Developmental Cell
Short Article

Cytokinin Modulates Endocytic Trafficking of PIN1 Auxin Efflux Carrier to Control Plant Organogenesis

Peter Marhavý,[1,2] Agnieszka Bielach,[1,2] Lindy Abas,[3] Anas Abuzeineh,[1,2] Jerome Duclercq,[1,2] Hirokazu Tanaka,[1,2,7] Markéta Pařezová,[4] Jan Petrášek,[4,5] Jiří Friml,[1,2,6] Jürgen Kleine-Vehn,[1,2,3] and Eva Benková[1,2,*]
[1]Department of Plant Systems Biology, VIB, Technologiepark 927, B-9052 Gent, Belgium
[2]Department of Plant Biotechnology and Genetics, Ghent University, Technologiepark 927, B-9052 Gent, Belgium
[3]Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences Vienna, Muthgasse 18, 1190 Vienna, Austria
[4]Institute of Experimental Botany, ASCR, Rozvojová 263, 16502 Prague, Czech Republic
[5]Department of Experimental Plant Biology, Faculty of Science, Charles University, Viničná 5, 128 44 Prague 2, Czech Republic
[6]Department of Functional Genomics and Proteomics, Faculty of Science, and Central European Institute of Technology (CEITEC), Masaryk University, 62500 Brno, Czech Republic
[7]Present address: Department of Biological Science, Graduate School of Science, Osaka University, Machikaneyama-cho 1-1, Toyonaka, Osaka 560-0043, Japan
*Correspondence: evben@psb.vib-ugent.be
DOI 10.1016/j.devcel.2011.08.014

SUMMARY

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 (Müller 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 toward 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 (Müller and Sheen, 2008; Dello Ioio et al., 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 (Gälweiler 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 hr, 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 available online). 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 (Benková 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 (Benková 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 hr 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 (Benková 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; Figures S1F, 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 hr of BA treatment, the membrane PIN1-GFP signal was reduced by 45%–50% and almost completely absent within 5 hr (Figure 2A). These results indicate that, besides the previously shown transcriptional regulation (Dello Ioio et al., 2008; Růžička 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.

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 hr, 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). Pretreatment with 400 μM 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 hr 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 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 downregulation (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 a 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 hr after cytokinin application and, from 3 hr on, the signal gradually increased (Figure 2A), most likely following the onset of the cytokinin-mediated transcriptional stimulation, as previously described for *PIN7* (Růžička 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 hr 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

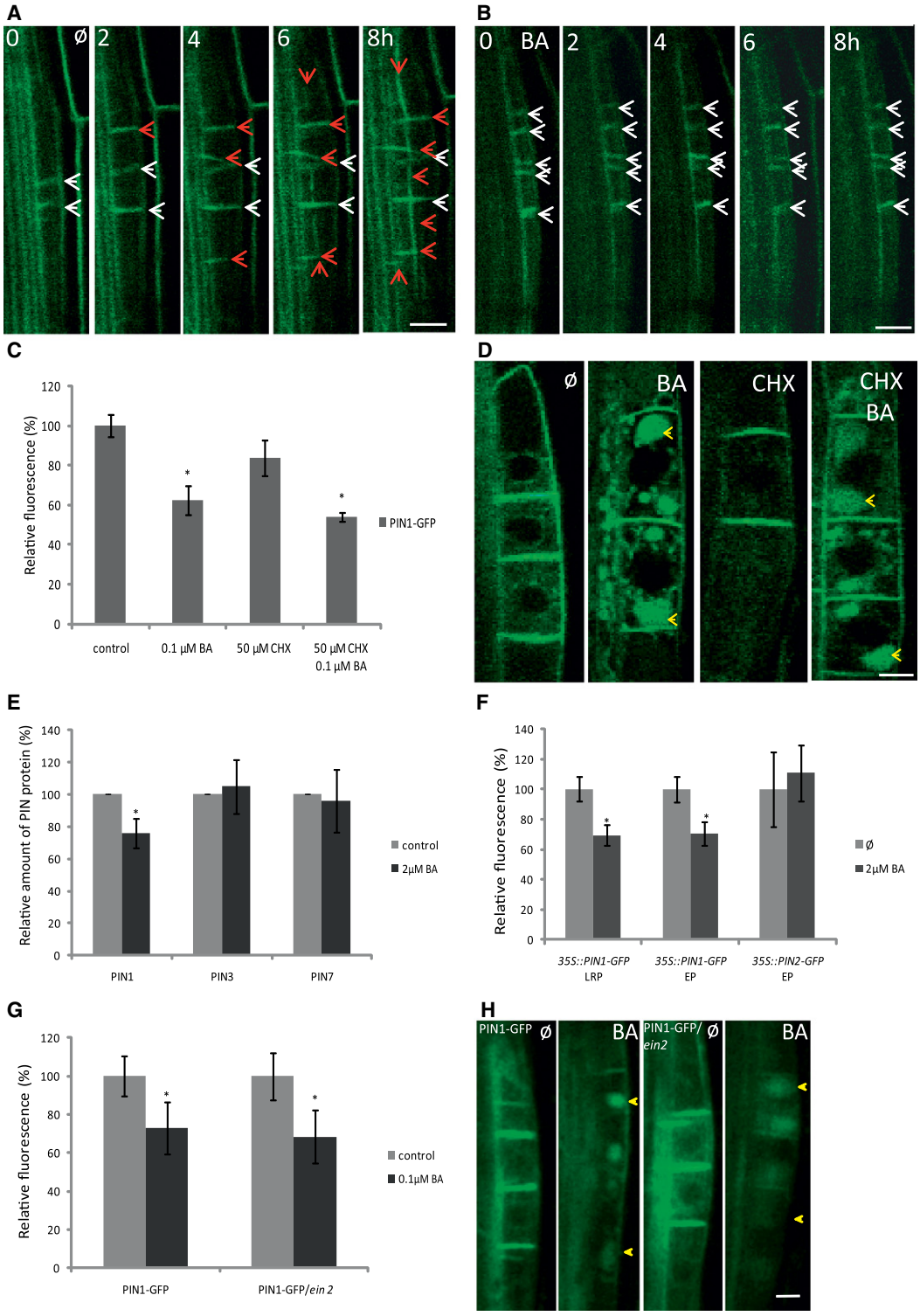

Figure 1. Cytokinin Inhibits LRP Development and Rapidly Depletes PIN1 from the Plasma Membranes

(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).

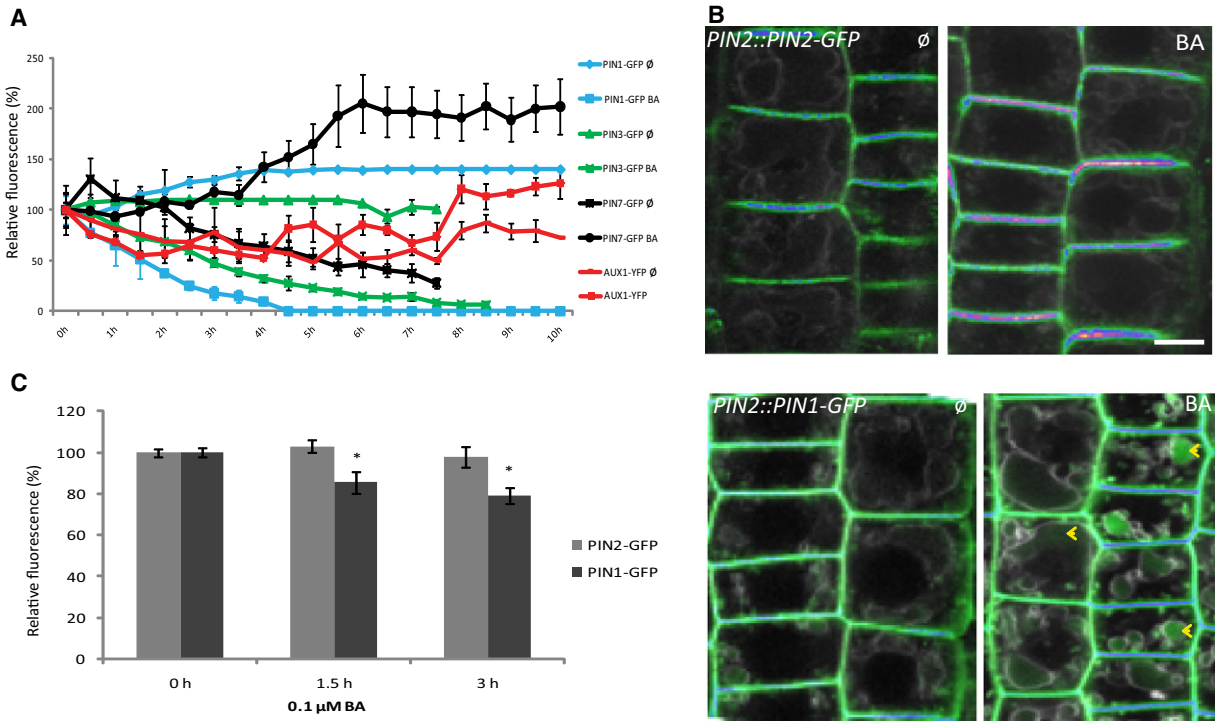

Figure 2. PIN1 Exhibits High Sensitivity to Cytokinin

(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 hr 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.

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.

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.

(F) PIN1-GFP, but not PIN2-GFP, expressed under the control of the *35S* promoter is downregulated after BA treatment in both LRP and root epidermal cells (*$p < 0.05$, n = 10).

(G and 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, and G) and root epidermal cell (EP) 1.5 hr after BA treatment (F). Error bars mark standard error of the mean. See also Figure S1.

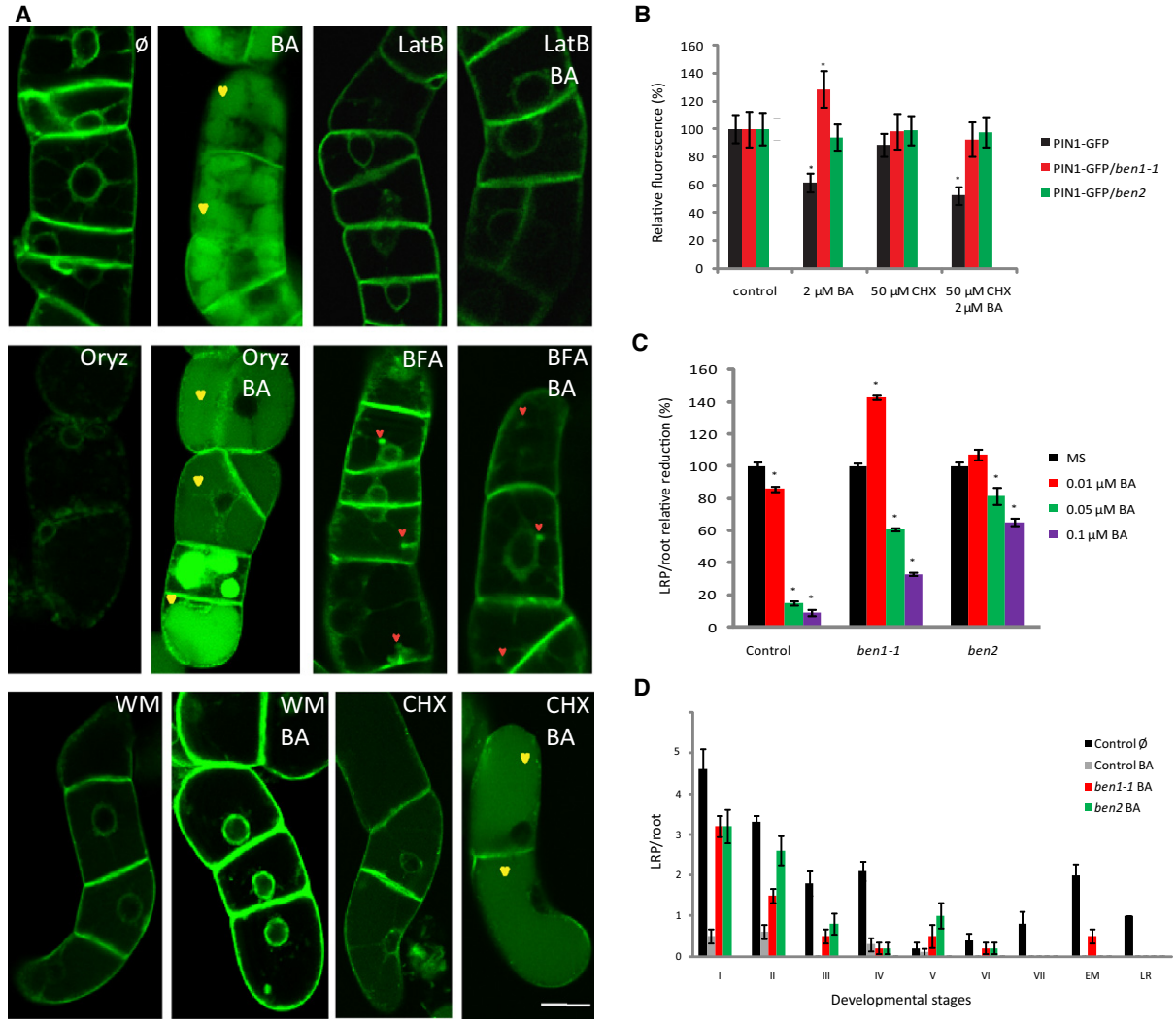

Figure 3. Cytokinin Activity Depends on the Functional Endocytic Trafficking

(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.

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 or 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 PIN1 Degradation

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.

To examine the developmental consequences of the modified cytokinin sensitivity toward 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).

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 (Figures 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; Růžička 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 a 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

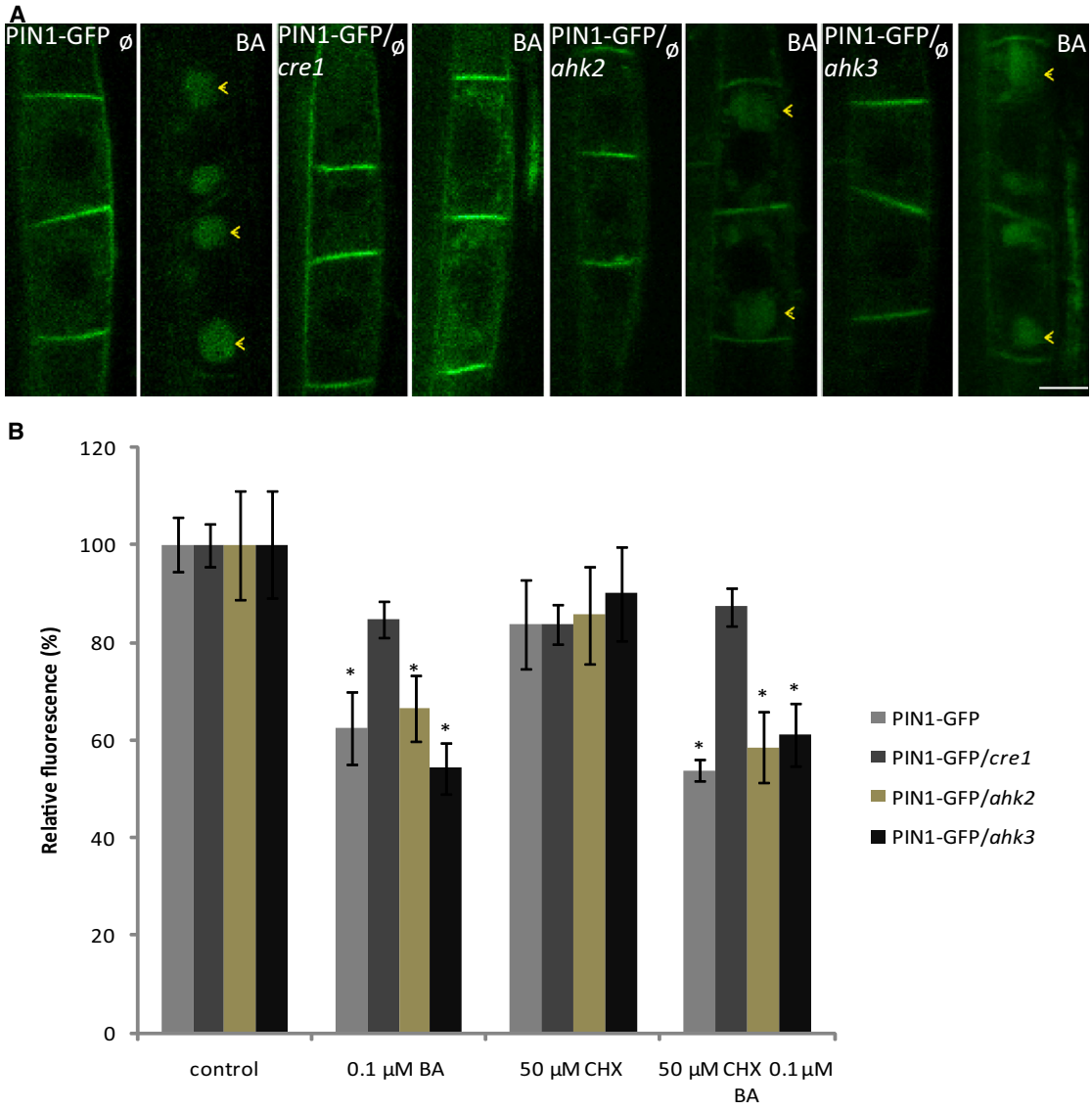

Figure 4. Cytokinin-Induced PIN1 Degradation Requires a Functional Cytokinin Perception

(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 hr after BA treatment. Yellow arrows indicate vacuoles with GFP accumulation. Scale bar, 8 µm. Error bars mark standard error of the mean. See also Figure S4.

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* (Benková et al., 2003); *PIN2:PIN2-GFP* (Xu and Scheres, 2005); *PIN7::PIN7-GFP* (Blilou et al., 2005); *PIN3::PIN3-GFP* (Zádníková et al., 2010); *PIN2::PIN1-GFP* (Wiśniewska et al., 2006); *AUX1::AUX1-YFP* (Swarup et al., 2004); *35S::PIN1-GFP* (Růžička 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), *arr12-1* (N6978) (Mason et al., 2005), and *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 and 1% agar (pH 5.7) and stratified for 2 days at 4°C. Seedlings were grown on vertically oriented plates in growth chambers under a 16-hr-light/8-hr-dark photoperiod at 18°C. Tobacco VBI-0 cells were grown in liquid media and stably transformed with *Arabidopsis*

PIN1::PIN1-GFP (Benková et al., 2003) and *PIN7::PIN7-GFP* (Blilou et al., 2005). For transformation details see Supplemental Experimental Procedures.

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 hr in the dark at 22°C. Drugs and hormones used were as follows: 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 hr 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 Supplemental Experimental Procedures. The fluorescence intensity of the membrane PIN-GFP signal was quantified with ImageJ (NIH; http://rsb.info.nih.gov/ij) as described (Zádníková 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 hr 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 (Benková 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 Supplemental Experimental Procedures.

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 hr. 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 et al., 2003) were used. The statistical significance was evaluated with Student's t test (paired, 2-tailed, n = 7 independent biological repeats). For details see Supplemental Experimental Procedures.

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

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.devcel.2011.08.014.

ACKNOWLEDGMENTS

We thank Professor Tatsuo Kakimoto for sharing published material, Christian Luschnig for the PIN3 serum, Candela Cuesta Moliner for technical assistance, Stephanie Robert and Satoshi Naramoto for discussions, and Martine De Cock for help in preparing the manuscript. This work was supported by the ERC starting independent research grant ERC-2007-Stg-207362-HCPO (P.M., A.A., J.D., E.B.), Austrian Science Fund FWF project P21533 (L.A.), GA ASCR IAA601630703 and MSM0021622415 (to J.F.), and the research programs of the Ministry of Education of the Czech Republic, projects LC06034 (M.P. and J.P.) and MSM 0021620858 (J.P.). H.T. is indebted to the MEXT Grant-in-Aid for Scientific Research on Priority Areas (23012026).

Received: February 22, 2011
Revised: June 9, 2011
Accepted: August 15, 2011
Published online: September 29, 2011

REFERENCES

Abas, L., and Luschnig, C. (2010). Maximum yields of microsomal-type membranes from small amounts of plant material without requiring ultracentrifugation. Anal. Biochem. *401*, 217–227.

Abas, L., Benjamins, R., Malenica, N., Paciorek, T., Wiśniewska, J., Moulinier-Anzola, J.C., Sieberer, T., Friml, J., and Luschnig, C. (2006). Intracellular trafficking and proteolysis of the *Arabidopsis* auxin-efflux facilitator PIN2 are involved in root gravitropism. Nat. Cell Biol. *8*, 249–256.

Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., and Friml, J. (2003). Local, efflux-dependent auxin gradients as a common module for plant organ formation. Cell *115*, 591–602.

Bennett, M.J., Marchant, A., Green, H.G., May, S.T., Ward, S.P., Millner, P.A., Walker, A.R., Schulz, B., and Feldmann, K.A. (1996). *Arabidopsis AUX1* gene: a permease-like regulator of root gravitropism. Science *273*, 948–950.

Blilou, I., Xu, J., Wildwater, M., Willemsen, V., Paponov, I., Friml, J., Heidstra, R., Aida, M., Palme, K., and Scheres, B. (2005). The PIN auxin efflux facilitator network controls growth and patterning in *Arabidopsis* roots. Nature *433*, 39–44.

Cary, A.J., Liu, W., and Howell, S.H. (1995). Cytokinin action is coupled to ethylene in its effects on the inhibition of root and hypocotyl elongation in *Arabidopsis thaliana* seedlings. Plant Physiol. *107*, 1075–1082.

Dello Ioio, R., Nakamura, K., Moubayidin, L., Perilli, S., Taniguchi, M., Morita, M.T., Aoyama, T., Costantino, P., and Sabatini, S. (2008). A genetic framework for the control of cell division and differentiation in the root meristem. Science *322*, 1380–1384.

Dharmasiri, N., Dharmasiri, S., and Estelle, M. (2005). The F-box protein TIR1 is an auxin receptor. Nature *435*, 441–445.

Friml, J., Vieten, A., Sauer, M., Weijers, D., Schwarz, H., Hamann, T., Offringa, R., and Jürgens, G. (2003). Efflux-dependent auxin gradients establish the apical-basal axis of *Arabidopsis*. Nature *426*, 147–153.

Gälweiler, L., Guan, C., Müller, A., Wisman, E., Mendgen, K., Yephremov, A., and Palme, K. (1998). Regulation of polar auxin transport by AtPIN1 in *Arabidopsis* vascular tissue. Science *282*, 2226–2230.

Geldner, N., Friml, J., Stierhof, Y.-D., Jürgens, G., and Palme, K. (2001). Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. Nature *413*, 425–428.

Geldner, N., Dénervaud-Tendon, V., Hyman, D.L., Mayer, U., Stierhof, Y.-D., and Chory, J. (2009). Rapid, combinatorial analysis of membrane compartments in intact plants with a multicolor marker set. Plant J. *59*, 169–178.

Higuchi, M., Pischke, M.S., Mähönen, A.P., Miyawaki, K., Hashimoto, Y., Seki, M., Kobayashi, M., Shinozaki, K., Kato, T., Tabata, S., et al. (2004). *In planta* functions of the *Arabidopsis* cytokinin receptor family. Proc. Natl. Acad. Sci. USA *101*, 8821–8826.

Holtorf, H., Schöb, H., Kunz, C., Waldvogel, R., and Meins, F., Jr. (1999). Stochastic and nonstochastic post-transcriptional silencing of chitinase and β-1,3-glucanase genes involves increased RNA turnover-possible role for ribosome-independent RNA degradation. Plant Cell *11*, 471–484.

Hwang, I., and Sheen, J. (2001). Two-component circuitry in Arabidopsis cytokinin signal transduction. Nature *413*, 383–389.

Kleine-Vehn, J., Leitner, J., Zwiewka, M., Sauer, M., Abas, L., Luschnig, C., and Friml, J. (2008). Differential degradation of PIN2 auxin efflux carrier by

retromer-dependent vacuolar targeting. Proc. Natl. Acad. Sci. USA *105*, 17812–17817.

Laplaze, L., Benkova, E., Casimiro, I., Maes, L., Vanneste, S., Swarup, R., Weijers, D., Calvo, V., Parizot, B., Herrera-Rodriguez, M.B., et al. (2007). Cytokinins act directly on lateral root founder cells to inhibit root initiation. Plant Cell *19*, 3889–3900.

Malamy, J.E., and Benfey, P.N. (1997). Down and out in *Arabidopsis*: the formation of LRs. Trends Plant Sci. *2*, 390–396.

Mason, M.G., Mathews, D.E., Argyros, D.A., Maxwell, B.B., Kieber, J.J., Alonso, J.M., Ecker, J.R., and Schaller, G.E. (2005). Multiple type-B response regulators mediate cytokinin signal transduction in Arabidopsis. Plant Cell *17*, 3007–3018.

Müller, B., and Sheen, J. (2008). Cytokinin and auxin interaction in root stem-cell specification during early embryogenesis. Nature *453*, 1094–1097.

Ongaro, V., and Leyser, O. (2008). Hormonal control of shoot branching. J. Exp. Bot. *59*, 67–74.

Opatrný, Z., and Opatrná, J. (1976). The specificity of the effect of 2,4-D and NAA on the growth, micromorphology, and occurrence of starch in long-term *Nicotiana tabacum* L. cell strains. Biol. Plant. *18*, 359–365.

Paciorek, T., Zazímalová, E., Ruthardt, N., Petrásek, J., Stierhof, Y.-D., Kleine-Vehn, J., Morris, D.A., Emans, N., Jürgens, G., Geldner, N., and Friml, J. (2005). Auxin inhibits endocytosis and promotes its own efflux from cells. Nature *435*, 1251–1256.

Roman, G., Lubarsky, B., Kieber, J.J., Rothenberg, M., and Ecker, J.R. (1995). Genetic analysis of ethylene signal transduction in *Arabidopsis thaliana*: five novel mutant loci integrated into a stress response pathway. Genetics *139*, 1393–1409.

Růžička, K., Ljung, K., Vanneste, S., Podhorská, R., Beeckman, T., Friml, J., and Benková, E. (2007). Ethylene regulates root growth through effects on auxin biosynthesis and transport-dependent auxin distribution. Plant Cell *19*, 2197–2212.

Růžička, K., Simásková, M., Duclercq, J., Petrásek, J., Zazímalová, E., Simon, S., Friml, J., Van Montagu, M.C.E., and Benková, E. (2009). Cytokinin regulates root meristem activity via modulation of the polar auxin transport. Proc. Natl. Acad. Sci. USA *106*, 4284–4289.

Sauer, M., Paciorek, T., Benková, E., and Friml, J. (2006). Immunocytochemical techniques for whole-mount *in situ* protein localization in plants. Nat. Protoc. *1*, 98–103.

Swarup, R., Kargul, J., Marchant, A., Zadik, D., Rahman, A., Mills, R., Yemm, A., May, S., Williams, L., Millner, P., et al. (2004). Structure-function analysis of the presumptive Arabidopsis auxin permease AUX1. Plant Cell *16*, 3069–3083.

Tanaka, H., Kitakura, S., De Rycke, R., De Groodt, R., and Friml, J. (2009). Fluorescence imaging-based screen identifies ARF GEF component of early endosomal trafficking. Curr. Biol. *19*, 391–397.

Vanneste, S., De Rybel, B., Beemster, G.T.S., Ljung, K., De Smet, I., Van Isterdael, G., Naudts, M., Iida, R., Gruissem, W., Tasaka, M., et al. (2005). Cell cycle progression in the pericycle is not sufficient for SOLITARY ROOT/IAA14-mediated lateral root initiation in Arabidopsis thaliana. Plant Cell *17*, 3035–3050.

Wiśniewska, J., Xu, J., Seifertová, D., Brewer, P.B., Růžička, K., Blilou, I., Rouquié, D., Benková, E., Scheres, B., and Friml, J. (2006). Polar PIN localization directs auxin flow in plants. Science *312*, 883.

Xu, J., and Scheres, B. (2005). Dissection of Arabidopsis ADP-RIBOSYLATION FACTOR 1 function in epidermal cell polarity. Plant Cell *17*, 525–536.

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

Zhao, Z., Andersen, S.U., Ljung, K., Dolezal, K., Miotk, A., Schultheiss, S.J., and Lohmann, J.U. (2010). Hormonal control of the shoot stem-cell niche. Nature *465*, 1089–1092.