times are relative to root bending. (F-H) NAA (10 µM) treatment induces PIN3-YFP lateralization in the DZ but not in the root meristem (RM). Fluorescent images (F and G) and graph (H) displaying the ratio between relative fluorescence intensity at the inner and outer cell membranes in the root meristem (RM) (F) and differentiation zone (DZ) (G) in control and 5 hr NAA treated seedlings. White arrowheads indicate the lateral membranes in the endodermis and red arrowheads indicate divided nuclei in the stage I LRP. White asteriks indicate the neighboring pericycle cell file. Error bars represent SE (n = 30), P values are $^{**}P<0.001$; Student's t-test. Scale bars represent 40 µm(A and B), 20 µm (C and D) and 50 µm (E-G).

DISCUSSION

As LRPs start to develop, they are concealed under several root tissue layers encompassing the endodermis, cortex and epidermis. This puts special demands on the adaptation of neighboring tissues to the expansion of the new rapidly developing organ. Proper interaction between the LRP and the adjacent cells is particularly critical for controlled non-invasive emergence of primordia through neighboring tissue layers. An important regulatory component of this tissue interaction is auxin, shown to promote cell separation in advance of the developing LRP through upregulation of cell wall remodeling genes (Swarup *et al*., 2008).

Here, we reveal that controlled auxin transport between the site of LR formation and the overlaying tissues is instructive for LR formation at a much earlier stage. Expression of *PIN3* detected in the endodermal cells before LRI indicates a function in the developmental phase which precedes the massive LRP body expansion.

During pre-initiation phases, *PIN3* was transiently induced in the adjacent endodermal cells overlaying FCs, till LRs were initiated by several rounds of anticlinal divisions. At the same time, the auxin response marker was active in the same endodermal cells (Figure 1G; 180'). *PIN3* is auxin inducible (Vieten *et al*., 2005) and auxin treatment induces *PIN3-GFP* in the endodermal and cortical cell files of the root (data not shown). Altogether these data suggest that after FC establishment, auxin induces *PIN3* expression specifically in the overlaying endodermal cell. Laterally localized PIN3 in these endodermal cells subsequently reinforces auxin movement towards the FCs and thus provides a local auxin reflux circuit, which contributes to auxin accumulation in FCs important for further progress to LRI.

The identification of mutants in which, despite defects in LRI (De Rybel *et al*., 2010; Dubrovsky *et al*., 2008), FC establishment still occurs, indicates that specification of FCs is not ultimate for LRI and that these two events are not directly coupled. In addition, real time imaging of LRI has shown that auxin continues to accumulate in the FCs, until it reaches a maximum just prior to the actual initiation event (De Rybel *et al*., 2010). These observations indicate that LRI might require additional input upon FC establishment and that there are extra checkpoints, requirements to be met before pericycle cells actually divide. Our data support a regulatory function for the endodermis in LRI and indicate that interaction between FC and the adjacent endodermal cells defines a developmentally specific auxin re-flux that enables FCs to reach auxin threshold levels to initate lateral root formation.

EXPERIMENTAL PROCEDURES

Plant Material

The transgenic *Arabidopsis thaliana* (L.) Heynh. lines have been described elsewhere: *PIN7::PIN7-GFP* (Blilou *et al*., 2005a), *PIN3::PIN3-GFP* (Žádníková *et al*., 2010), *pin3-4/DR5::GFP* (Ding *et al*., 2011), *DR5rev::GFP* (Friml *et al*., 2003), *DR5pro::N7:Venus* (Heisler *et al*., 2005), *pin7-1* (Benková *et al*., 2003).

Growth Conditions

Seeds of *Arabidopsis* (ecotype Columbia-0) were plated on 0.5 MS medium (Duchefa) with 1% sucrose and 0,8% 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-hr-light/8-hr-dark photoperiod at 18°C.

Plasmid Construction

Construction of the *PIN3-YFP* (Rakusová *et al*., 2011) and *CASP1* (Roppolo *et al*., 2011) promoter entry vectors and the *SCR::PIN3-YFP* (Rakusová *et al*., 2011) expression vector has been described elsewhere. Detailed information on the construction of additional expression clones are described in the Supplemental Experimental Procedures.

Pharmacological and Hormonal Treatments

5- or 6-day-old seedlings were transferred onto solid MS media with or without 1-naphthalene acetic acid (NAA) (10 μM) and were further incubated for the time indicated.

Confocal Imaging and Image Analysis

For confocal microscopy images, the Zeiss LSM 510 or Olympus FV10 ASW confocal scanning microscopes were used. Fluorescence signals for GFP (excitation 488 nm, emission 507 nm) and PI (excitation 536 nm, emission 617 nm) were detected. YFP signals were observed using GFP settings. Sequential scanning was used to avoid any interference between fluorescence channels.

Timing of the transition from FC to LRI and relative fluorescence intensity measurements on lateral plasma membranes are described in detail in the Supplemental Experimental Procedures. The statistical significance was evaluated with Student's t test.

Phenotypic Analysis of LRI

For stage distribution of lateral root development, the LRP density was analyzed as described (Malamy *et al*., 1997). Root growth parameters (root length) were analyzed with the ImageJ software (NIH; http://rsb.info.nih.gov/ij). Phenotypic analyses of FC and LRI using the *DR5rev::GFP* marker are described in detail in the Supplemental Experimental Procedures. The statistical significance was evaluated with Student's t test.

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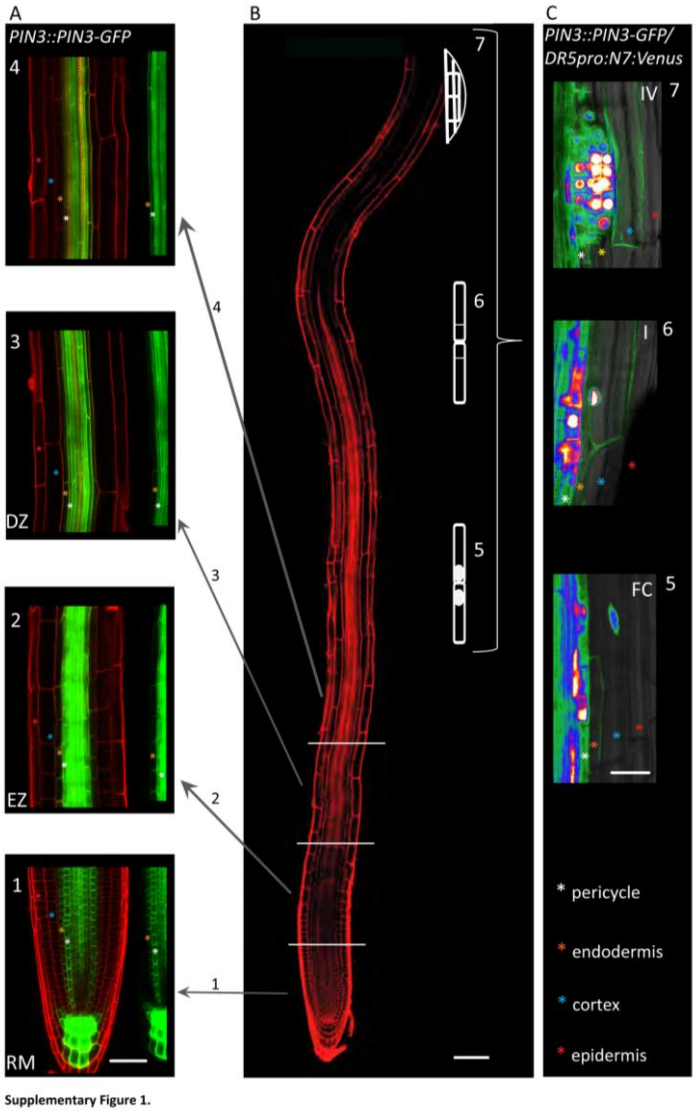
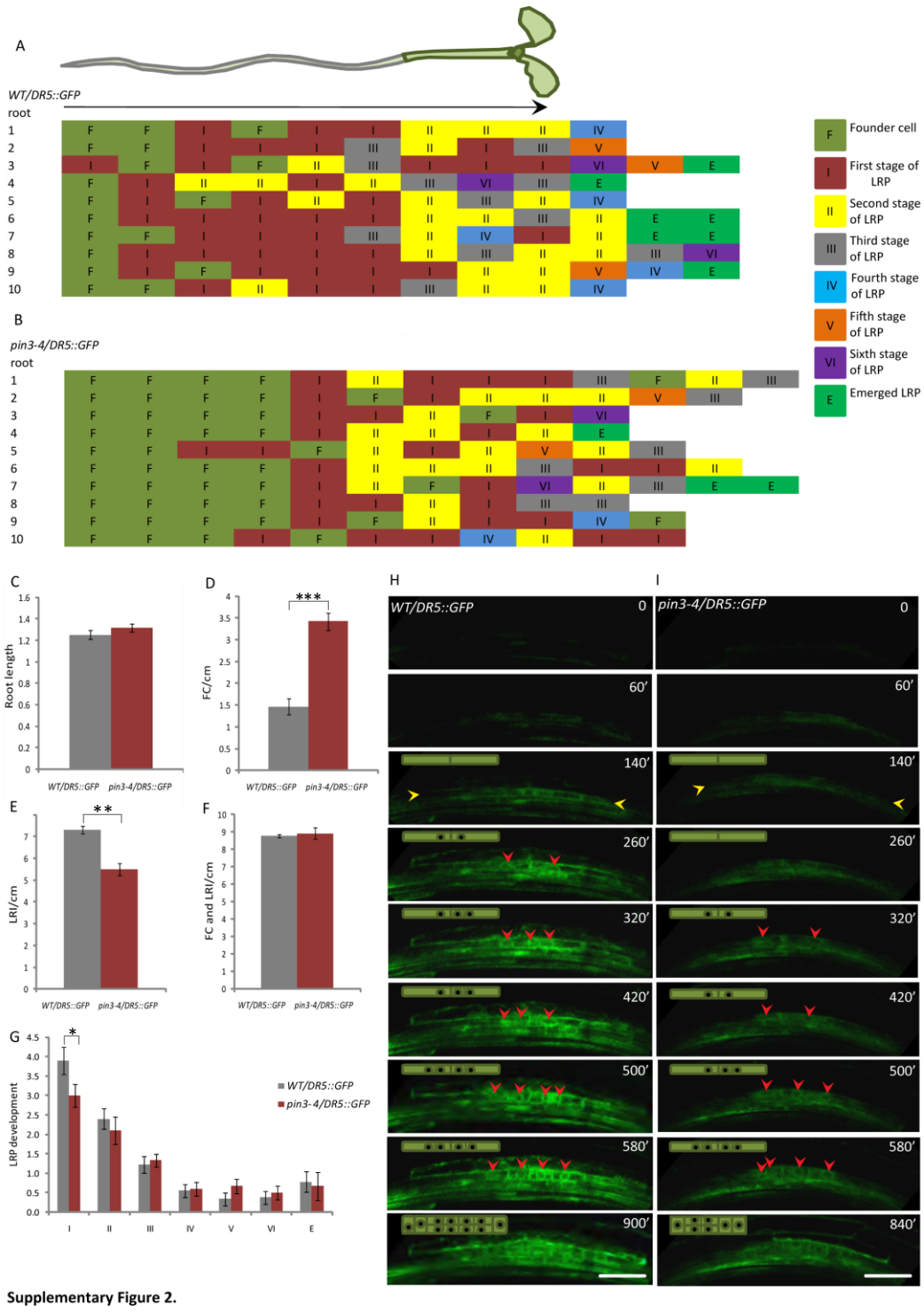
SUPPLEMENTARY FIGURES



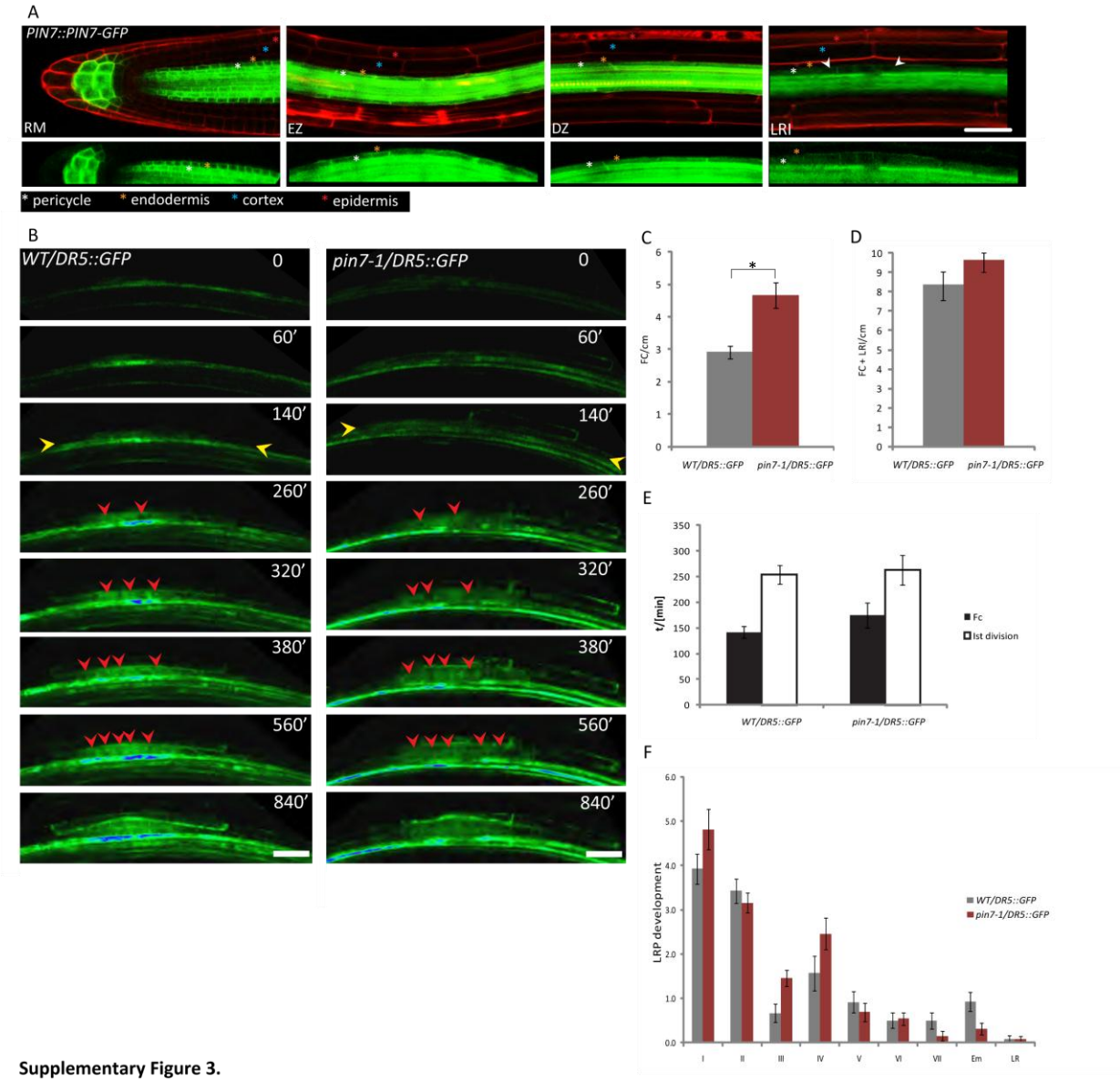
Supplementary Figure 1.

Figure S1. *PIN3::PIN3-GFP* expression along the root and during LR development (related to Figure 1)
(A-C) PIN3::PIN3-GFP expression along the root and during LR formation. (B) PI stained root with the relative positions of the images taken in (A) and (C) indicated. The PIN3-GFP signal was continuous in the endodermis of the root meristem (RM) (1) and elongation zone (EZ) (2). Onset of xylem strand differentiation, as observed using PI stain, was taken as the transition between the elongation zone (EZ) and differentiation zone (DZ). PIN3-GFP was still present in the endodermis at this transition zone (3) but was absent from the remaining DZ (4). Here, PIN3-GFP was only detected in the endodermis during FC establishment (5) and LRI (6). During further primordium development, the PIN3-GFP signal disappeared from the endodermis and was induced in the overlaying cortex (7). Relative positions of the different stages (indicated in the upper right corner) of LR formation that occur in the pericycle of the DZ are also shown in (B). Color-coded asterisks indicate the different root cell files. Scale bars: 20 μm (A), 50 μm (B) and 30 μm (C).



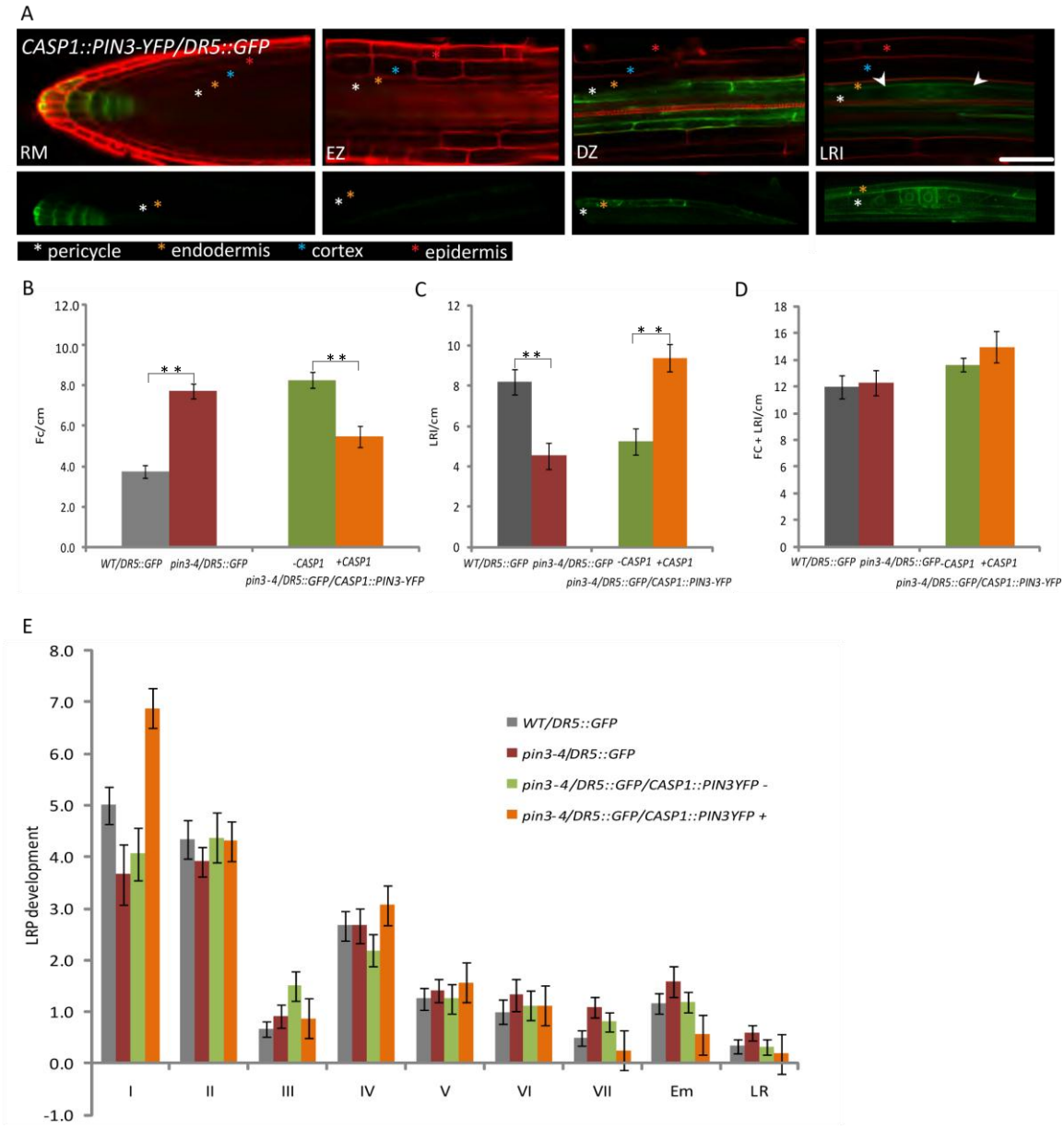
Supplementary Figure 2.

Figure S2. LRI and development in 7 days old *pin3* roots (related to Figure 1)
(A and B) Scheme of FC and LRP distribution along the longitudinal root growth axis in 7 day old roots of WT (A) and *pin3* (B). FCs accumulate mainly at the distal root end of the *pin3* mutant. FCs and the individual stages of lateral root development were monitored using the DR5rev::GFP reporter.
(C-G) Root length (C), FC (D), LRI (E) and FC+LRI (F) densities and the stage distribution of LRP (G) were calculated from the same experiment and confirmed the observations made in 5 day old roots.(H-I) Real time analysis of FC establishment and LRI in WT/*DR5::GFP* and *pin3/DR5::GFP* mutants after bending. FC establishment was followed by the appearance of the *DR5rev::GFP* signal in the pericycle (orange arrowhead) and LRI was scored when nuclear (red arrowhead) division could be observed. Schematic representation of the observed cell divisions are shown in the left upper corner. Scale bar, 30 µm.



Supplementary Figure 3.

Figure S3. PIN7 is not required for FC progression to LRI (related to Figure 1)
(A) *PIN7::PIN7-GFP* expression (green) in the stele of the root meristem (RM), elongation zone (EZ), and differentiation zone (DZ) and during LRI (arrowheads), with PI counterstain (red) in the upper panel. Color-coded asterisks indicate the different root cell files.(B) Real time analysis of FC establishment and LRI in *WT/DR5::GFP* and *pin7/DR5::GFP* mutants after root bending. FC establishment (yellow arrowhead) was followed by the appearance of the *DR5::GFP* signal in the pericycle and LRI was scored when nuclear divisions (red arrowheads) could be observed. Time points (in min) are relative to root bending. (C) FC density is increased in *pin7* mutants compared to WT.(D) The total number of FC+LRI density is slightly higher in *pin7* mutants compared to WT.(E) Time of FC establishment and LRI after root bending indicates that FCs proceed normally to LRI in *pin7* mutants compared to WT.(F) Stage distribution of LRP shows an increase in stage I LRP in *pin7* mutants. Error bars represent SE (n = 20). P values are *P<0.05, ** P<0.01; Student's t-test. Scale bars: 50 µm (A) and 20 µm (B).



Supplementary Figure 4.

Figure S4. *PIN3* expression in the endodermis of the DZ restores the *pin3* LRI defect (A) Fluorescence images of *PIN3-GFP* (green) expression driven by the *CASP1* promoter in the stele of the root meristem (RM), elongation zone (EZ), and differentiation zone (DZ), and during LRI in the *pin3/DR5::GFP* background, with PI counterstain (red) in the upper panel. Color-coded asterisks indicate the different root cell files. The fluorescent signal in the root cap is derived from the DR5::GFP marker. Scale bar, 50 μm. (B-D) FC (B) and LRI (C) densities are restored in *pin3/DR5::GFP* mutants when *PIN3-YFP* is expressed in the endodermis by the *CASP1* promoter, while the total number of FC+LRI remain similar (D). (E) Stage distribution of LR development shows that the decrease in stage I primordia is rescued in *pin3/DR5::GFP/CASP1::PIN3-YFP* fluorescent positive seedlings. +CASP1 and –CASP1 refer to the pooled fluorescent positive and negative seedlings, respectively, from a segregating population in the stable *pin3/DR5::GFP* background (B-E). Error bars represent SE (n = 20). P values are $^{**}P<0.01$; Student's t-test.

SUPPLEMENTARY EXPERIMENTAL PROCEDURES

Plasmid construction

Entry clones containing the 2500-bp SHR promoter were cloned into the donor vector *pDONRP4P1R*. Expression clones were generated by recombining the promoter and PIN3-YFP fragments into the expression vector *pB7m24GW*, which were then directly transformed to *pin3-4/DR5rev::GFP* plants.

Confocal imaging and image analysis

Timing of the transition from FC to LRI was done after root bending. 5- or 6-day-old seedlings were placed on chambered cover glass (Nunc Lab-Tek) and manually curved by bending the root tip just above the root meristem using forceps. All the samples were analyzed in the same area of the bended root with a focus on the beginning of xylem strains formation. The seedlings were then covered with 0.2 mm thin square blocks of solid MS media. LRPs were scanned in 3, 5, 10, or 20 min time intervals for 8–17 hr by the FV10 ASW confocal microscope (Olympus) with a 20x or 60x (water immersion) objective.

For relative fluorescence intensity measurements on lateral plasma membranes, between 30 and 40 cells corresponding to the RM and DZ, where PIN3 is normally non-polarly distributed, were imaged with both inner and outer lateral membranes in focus. Later, images were analyzed using the ImageJ software (NIH; http://rsb.info.nih.gov/ij), where the absolute fluoresence intensities of both lateral membranes were measured to calculate the relative fluorescence intensity of the inner lateral membrane over the outer lateral membrane.

Phenotypic analysis of LRI

For phenotypic analyses of FC and LRI, 10 to 20 roots of 5- and 7 day-old seedlings were analyzed. The counting process was conducted in the direction from the root tip towards the root base.

GFP settings were used to identify and count *DR5rev::GFP* positive signals in the pericycle. GFP signals accompanied with cell divisions were scored as LRI, whereas FCs were counted when no cell divisions were observed.

For all experiments, independent experiments were performed in triplicate with the same results, and representative images are shown.

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Chapter 5.

Conclusions and Perspectives

Conclusions and perspectives

Already for a long time cytokinin (CK) and auxin have been recognized as crucial signaling molecules controling plant growth and development. Recent studies using *Arabidopsis thaliana* provide more insights about the role of CK and auxin in plants (Bishopp *et al*., 2009). They both act either synergistically or antagonistically to control developmental processes, such as growth and development including branching (Ongaro and Leyser, 2008; Dello Ioio *et al*., 2008), establishment of the root pole during early embryogenesis (Müller and Sheen, 2008), shoot apical meristem maintenance (Zhao *et al*., 2010), vascular differentiation, leaf senescence (Mahonen *et al*., 2006, Werner *et al*., 2009; Argueso *et al*., 2009) and lateral root (LR) organogenesis (Laplaze *et al*., 2007).

Previous reports have revealed that CK can regulate *PIN* gene expression, resulting in an auxin maximum redistribution and cell differentiation, which occurs at the transcriptional regulation level (Dello Ioio *et al*., 2008; Ruzicka *et al*,., 2009, Pernisova *et al*., 2009). These results prompted us to study the CK effect on PINs and the impact of this regulation on the lateral root primordia (LRP) development.

We have revealed that CK, besides the reported transcriptional regulation of *PIN* genes, interferes with PIN protein endocytotic trafficking and promotes lytic degradation of PIN1. We show that this CK effect might play a role in PIN polarity establishment and might contribute to PIN polarity re-establishment in developmental processes when auxin flow redirection is required.

One of the surprising findings of this thesis is the identification of a regulatory function of the endodermis in lateral root initiation (LRI), which might act as one of the check points in lateral root (LR) organogenesis after founder cells (FCs) are specified.

Cytokinin modulates PIN1 trafficking to lytic degradation to control plant organogenesis

After a short-term CK application on the first stage of the LRP we observed a rapid decrease of PIN1 protein abundance from the plasma membrane. Cytokinin-dependent activation of AHK4/CRE1 specifically affects endocytic trafficking of the PIN1 auxin efflux carrier

(Figure 1). To dissect the transcriptional and transcription-independent control of auxin efflux carriers stimulated by CK, cycloheximide (CHX), an inhibitor of new protein synthesis, was used, as well as cordycepin (COR), an inhibitor of RNA transcription. Neither inhibitor blocked the CK effect on the PIN1 lytic degradation. Moreover, a robust reduction of PIN1 upon CK application was detected, when *PIN1* was expressed under the control of the 35S promoter as well. Those observations support the existence of a transcription-independent CK signaling cascade downstream of the AHK4/CRE1 receptor. The downregulation of *PIN1* after CK treatment in several single B-type Arabidopsis Response Regulators (ARRs) knockouts suggests that the action between auxin and CK takes place at the level of ARRs or further downstream. There remain many unanswered questions, for instance what are the molecular components and unknown regulatory mechanisms that are involved in the CK effect on PIN1 degradation, what is the CK receptor for this pathway? To answer these and other questions targeted forward genetic screens might be helpful.

However, it will be very interesting to reveal the exact function of CK in the context of the constitutive recycling regulated by the endosomal trafficking regulator GNOM-ARF-GEF.

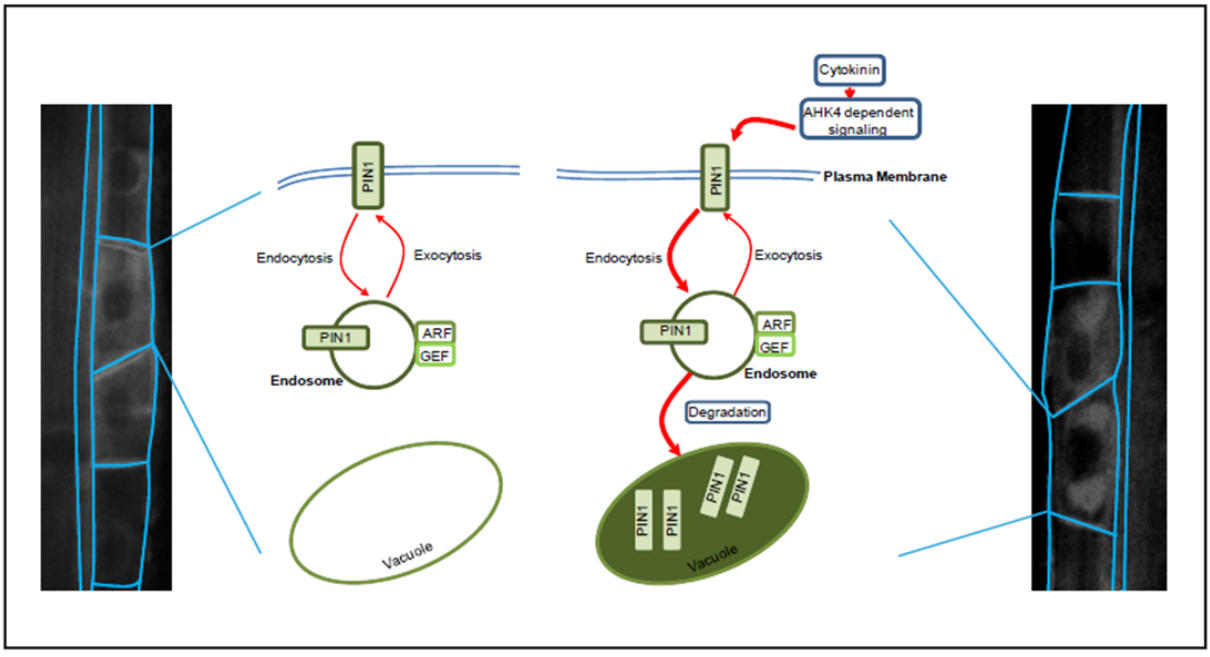

Figure 1. Cytokinin contribution to the regulation of PIN trafficking
Alternative mechanism proposed for the cytokinin-auxin crosstalk by rapid auxin regulation. Cytokinin regulates PIN1 trafficking through endocytosis processes by redirecting the protein for lytic degradation to the lytic vacuoles. A functional cytokinin perceptionis required for this cytokinin-induced PIN1 degradation.

Role of cytokinin in PIN polarity regulation

In the first part of our studies we demonstrated that CK is able to redirect PIN1 for lytic degradation into the vacuoles and thereby regulates PIN activity, auxin transport and auxin-dependent development. Those results implicated an alternative mechanism for the rapid control of auxin availability in root tissues (Figure 2). Following studies showed that in root epidermal cells PIN1, which is located at basal membranes, was targeted for lytic degradation upon CK application, while the apically located PIN1-GFP3 as well as PIN2 stayed unaffected. Consequently, in the *PIN2::PIN1-HA* agravitropic line the local degradation of PIN1 selectively from the basal membranes restored the basipetal auxin transport and root response to the gravistimulus.

PIN polarity establishment was found to be the determinant of the phosphorylation state. Ser residues in the conserved TPRXS(N/S) motifs within the PIN1 hydrophilic loop were recognized as an important targets that are phosphorylated by PINOID kinase in vitro (Huang *et al*., 2010; Zhang *et al*, 2010). Analysis of mutant loss-of-phosphorylation and phosphomimic PIN1:GFP alleles revealed that phosphomimic PIN1 with apical localization was insensitive to CK treatment, in contrast to the loss-of-phosphorylation PIN1 allele. To dissect the role of phosphorylation in CK-mediated PIN1 degradation further, we analyzed mutants defective in phosphorylation (*pid*) and dephosphorylation (*ppa2*). These analyses brought further support for our hypothesis that CK sensitivity of the PIN1 protein might be determined by the phosphorylation status of PIN1.

Altogether, these data indicate that CK interferes preferentially with trafficking pathways directing PIN from the basal membranes into the vacuole, which leads to a significant extinction of the PIN proteins from basal, but not apical membranes. The molecular mechanism by which CK regulates the degradation and helps to maintain apical-basal PIN polarity remains elusive. Nevertheless, many questions still need to be answered. For instance, does (and how does) CK distinguish phosphorylated over non-phosphorylated PIN proteins before recruiting it to apical or basal polar trafficking pathways? What are the other elements acting on PIN phoshorylation, and besides PID, WAG1 and WAG2? Does CRE modulate the phosphorylation degree of PIN? Is there a direct or indirect effect of CRE? Further experimental work can bring the answers to these questions.

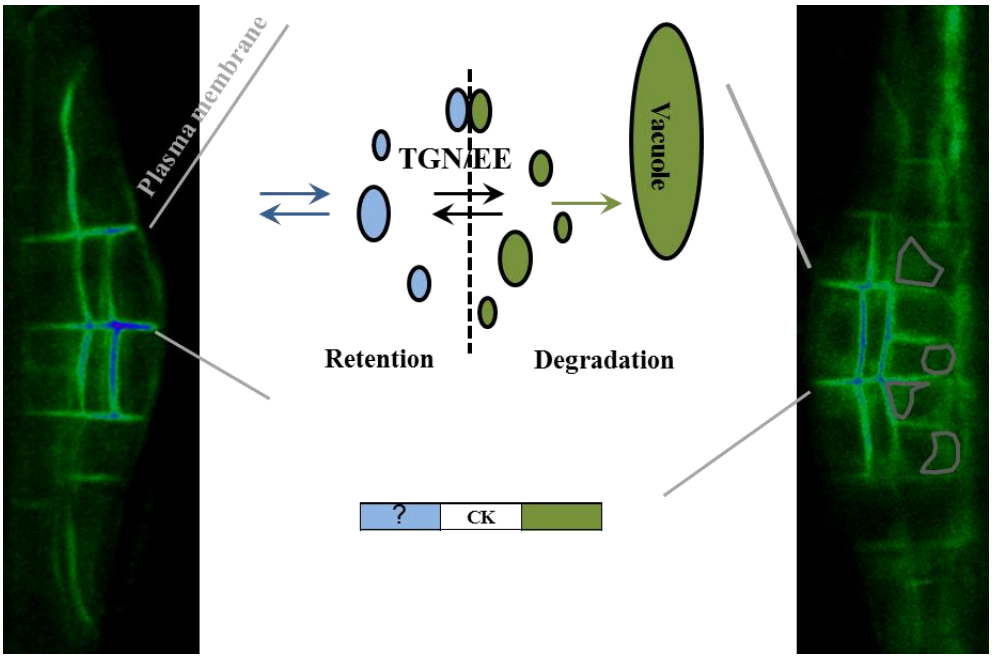

Figure 2. Cytokinin contribution to the regulation of PIN trafficking.
Cytokinin participates in mechanisms regulating PIN polarity re-establishment in developmental processes when auxin flow redirection is required. The PIN protein sensitivity to cytokinin is dependent on its membrane localization. Cytokinin acts on degradation/vacuolar targeting (green arrows), and might be involved on PIN retention/recycling (blue arrows). However, the signal perception molecular mode of action on the level of vesicle trafficking is still unknown.

PIN3 plays an important role in LRP initiation

The intracellular auxin flow important during LRP initiation is provided by PIN-dependent auxin transport (Casimiro *et al*., 2001, Benkova *et al*., 2003, Laskowski *et al*., 2007). In the last part of our work we reveal a developmentally specific auxin re-flux important for LRP initiation. This auxin re-flux is established between the endodermal and FCs and is mediated by the PIN3 auxin efflux carrier. Multiple independent genetic and pharmacological manipulations demonstrated that a lack of this re-flux causes dramatic defects in the progress of FCs towards the next initiation phase. We propose that PIN3 mediates an interaction between FCs and the adjacent endodermal cells and defines a developmentally specific auxin re-flux that enables FCs to reach the required auxin threshold levels to initiate LR formation (Figure 3). Although we clarified that the PIN3 auxin efflux carrier in the endodermis is required for the progress from the FC to the initiation of LR, there are remaining questions related to the mechanism by which such a model exactly works. In order to identify the molecular component involved in the regulation of transient *PIN3* expression and the

asymmetric localization in the endodermis before the initiation phase, a forward genetic screen for regulators of *PIN3* expression and relocalization might be relevant.

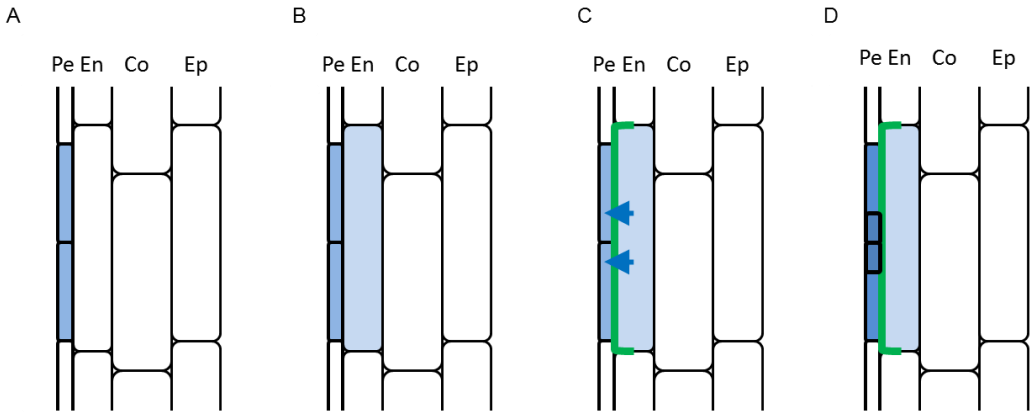

Figure 3. Model for endodermal PIN3 controlled transition from the FC stage to lateral root initiation. (**A**) Auxin response activity (blue) in a restricted number of pericycle cells indicates FC establishment. (**B** and **C**) Soon after, auxin signalling is activated in the overlaying endodermal cell (**B**), inducing local and transient expression of PIN3 (green) (**C**). In the endodermis cell, the PIN3 protein is laterally localized to the inner membrane, thereby transporting auxin towards the FCs, providing a local auxin reflux circuit. (**D**) This PIN3 driven auxin reflux contributes to auxin accumulation in FCs important for further progress to LRI.

Potential applications of acquired knowledge

The plant development is characterized by continuous growth, and high flexibility to adjust their architecture to ever changing environmental conditions. Plants during their live cycle are able to maintain permanent stem cell populations, undifferentiated and committed cells perpetuate the existence of bodies and train them de novo (Benkova *et al*. 2003). All those development processes are directed and coordinated by plant hormones such as auxin (involved in growth and root development), abscisic acid (response to abiotic stress), brassinosteroids (division and cell elongation), cytokinins (cell differentiation meristem), ethylene (geotropic response), gibberellins (expansion and cell growth) and jasmonic acid (biotic stress response) (Teale *et al*., 2008).

Important processes such as rooting are of high agronomic interest, and after considering the current situation of environmental change, the study of the mechanisms that regulate the root system (mainly by the lateral roots formed throughout the life cycle of the plant) is indispensable (Laplaze et al. 2007). In this sense, and by using lateral root formation as a model for studying the physiological and molecular mechanisms that regulate root

organogenesis in plants, more productive crops, both in quality and quantity could be performed.

We have demonstrated that cytokinin might be an important player to control auxin flux during plant growth by promoting PIN degradation during the lateral root initiation,. This research may reveal novel tools for auxin manipulation, which can be developed for altering plant architecture, such as optimization of lateral root branching for high density planting, or even decrease competition between plants on the field.

Additionally, lateral roots are essential for efficient nutrient and moisture retention, so increasing their number, might affect a plant productivity. Our research might be also useful for transgenic strategies and breeding programs to improve properties of crops, such as the adaptability of plants in the high-density fields to absorb the light source for photosynthesis, carbon partitioning, flower development or seed production. Indeed, Yoshida *et al*., 2011 demonstrated that light controls the shoot apical meristem activity of lateral organs by regulating two key hormones: auxin and cytokinin, where CK is required for meristem propagation, and auxin redirects cytokinin-inducible meristem growth toward organ formation. In such scenario, cytokinins might also increase grain yield; for example, by activating inflorescence meristem activity in rice (*Oryza sativa*), one of the world most important crop species (Kurakawa *et al., 2007;* Ashikari *et al.,* 2005).

Moreover the progress made in agriculture have been preceded by the study of various processes of interest such as flowering, fruiting or adaptation to drought conditions, e.g. cytokinin accumulation in tobacco (*Nicotiana tabacum*) leads to tolerance against extreme drought stress (Rivero *et al.,* 2007). Furthermore plant hormone signaling is also known to regulate other morphogenic processes, including the flowering-time. All these features highlight the importance of the basic studies with a long-term agricultural applications.

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Summary

The ability to form and develop new organs throughout their life is a unique feature of plants. These newly developed organs include lateral organs, such as roots and shoots, as well as reproductive organs (Shkolnik-Inbar and Bar-Zvi 2010). Plants are able to maintain a permanent stem cell population and form new organs during their entire life cycle. As endogenous factors plant hormones largely participate on shaping plant architecture and modulating their growth. Over the last few years, our knowledge increased on how these signaling molecules can act in single cells, different tissues, organs and whole developmental processes (Benkova *et al*., 2003; Fukaki *et al*., 2007; Laplaze *et al*., 2005, 2007; Peret *et al*., 2009). Moreover it was proven, that different plant hormones affect overlapping processes. Evidences of 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 significantly contributing to shaping the root system architecture (Laplaze *et al*., 2005, 2007; Peret *et al*., 2009; Benková and Bielach, 2010). The plant root system is a complex structure composed of the main root and LRs, which, apart from anchoring the plant in the soil, mediate a vitally important interaction between the plant and the soil to provide water and nutrients. Thus understanding of molecular mechanisms controling root architecture is of high agronomical importance (Fitter *et al*., 2002; Fukaki *et al*., 2007; Osmont *et al*., 2007; MacGregor *et al*., 2008).

In this PhD thesis, we reveal novel mechanisms of the CK – auxin interaction contributing to control root growth and branching. We demonstrate that CK interferes with endocytotic trafficking of auxin efflux carrier AtPIN1 and redirects it for lytic degradation into vacuoles. Such a rapid depletion of a membrane-localized auxin efflux carrier, which is independent of transcription, provides a rapid mechanism to efficiently control auxin fluxes and distribution in developmental processes such as LR organogenesis and root meristem differentiation. Moreover, our results indicate a potential mechanism on how endocytic trafficking plays a role in CK-controlled development.

Furthermore, we show that CK preferentially targets PIN1 localized at the basal membranes in root epidermal and cortex cells, while apically targeted PIN1 is largely insensitive to the CK treatment. Accordingly, genetic manipulations increasing the proportion of phosphorylated PIN1 and promoting its apical localization reduced the sensitivity of PIN1 to CK and led to CK insensitive root development.
We propose that this selective interaction of CK with a subset of PIN1 proteins might provide a mechanism for rapid, membrane-specific elimination of auxin efflux carriers, which could be employed in developmental processes requiring PIN polarity re-establishment and redirection of the auxin flow.

Another aspect of our research addresses the function of the PIN3 auxin efflux carrier transiently expressed in endodermal cells adjacent to the site of LR initiation (LRI). We demonstrate that controlled auxin transport between the site of LR formation and the overlaying tissues is instructive for LRI. Our results suggest that after founder cell establishment, auxin induces *PIN3* expression specifically in the overlaying endodermal cell. Laterally localized PIN3 in these endodermal cells subsequently reinforces auxin movement towards the founder cells (FCs) and thus provides a local auxin reflux circuit, which contributes to auxin accumulation in FCs, which is important for further progress to LRI.

Altogether, this PhD research brings novel insights into the mechanisms of hormonal control of the root system architecture.

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Resume

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Specialization on: *Characterisation and Taxonomy of Acetobacteria*
Supervisor: Assoc, Prof. Jozef Grones, PhD

2005-2006 Canterbury Business College
Sydney, Australia
Business Marketing

2001 – 2002 Sydney Business and Travel Academy
Sydney, Australia
Certificate III in Information Technology

Work Experience

Jun – Sep 2007 University of Glasgow
Glasgow, UK

In summer 2007 I spent eleven weeks working in lab 61 in the Pontecorvo building (a part of the Division of Molecular Genetics). The aim of this project was the investigation of properties of zinc finger-resolvase fusion proteins and assessment of the efficiency of recombination catalysis performed by these recombinant proteins. The work was sponsored by the I.A.E.S.T.E foundation.

Supervisor: Prof. Marshall Stark, PhD

Jul – Sep 2004 Institute of Forest Ecology at Slovak Academy of Sciences
Nitra, Slovakia

Over summer 2004 I spent ten weeks working in field of Caryotaxonomy. I was preparing microscopics preparates of structure of flowers with undeveloped organum in rate Rhamus cathartica and R. saxatilis. I was also studing and preparing caryologic preparates in the rate Corylus avellana.

Supervisor: Dr. František Mercel, PhD

Publications

- *Cytokinin modulates endocytic trafficking of PIN1 auxin efflux carrier to control plant organogenesis*. **Marhavý, P**, Bielach A, Abas L, Abuzeineh A, Duclercq J, Tanaka H, Pařezová M, Petrášek J, Friml J, Kleine-Vehn J, Eva Benková (Dev Cell 2011, 21:796-804)

- *Role of PIN-mediated auxin efflux in apical hook development of Arabidopsis thaliana.* Žádníková, P., Petrášek, J., **Marhavý, P**., Raz, V., Vandenbussche, F., Ding, Z., Schwarzerová , K., Morita, M.T., Tasaka, M., Hejátko, J., Eva Benková (Development 2010, 137, 607–617).

- *Genetic approach towards the identification of auxin-cytokinin crosstalk components involved in root development.* Bielach A, Duclercq J, **Marhavý P**, Benková E. (2011) (Philos Trans R Soc Lond B Biol Sci). (Articles in press).

Manuscripts in preparation

- *Cytokinin promotes PIN1 polarity establishment*. **Marhavý P**, Duclercq J, Feraru E, Offringa R, Friml J and Benkovà , E.

- *Auxin Re-flux between Endodermis and Pericycle Controls Lateral Root Initiation*. **Marhavý P**,[$] Vanstraelen M,[$,*] De Rybel B, Bennett M, Beeckman T and Benková E[*].

- *Spatiotemporal regulation of lateral root organogenesis by cytokinin.* Bielach A, Václavíková K, **Marhavý P**, Duclercq J, Tarkowski P, Benková E.

- *Auxin redistribution in lateral root columella cells orchestrates root system architecture.* Ruiz Rosquete M, Wangenheim D**, Marhavý** P, Barbez E, Stelzer E, Benková E, Maizel A, Kleine-Vehn J.

- *CLE peptides counteract cytokinin-induced PIN-FORMED 1 degradation through clathrin-mediated endocytosis modulation.* Duclercq J, **Marhavý P**, Abuzeineh A, Simon R, Van Leene J, De Jaeger G, Friml J and Benkova E.

Presentation

Cytokinin effect on PIN1 trafficking. Annual ENPER Meeting; Marienburg, Zell an der Mosel, Germany, 2010.

Poster presentation

Hormonal interactions regulating lateral root development of plants; VIB seminar, Blankenberge, Belgium, 2009.

Cytokinin effect on PIN1 trafficking. Membrane Dynamics of the Cell; Dusseldorf, Germany, 2010.

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