SCF^{TIR1/AFB}-auxin signalling regulates PIN vacuolar trafficking and auxin fluxes during root gravitropism

Paweł Baster¹, Stéphanie Robert^{1,2},
Jürgen Kleine-Vehn^{1,3}, Steffen Vanneste¹,
Urszula Kania¹, Wim Grunewald¹,
Bert De Rybel^{1,4}, Tom Beeckman¹
and Jiří Friml^{1,5,*}

¹Department of Plant Systems Biology, VIB and Department of Plant Biotechnology and Genetics, Ghent University, Ghent, Belgium,

²Department of Forest Genetics and Plant Physiology, Swedish University of Agricultural Sciences/Umeå Plant Science Center, Umeå, Sweden, ³Department of Applied Genetics and Cell Biology, University of Applied Life Sciences and Natural Resources (BOKU), Vienna, Austria, ⁴Laboratory of Biochemistry, University of Wageningen, Wageningen, The Netherlands and ⁵Institute of Science and Technology Austria (IST Austria), Klosterneuburg, Austria

The distribution of the phytohormone auxin regulates many aspects of plant development including growth response to gravity. Gravitropic root curvature involves coordinated and asymmetric cell elongation between the lower and upper side of the root, mediated by differential cellular auxin levels. The asymmetry in the auxin distribution is established and maintained by a spatio-temporal regulation of the PIN-FORMED (PIN) auxin transporter activity. We provide novel insights into the complex regulation of PIN abundance and activity during root gravitropism. We show that PIN2 turnover is differentially regulated on the upper and lower side of gravistimulated roots by distinct but partially overlapping auxin feedback mechanisms. In addition to regulating transcription and clathrin-mediated internalization, auxin also controls PIN abundance at the plasma membrane by promoting their vacuolar targeting and degradation. This effect of elevated auxin levels requires the activity of SKP-Cullin-F-box^{TIR1/AFB} (SCF^{TIR1/AFB})-dependent pathway. Importantly, also suboptimal auxin levels mediate PIN degradation utilizing the same signalling pathway. These feedback mechanisms are functionally important during gravitropic response and ensure fine-tuning of auxin fluxes for maintaining as well as terminating asymmetric growth.

The EMBO Journal (2013) 32, 260–274. doi:10.1038/emboj.2012.310; Published online 4 December 2012

Subject Categories: signal transduction; plant biology

Keywords: auxin; degradation; gravitropism; PIN

Introduction

The phytohormone auxin is an important regulator of cell morphogenesis shaping and directing growth of organs within different developmental contexts and in response to environmental signals (Vanneste and Friml, 2009). To ensure optimal growth and development, plants have acquired elaborate mechanisms to control the local auxin homeostasis, including control of auxin metabolism (Cheng *et al*, 2006, 2007; Stepanova *et al*, 2008; Tao *et al*, 2008), subcellular compartmentalization (Mravec *et al*, 2009; Barbez *et al*, 2012; Ding *et al*, 2012) and directional auxin transport mediated by plasma membrane-resident transporters, such as ABCB, PIN-FORMED (PIN) and AUXIN-RESISTANT 1 (AUX1) (Bennett *et al*, 1996; Geisler *et al*, 2005; Petrášek *et al*, 2006; Cho *et al*, 2007; Swarup *et al*, 2008; Jones *et al*, 2009). One of the prominent growth responses mediated by auxin transport is root gravitropism. Changes of the orientation relative to the gravity vector are perceived in the root tip, by the sedimentation of statoliths, defined as gravity-sensing organelles (Harrison and Masson, 2008; Leitz *et al*, 2009; Morita, 2010). This process appears to induce the relocation of the auxin efflux carriers (Petrášek *et al*, 2006) PIN3 and PIN7 to the lower side of the gravity-sensing cells, which presumably aligns auxin flux with gravity vector towards the lower side of the root tip (Friml *et al*, 2002; Harrison and Masson, 2008; Kleine-Vehn *et al*, 2010). From there, another auxin efflux carrier, PIN2, which is apically (shootward, upper cell side) localized in the lateral root cap and epidermal cells, mediates the directional auxin flow from the root tip to the elongation zone where control of elongation occurs (Luschnig *et al*, 1998; Müller *et al*, 1998; Abas *et al*, 2006; Wiśniewska *et al*, 2006). Hence, the PIN-mediated establishment of the asymmetric auxin distribution leads to a differential growth between the lower and the upper side of the root. As a consequence, root bends and re-orient in respect to the gravity vector, allowing the efficient exploration of the soil (Firn *et al*, 2000; Swarup *et al*, 2005).

The mechanisms underlying the PIN3 and PIN7 polarization in gravity-sensing columella cells and control of the PIN2 abundance at the plasma membrane for defined gravitropic response remain largely elusive. Nevertheless, some of the molecular processes controlling the subcellular localization of PIN proteins have been characterized (Grunewald and Friml, 2010). PIN proteins internalize continuously via a clathrin-mediated endocytotic pathway (Dhonukshe *et al*, 2007; Kitakura *et al*, 2011) and cycle back to the plasma membrane as shown by pharmacological approaches with a vesicle-budding inhibitor, Brefeldin A (BFA) (Geldner *et al*, 2001). This permanent cycling leads to a dynamic control of their polar localization and abundance at the plasma membrane (Kleine-Vehn *et al*, 2008a), which in turn, determines the rate and direction of the auxin flow (Paciorek *et al*, 2005; Wiśniewska *et al*, 2006). The

*Corresponding author. Department of Plant Systems Biology, VIB-Ghent University, Technologiepark 927, 9052 Ghent, Belgium. Tel.: +32 9 3313913; Fax: +32 9 3313809; E-mail: jiri.friml@psb.vib-ugent.be

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (<http://www.embojournal.org>) is Jiří Friml (jiri.friml@psb.vib-ugent.be)

Received: 27 September 2012; accepted: 19 October 2012; published online: 4 December 2012

constitutive endocytic recycling enables also rapid switches in PIN polarity and, consequently, directionality of auxin fluxes in response to environmental signals, including light and gravity (Friml *et al*, 2002; Kleine-Vehn *et al*, 2010; Ding *et al*, 2011; Rakusová *et al*, 2011).

Besides the control of the polar localization, PIN protein activity can be also regulated by degradation. Numerous studies reported the occurrence of PIN degradation in the vacuoles (Abas *et al*, 2006; Laxmi *et al*, 2008; Kleine-Vehn *et al*, 2008b; Shirakawa *et al*, 2009; Leitner *et al*, 2012; Marhavý *et al*, 2011), to which they are targeted via a BFA-sensitive canonical retrograde trafficking pathway, involving the retromer complex (Kleine-Vehn *et al*, 2008b). Moreover, PIN2 turnover depends on the proteasomal activity (Sieberer *et al*, 2000; Abas *et al*, 2006) and sorting for vacuolar delivery was recently associated with the formation of the polyubiquitin chains linked to the specific lysine residues at the PIN2 hydrophilic loop (Leitner *et al*, 2012). Together, this data highlights the importance of post-transcriptional regulations in auxin flux determination.

Notably, auxin itself modulates its own distribution by providing feedback on PIN biosynthesis and trafficking (Benjamins and Scheres, 2008). Short auxin treatments (≤ 2 h) activate the transcription of different PIN genes (Peer *et al*, 2004; Heisler *et al*, 2005; Vieten *et al*, 2005; Scarpella *et al*, 2006) and can stabilize PIN at the plasma membrane by inhibiting clathrin-mediated internalization (Paciorek *et al*, 2005; Robert *et al*, 2010). Recently, it was found that AUXIN-BINDING PROTEIN 1 (ABP1) is a positive regulator of clathrin-mediated endocytosis, which is inhibited upon auxin binding (Robert *et al*, 2010; Chen *et al*, 2012). In contrast, prolonged application of auxin also promotes the turnover of PIN proteins via an unknown mechanism (Sieberer *et al*, 2000; Vieten *et al*, 2005; Abas *et al*, 2006). How this duality of auxin action on endocytosis versus degradation is regulated is unknown.

The BFA fungal toxin is known to inhibit the activity of specific ADP-ribosylation factor GTP-exchange factors (ARF-GEFs) (Peyroche *et al*, 1999; Sata *et al*, 1999; Geldner *et al*, 2003). In plants, the secretory pathway is readily inhibited by BFA, resulting in the intracellular accumulation of endocytosed plasma membrane proteins such as PIN proteins (Geldner *et al*, 2001). Upon inhibition of endocytosis (at $\sim 25 \mu\text{M}$ of BFA), PIN proteins no longer end up in such a BFA compartments (Paciorek *et al*, 2005; Men *et al*, 2008; Kitakura *et al*, 2011). Interestingly, it has recently been discovered that at higher concentrations ($\sim 50 \mu\text{M}$), BFA also inhibits vacuolar targeting and degradation of PIN proteins (Kleine-Vehn *et al*, 2008b; Kleine-Vehn and Friml, 2008; Robert *et al*, 2010). Thus, different concentrations of BFA allow discriminating between effects on endocytosis for recycling and targeting for degradation (Robert *et al*, 2010). Notably, the aforementioned BFA concentration cutoff should not be taken precisely as most likely specificity of the BFA towards specific ARF-GEF's changes gradually.

Here, we show that PIN2 protein abundance is dynamically and differentially controlled at the upper and lower sides of a gravistimulated root. Both increased and decreased auxin levels change PIN2 stability by a post-transcriptional regulation of its vacuolar targeting. Moreover, we provide additional data to clarify the involvement of SCF^{TIR1/AFB}-based signalling in auxin-mediated PIN turnover. These findings

link auxin-mediated regulation of vesicle transport and asymmetric growth control during gravitropic response.

Results

Dynamic changes of auxin response and PIN2 abundance in gravistimulated roots

To better understand the regulation of auxin transport activity in response to gravity, we have investigated the dynamics of root bending, auxin redistribution and abundance of PIN2 in gravistimulated roots of *Arabidopsis thaliana*. We have indirectly visualized the auxin redistribution by monitoring the activity of the synthetic auxin-responsive promoter DR5rev (Ulmasov *et al*, 1997) driving expression of a nuclearly localized VENUS protein (DR5rev::3xVENUS-N7; Heisler *et al*, 2005). Consistent with previous observations, after 2 h of gravistimulation, *Arabidopsis* root bent visibly (Figure 1A–F) and an asymmetric increase in DR5, expression was observed at the less elongated (lower) root side (Ottenschläger *et al*, 2003; Paciorek *et al*, 2005), whereas at the upper side of the bending root, the DR5 response was reduced (Figure 1G–M). This asymmetry in auxin response was maintained throughout gravity-induced root bending (Figure 1A–M). In time, the growth angle of the root became progressively parallel to the gravity vector (Figure 1F) and with a delay, a balanced DR5 expression was re-establishing (Figure 1L and M). We have confirmed the formation of auxin lateral gradient in roots responding to the gravity with use of highly dynamic *DII-VENUS* reporter system (see Supplementary Figure 1; Brunoud *et al*, 2012). This system was previously used to precisely place the timing of auxin accumulation during root gravitropic response (Band *et al*, 2012). It is important to note that the timing of onset and disappearance of the DR5rev::3xVENUS-N7 signal lags behind the real kinetics of the auxin distribution due to the time needed for VENUS maturation and turnover. Nonetheless, in spite of the inherent shortcomings of this reporter, we were able to demonstrate a clear spatio-temporal regulation of the auxin distribution during gravitropic bending.

To further characterize the regulation of the efflux carrier activity in response to gravity, we have investigated PIN2 abundance at the plasma membrane of gravistimulated roots. As previously suggested, following gravistimulation, PIN2 distribution became asymmetric between the upper and lower sides of the root, in concordance with an asymmetrical auxin distribution (Paciorek *et al*, 2005; Abas *et al*, 2006; Kleine-Vehn *et al*, 2008b) (Figure 1N–T). We have quantified the plasma membrane-localized PIN2 abundance at the lower and upper sides of horizontally placed roots at different time points after gravistimulation. Within 2 h of gravistimulation, an increase in PIN2 at the plasma membrane of cells on the lower root side was detected, which spatially correlated with an increase in auxin response (Figure 1H, M, O and S). Following this temporal stabilization, the PIN2 level at the lower side of the root started to decrease to supposedly re-establish the pre-stimulation levels after 12 h of gravistimulation (Figure 1O–S). Thus, at the lower root side, the PIN2 levels transiently increased before gradually decreasing to the pre-stimulation values.

In parallel, at the upper side of the bending root, where auxin response initially decreases (Figure 1G–M), PIN2 protein levels at the plasma membrane steadily decreased

Figure 1 Localization of PIN2-GFP protein and auxin maxima during root gravitropic response. (A–E) Kinetics of the root bending in seedlings at 0 h (A), 2 h (B), 4 h (C), 8 h (D) and 12 h (E) after gravistimulation. (F) Angle of the root curvature in relation to horizon after gravistimulation. $n=3$ independent experiments with at least six roots analysed for each assay. (G–L) Activity of *DR5rev::3XVENUS-N7* promoter in seedlings at 0 h (G), 2 h (H), 4 h (I), 8 h (J), 12 h (K) and 24 h (L) after gravistimulation. Pictures represent maximum intensity projection of median root sections (10 Z-sections spaced ~4.5 μm). (M) Quantification of the *DR5rev::3XVENUS-N7* expressing nuclei in the epidermal cells of the gravistimulated root. $n=3$ independent experiments with at least six roots analysed for each assay. Note a minimum of *DR5rev::3XVENUS-N7* expression on the upper side as well as maximum on the lower side of the root 8 h after gravistimulation marked on (J) and graph (M) by red and green discontinuous lines, respectively. (N–S) PIN2-GFP protein localization in epidermal and cortical cells at 0 h (N), 2 h (O), 4 h (P), 8 h (Q) and 12 h (R) after gravistimulation. Pictures represent maximum intensity projection of median root sections (10 Z-sections spaced ~1 μm apart). (S) PIN2-GFP signal intensity in gravistimulated roots. $n=3$ independent experiments with at least six roots analysed for each assay. Error bars represent standard error of the mean (s.e.m.), P -value calculated according to Student's t -test. Signal intensities are coded white to black and blue to yellow corresponding to increasing intensity levels (see colour scale). cor, cortex; epi, epidermis; lower, lower side of gravistimulated root; upper, upper side of gravistimulated root. Scale bar = 10 μm.

in time (Figure 1N–P and S), probably because of higher rates of protein degradation due to an increased targeting to the vacuole (Kleine-Vehn *et al*, 2008b; Figure 4A and B). Notably, after 4 h of gravistimulation, PIN2 started to accumulate

again at the plasma membrane, reaching levels close to the initial pre-stimulation levels at ~12 h after gravistimulation (Figure 1P–S). Thus, at the upper root side, the PIN2 levels initially decrease, which is followed by an increase leading to

re-establishment of the pre-stimulation values. The observed changes in signal intensity infer ~12 and 14% change in PIN2 abundance on the lower and upper sides of gravistimulated root, respectively. Notably, the recovery of symmetry in PIN2 protein levels at the plasma membrane after 12 h of gravistimulation at both lower and upper sides of the root presumably reflects a re-established symmetric auxin flow, resulting in vertical root growth (Figure 1).

Overall, our data shows that a spatio-temporal regulation of the auxin distribution after gravistimulation correlates with complex and differential regulation of the PIN2 abundance at the lower and upper side of gravistimulated roots. Specifically, the increase in auxin response at the lower side of the root is accompanied with the initial increase in PIN2 abundance followed by its gradual decrease. On the other hand, at the upper side of the root, we have detected a decrease in auxin response that is accompanied with initial decrease in PIN2 abundance followed by its gradual increase. Importantly, the differential auxin accumulation in all observed cases preceded changes in PIN2 abundance at the plasma membrane. The above findings also complement the observation of Luschnig *et al* (1998) that a missense *pin2* allele fails to establish gravity-induced lateral auxin gradient in the root.

Auxin promotes PIN2 degradation in the vacuoles at the lower side of the root

First, we have addressed the mechanisms underlying the regulation of PIN2 abundance at the lower side of the gravistimulated root. The initial, transient stabilization of PIN2 at the plasma membrane is presumably a result of a documented transient (≤ 2 h) inhibitory effect of higher auxin levels on PIN internalization (Paciorek *et al*, 2005; Robert *et al*, 2010; Chen *et al*, 2012; Lin *et al*, 2012). On the other hand, the following decrease in PIN2 levels that still coincides with a DR5-visualized local increase in auxin response (Figure 1) might be result of the long-term effect of auxin on PIN stability (Sieberer *et al*, 2000; Vieten *et al*, 2005). Therefore, we have tested the effect of prolonged (≥ 3 h) exogenous auxin application on PIN2 abundance at the plasma membrane. Following NAA treatment, we have observed a reduction of PIN2-GFP levels (in *PIN2::PIN2-GFP* (*eir1-1*) transgenic seedlings) at the plasma membrane concomitantly with an increase in a diffused vacuolar GFP signal (Figure 2A–C; see Supplementary Figure 2). This observation was confirmed by a significant reduction of PIN2 abundance in membrane protein extracts from NAA-treated seedlings as detected by western blots (Figure 2D).

We have then addressed the cellular mechanism of the auxin effect on PIN2 abundance. In general, protein abundance at the plasma membrane is expected to reflect a sum of transcription, translation, targeting and proteolysis. It has been shown previously that *PIN2* transcription does not change dramatically in response to auxin (Sieberer *et al*, 2000; Shin *et al*, 2005). Consistently, *PIN2* mRNA levels were shown to be induced by auxin with low amplitude and much slower kinetics than other *PIN* genes or other auxin inducible genes (Vieten *et al*, 2005; Lee *et al*, 2009). In agreement with those findings, in our experimental conditions, auxin treatment only mildly affected *PIN2* transcription (see Supplementary Figure 3), suggesting that auxin regulates PIN2 levels via a post-transcriptional mechanism. Moreover, auxin-mediated decrease in PIN2

from the plasma membrane occurred regardless of whether *PIN2* was expressed under its endogenous (Figure 2A–C) or constitutive, heterologous 35S promoter (Figure 2E–G), suggesting that the increased downregulation of PIN2 is not an indirect effect of an excess of PIN2 protein in the cell's endomembrane system.

It is proposed that PIN proteins are degraded in the vacuoles (Laxmi *et al*, 2008; Kleine-Vehn *et al*, 2008b; Shirakawa *et al*, 2009; Marhavý *et al*, 2011), where GFP-tagged proteins can be visualized after an incubation in the dark (Tamura *et al*, 2003). In these conditions, we have found a decrease in PIN2-GFP at the plasma membrane and concomitant increase in fluorescence signal in the vacuoles in response to auxin treatment (Figure 2A–C; see Supplementary Figure 2K–M). This strongly suggests that auxin downregulates PIN2 abundance at the plasma membrane by enhancing PIN trafficking to the vacuole. Moreover, the auxin application destabilized both apical and basal PIN1 and PIN2 cargos from the plasma membrane (Figure 2H–J; see Supplementary Figures 2A–C and 4A–C) and to lesser extent also non-polar integral plasma membrane proteins such as BRASSINOSTEROID INSENSITIVE1 (BRI1)-GFP and PLASMA MEMBRANE INTRINSIC PROTEIN2 (PIP2)-GFP (see Supplementary Figure 4D–I). In addition, we could demonstrate that auxin reduces PIN2 protein levels (Figure 2D), thereby strongly suggesting that the observed vacuolar targeting of PINs is associated with protein degradation.

To further confirm the auxin effect on the degradation of plasma membrane proteins, we have genetically manipulated the endogenous auxin concentrations in *Arabidopsis* seedlings. We have constitutively overexpressed the *Agrobacterium tumefaciens* indoleacetic acid-tryptophan monooxygenase (*iaaM*) under the strong ribosomal promoter RPS5. The *iaaM* enzyme converts tryptophan into indole-3-acetamide, which is then hydrolysed to indole-3-acetic acid (IAA) in plant cells (Klee *et al*, 1987; Romano *et al*, 1995; Weijers *et al*, 2001, 2005). The transcription of *PIN2* was not altered in the *RPS5::iaaM* transactivated line (see Supplementary Figure 5). We have then analysed the abundance and intracellular distribution of PIN2-GFP marker crossed into the *iaaM* background. Similarly to exogenously applied, endogenously produced auxin promoted an increased PIN2 degradation as manifested by higher vacuolar GFP signal (Figure 2K–M). The *iaaM* expression was shown to elevate cellular auxin concentration 2- to 10-fold (Klee *et al*, 1987; Romano *et al*, 1995). Therefore, considering that we have not used additional media supplementation, neither with tryptophan nor with auxin, it can be expected that the physiological threshold of auxin effect on increased PIN degradation is placed in the aforementioned range of auxin concentration change above normal/physiological level.

Taken together, this data shows that exogenously applied or endogenously produced auxin mediates the PIN targeting to the vacuole and promotes PIN2 degradation. This auxin effect presumably accounts for the decrease in PIN2 level at the lower side of the gravistimulated root after 4 h.

Auxin promotes PIN2 degradation by SCF^{TIR1/AFB}-mediated signalling

Next, we have assessed by which signalling pathway auxin promotes PIN2 degradation. We have previously shown that the inhibitory effect of auxin on PIN endocytosis is mediated

Figure 2 Auxin effect on PIN protein degradation. (A, B) Intracellular localization of PIN2-GFP (*eir1-1*) protein in seedlings incubated with DMSO (A) or with 10 μ M NAA (B). (C) Relative PIN2-GFP abundance at the plasma membrane versus the intracellular signal in *PIN2::PIN2::GFP* (*eir1-1*) expressing line. $n = 3$ independent experiments with at least six roots analysed for each assay and 60 cells counted in total. (D) Total membrane protein fractions probed with anti-PIN2 antibody. PIN2 protein level decreased when seedlings were treated 3 h with 20 μ M NAA. PIN2-specific band at ~ 70 kDa is marked with the cross. (E, F) Intracellular localization of 35S::PIN2-EosFP (*eir1-1*) protein in seedlings incubated with DMSO (E) or with auxin (10 μ M/14 h) (F). The effect of auxin on 35S::PIN2-EosFP targeting to the vacuole was observed after an extended auxin treatment probably due to the stabilized expression under 35S promoter, similarly to what was observed with 35S::PIP2-GFP (see Supplementary Figure 4G–I). (G) Relative PIN2-EosFP abundance at the plasma membrane versus intracellular signal in 35S::PIN2-EosFP (*eir1-1*) expressing line. $n = 3$ independent experiments with at least six roots analysed for each assay and ten cells counted for each root. (H, I) Intracellular localization of PIN2::PIN1-GFP protein in seedlings incubated with DMSO (H) or with 10 μ M NAA (I). (J) Relative PIN1-GFP abundance at the plasma membrane versus intracellular signal in *PIN2::PIN1-GFP* expressing line. $n = 3$ independent experiments with at least six roots analysed for each assay and ten cells counted for each root. (K, L) Intracellular localization of PIN2-GFP in *eir1-1* background (F₁ generation after cross with Col-0) (K) compared to *RPS5>>iaaM* background (F₁ generation after cross with *PIN2::PIN2-GFP* (*eir1-1*)) (L). (M) Relative PIN2-GFP abundance at the plasma membrane versus intracellular signal. $n = 1$ with 60 cells analysed. Error bars represent standard error of the mean (s.e.m.), P -value calculated according to Student's t -test. Arrowheads highlight differences in PIN protein retention at the plasma membrane and accumulation in the vacuoles. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see colour scale). Scale bar = 10 μ m.

by an ABP1-dependent signalling. Whereas auxin inhibits endocytosis instantaneously without *de novo* protein biosynthesis and nuclear auxin signalling (Paciorek *et al*, 2005; Robert *et al*, 2010; Chen *et al*, 2012; Lin *et al*, 2012), the auxin-induced PIN2 translocation to the vacuole for degradation required prolonged (≥ 3 h) auxin treatments (Figure 2A–C, see Supplementary Figure 4A–C). Given the fact that the earliest auxin-induced response proteins are detectable after ~ 10 –15 min of auxin application (Badescu and Napier, 2006), the auxin effect on the vacuolar targeting might require transcriptional regulation and *de novo* protein synthesis mediated by the SCF^{TIR1/AFB} pathway (Kepinski and Leyser, 2005; Dharmasiri *et al*, 2005a, b; Badescu and Napier, 2006; Tan *et al*, 2007). To test this hypothesis, we have used structural auxin analogues that can discriminate between ABP1- and SCF^{TIR1/AFB}-mediated signalling (Robert *et al*, 2010). We have observed that treatment

with IAA and all the synthetic auxin analogues, which induced transcriptional auxin response (as monitored by *DR5rev::GFP*), also promoted the degradation of PIN proteins. Moreover, the compounds, which did not induce *DR5rev::GFP* expression, did not cause a decrease in PIN abundance at the plasma membrane (Figure 3A–J; see Supplementary Figures 6 and 7). This data suggests that the same auxin perception mechanism and downstream effectors mediates regulation of gene transcription and control the PIN stability at the plasma membrane.

Indeed, in the quadruple *tir1afb1,2,3* mutant, auxin did not downregulate the PIN protein levels, showing a resistance to the auxin effect on PIN degradation (Figure 4A, B, D, E and G). Importantly, resistance could also be observed in double *tir1afb1, tir1afb2, tir1afb3* and partially in the single *tir1-1* mutant background, which all show comparable PIN protein levels to the wild type in control (untreated) conditions (see

Figure 3 Auxin analogues affect the SCF^{TIR1/AFB}-mediated signalling pathway and induce PIN protein turnover. (A–E) Activity of auxin-responsive promoter *DR5rev::GFP* (note the absence of induction in the elongation zone of the root marked in the internal panels by green arrowheads) and PIN protein turnover is not induced by DMSO (A), BA (B), naphthalene (C), IAA (D), or I3CA (E). (F–J) Structural auxin analogues, such as NAA (F), 5-F-IAA (G), 5-Br-IAA (H), 2,4,5-T (I) and 5-Cl-IAA (J) are effective in both inducing auxin-responsive promoter *DR5rev::GFP* (note the induction in the elongation zone of the root marked in the internal panels by red arrowheads) and promoting degradation of PIN proteins. Immunolocalization pictures represent maximum intensity projection of 20 Z-sections spaced $\sim 3.5 \mu\text{m}$ apart through the whole root. For quantitative analysis, see Supplementary Figure 7. Green and red arrowheads highlight the absence and presence of the induction in the elongation zone, respectively. Effect of IAA on PIN degradation and induction of *DR5rev::GFP* expression is presented in Supplementary Figure 6. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see colour scale). Scale bar = $10 \mu\text{m}$.

Supplementary Figure 8). We have also tested the *abp1-5* allele that contained a point mutation in the auxin-binding domain of ABP1 (Napier *et al*, 2002), and thus exhibited reduced auxin sensitivity (Robert *et al*, 2010; Xu *et al*, 2010). The auxin effect on PIN degradation in the *abp1-5* mutant was comparable to the one observed in the wild type (Figure 4C, F and G).

Next, we have attempted to identify downstream molecular components of the SCF^{TIR1/AFB} pathway that are involved in the control of PIN2 degradation process. We have analysed the promoter expression of 23 *ARF* genes in the root meristem using transcriptional nuclear GFP fusions (Rademacher *et al*, 2011). We have identified *ARF*'s 1, 2, 6, 9, 10, 16 and 19 as prominently expressed in epidermis of the root meristematic region where PIN2 is also specifically expressed (see Supplementary Figure 9). Subsequently, we have employed the $50 \mu\text{M}$ BFA and $20 \mu\text{M}$ NAA co-treatment on the *arf2*, *arf6*, *arf10arf16*, *arf19* and *arf7arf19* mutant lines. We were able to observe an increased PIN2 accumulation in BFA induced agglomeration in the *arf2* mutant when compared to the wild-type control. This effect was not observed after treatment with lower concentration of BFA (see Supplementary Figure 10). This suggests that the mutation in the *ARF2* gene disturbs vacuolar trafficking of PIN2 protein. We therefore propose that this transcription factor could be more specifically involved in the control of PIN2 vacuolar targeting.

Overall, these data suggest that SCF^{TIR1/AFB}-dependent signalling is required for auxin-induced PIN2 degradation. Thus, at the lower side of the gravistimulated root, overlapping auxin effects on PIN2 endocytosis (ABP1-mediated) and PIN2 vacuolar targeting (SCF^{TIR1/AFB}-mediated) presumably account for a transient increase in a PIN2-mediated auxin flow as well as for its subsequent decrease to the pre-stimulation levels.

Auxin depletion promotes PIN2 degradation at the upper side of the root

Next, we have examined the mechanisms underlying the regulation of PIN2 abundance at the upper side of the gravistimulated root. Here, PIN2 levels steadily decreased coinciding with reduced DR5-visualized auxin response (Figure 1). As seen for endogenous PIN2 (Figure 1), a similar asymmetric distribution with decreased levels at the upper epidermal cell file was observed also for PIN2-EosFP expressed under control of constitutive 35S promoter (see Supplementary Figure 11), suggesting that this decrease occurs independently of *PIN* transcriptional regulation. Furthermore, this decrease correlated with the increased PIN2 vacuolar targeting (Kleine-Vehn *et al*, 2008b; Figure 4A and B) also consistent with post-transcriptional regulation.

Figure 4 PIN protein degradation induced by auxin via the SCF^{TIR1/AFB}-mediated signalling pathway. (A–F) Immunolocalizations of PIN1 and PIN2 proteins after 14-h treatment with 20 μ M NAA. Auxin induced PIN protein degradation in the wild type (compare A to D), whereas *tir1afb1,2,3* mutant is resistant to the auxin effect on PIN degradation (compare B to E). Auxin induced PIN protein degradation in the *abp1-5* mutant (compare C to F). Immunolocalization pictures represent maximum intensity projection of the sections through the whole root (20 Z-sections spaced $\sim 3.5 \mu$ m). (G) Quantification of PIN1 and PIN2 signals at the plasma membrane. $n = 3$ independent experiments with at least six roots analysed for each assay. Error bars represent standard error of the mean (s.e.m.), P -value calculated according to Student's t -test. For the analysis of auxin-induced degradation in *tir1-1* single and double *tir1afb1*, *tir1afb2*, *tir1afb3* receptor mutant backgrounds, see Supplementary Figure 8. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see colour scale). Scale bar = 10 μ m.

We have tested whether decrease in PIN2 abundance at the plasma membrane and increased vacuolar targeting might be possibly a consequence of prolonged reduction in auxin levels. In *Arabidopsis* seedlings, not only the young leaves but also the cotyledons have a high capacity for auxin biosynthesis (Ljung *et al*, 2001). We have therefore reduced auxin biosynthetic capacity of the seedlings by removal of the cotyledons and shoot apical meristem (decapitation). We have observed that 14 h after such a decapitation, the growth rate of the roots was decreased but roots were still graviresponsive (see Supplementary Figure 12; Rashotte *et al*, 2000). By using *DR5rev::3XVENUS-N7*, we have detected a significant decrease in DR5-monitored auxin response in the PIN2 expression domain 14 h after decapitation (Figure 5A–C). These results are in line with previously reported findings showing that the auxin maximum in the root tip is highly stable and a decrease in auxin levels in the elongation zone can be detected only when auxin depletion by decapitation is prolonged (Grieneisen *et al*, 2007). Importantly, as a consequence of decapitation, we have observed a decreased PIN2 abundance at the plasma membrane and enhanced targeting to the vacuole (Figure 5D–F). This effect was independent of transcriptional control (see Supplementary Figure 13) and could be reversed by exogenous auxin application (see Supplementary Figure 14). What is more, we have confirmed the reduction of PIN2 level by western blot analysis of membrane fractions isolated 14 h after decapitation (Figure 5G).

To further simulate auxin depletion, we have used two independent chemical biology-based approaches. First, we have used the auxin-antagonist α -(phenyl ethyl-2-one)-indole-3-acetic acid (PEO-IAA) (Hayashi *et al*, 2008) that counteracts the auxin effect on transcription presumably by binding to the SCF^{TIR1} receptor (Nishimura *et al*, 2009). After 3 h of treatment, PEO-IAA caused a drop in auxin signalling as reflected by reduced expression of *DR5rev::3XVENUS-N7* reporter in epidermal and lateral root cap cells of the root apical meristem (Figure 6A–C). Similar treatment increased vacuolar targeting of PIN2 protein (Figure 6D–F) and, to lesser extent also non-polar integral plasma membrane proteins BRI1-GFP and PIP2-GFP (see Supplementary Figure 15). We have additionally observed that PEO-IAA disturbed the formation of the lateral gradient of PIN2 and consequently gravitropic response of the roots (see Supplementary Figure 16). Importantly, the PEO-IAA-induced destabilization of PIN2 from the plasma membrane could be counteracted by exogenous auxin application (see Supplementary Figure 17). Western blot analysis of membrane protein fractions confirmed reduced PIN2 levels after PEO treatment (Figure 6G). This suggests that a lower throughput of SCF^{TIR1/AFB}-mediated transcriptional auxin pathway decreases PIN stability at the plasma membrane. Second, we have interfered with Trp-dependent auxin biosynthesis by compromising the activity of key enzymatic components of this pathway. We have used L-Kynurenine, a competitive and specific inhibitor of TAA1/TAR enzymatic activity, which was

Figure 5 The effect of auxin depletion by decapitation on PIN2 protein turnover. (A, B) Activity of the auxin-responsive promoter *DR5rev::3XVENUS-N7* 14 h after the decapitation (B) compared to the untreated control (A). Note a decreased number of nuclei positive for *DR5rev::3XVENUS-N7* expression in epidermis and lateral root cap tissue, marked by the white line. (C) Quantification of *DR5rev::3XVENUS-N7* expression in epidermal tissue of seedlings 14 h after decapitation. $n = 3$ independent experiments with at least ten roots analysed for each assay. (D, E) Auxin depletion after decapitation in *PIN2::PIN2-GFP (eir1-1)* expressing seedlings resulted in increased vacuolar accumulation of PIN2 protein (E) than that of the untreated control (D). (F) Relative PIN2-GFP abundance at the plasma membrane versus intracellular signal in decapitated *PIN2::PIN2-GFP (eir1-1)* expressing seedlings. $n = 3$ independent experiments with at least six roots analysed for each assay and eight cells counted for each root. (G) Total membrane protein fractions were probed with anti-PIN2 antibody. PIN2 protein levels were decreased 14 h after decapitation. PIN2-specific band at ~70 kDa is marked with the cross. Error bars represent standard error of the mean (s.e.m.), P -value calculated according to Student's t -test. Arrowheads highlight differences in vacuolar accumulation of the PIN proteins. White line highlights differences in *DR5rev::3XVENUS-N7* expression in epidermis and lateral root cap tissues. Red fluorescence represents propidium iodide staining. decap, decapitated; untr, untreated. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see colour scale). Scale bar = 10 μ m.

shown to reduce *DR5-GUS* expression in the *Arabidopsis* roots (He *et al*, 2011). We have observed increased vacuolar accumulation coinciding with decreased plasma membrane abundance of PIN2-derived GFP signal after 24-h treatment with L-Kynurenine. Importantly, this effect could be reversed by co-incubation with auxin (Figure 6H–K).

As a complementary approach, we have genetically reduced the transcriptional auxin signalling. We have used a *HS::axr3-1* that expresses a stabilized allele of IAA17 after heat shock, resulting in a strong dominant repression of SCF^{TIR1/AFB}-regulated transcripts (Knox *et al*, 2003). Importantly, while *PIN2* transcript levels were unaffected (Figure 7A), heat shock diminished PIN2 levels in membrane protein fractions (Figure 7B) as revealed by western blot analysis. Consistently, heat shock caused an increase in vacuolar PIN2-GFP fluorescence signal along with the decrease in the fluorescence levels at the plasma membrane and caused root agravitropism (Figure 7C–E; Robert *et al*, 2010). These data imply that the genetic interference with SCF^{TIR1/AFB} auxin signalling promotes PIN protein degradation. We have also analysed the stability of PIN2 protein in decapitated *HS::axr3-1* seedlings. We could not observe an additive effect of decapitation on vacuolar targeting of PIN2 protein in this genetic background (see Supplementary Figure 18). This suggests that increased vacuolar targeting (and degradation as shown by western blot analysis) of PIN2 efflux carrier triggered by the removal of cotyledons and shoot apical meristem is most likely caused by the changes in auxin levels rather than by possible secondary effects of tissue wounding like changes in cytokinin or jasmonate activity (Crane and Ross, 1986; Wasternack, 2007; Sun *et al*, 2009; Marhavý *et al*, 2011).

Thus, decreasing auxin levels or interfering with SCF^{TIR1/AFB} auxin signalling leads to destabilization of PIN2 from the plasma membrane and higher rate of its vacuolar targeting. Overall, our data suggest that both the auxin decrease below optimal as well as increase above optimal levels can destabilize PIN proteins at the plasma membrane and, subsequently, induce PIN trafficking to the vacuole for degradation. Hence, 'optimal' auxin levels are required to stabilize PIN2 proteins for their action in gravitropic response.

Discussion

Dual regulation of PIN vacuolar targeting and degradation by auxin levels

The data presented in this study indicate that both, a prolonged increase or decrease in cellular auxin levels induce targeting of PIN auxin transporters (Petrásek *et al*, 2006) to the vacuole, thereby regulating the abundance of the auxin carriers at the plasma membrane. It appears that an 'optimal' auxin concentration is required to maintain PIN protein levels and thus auxin transport capacity at the plasma membrane. These effects of opposite auxin concentrations on PIN trafficking to the vacuole apparently depend on the canonical auxin signalling pathway, involving auxin-dependent degradation of Aux/IAA transcriptional repressor proteins (Kepinski and Leyser, 2005; Dharmasiri *et al*, 2005a). Given the known outlines of the PIN subcellular trafficking (Kleine-Vehn and Friml, 2008), auxin acts most likely in the regulation of the balance between recycling of PIN proteins

Figure 6 The effect of chemically induced auxin depletion on PIN2 protein turnover. (A, B) Activity of the *DR5rev::3XVENUS-N7* promoter after 3-h treatment with 50 μ M PEO-IAA (B) compared to the DMSO-treated control (A). Note a decreased number of nuclei positive for *DR5rev::3XVENUS-N7* expression in epidermis and lateral root cap tissue, marked by the white line. (C) Quantification of *DR5rev::3XVENUS-N7* expression level in epidermal tissue of seedlings after 3-h treatment with 50 μ M PEO-IAA. $n = 3$ independent experiments with at least six roots analysed for each assay. (D, E) Chemical auxin depletion by treatment with 50 μ M of PEO-IAA for 3 h resulted in higher vacuolar accumulation of PIN2 protein (E) when compared to DMSO-treated control (D). (F) Relative PIN2-GFP abundance at the plasma membrane versus intracellular signal in *PIN2::PIN2-GFP (eir1-1)* expressing seedlings treated with 50 μ M PEO-IAA. $n = 3$ independent experiments with at least six roots analysed for each assay and ten cells counted for each root. (G) Total membrane protein fractions were probed with anti-PIN2 antibody. PIN2 protein levels were decreased after 3-h treatment with 50 μ M PEO-IAA. PIN2-specific band at ~ 70 kDa is marked with the cross. (H, I) Increased vacuolar accumulation and decreased plasma membrane abundance of PIN2-GFP after treatment with 1 μ M ι -Kynurenine (24 h/dark) (I) compared to DMSO-treated control (H). (J) Destabilization from the plasma membrane and vacuolar targeting of PIN2-GFP upon ι -Kynurenine (see H and I) is reversed when co-treated with 0.1 μ M NAA. (K) Relative PIN2-GFP abundance at the plasma membrane versus intracellular signal in *PIN2::PIN2-GFP (eir1-1)* expressing seedlings treated with ι -Kynurenine. $n = 3$ independent experiments with at least six roots analysed for each assay and ten cells counted for each root. Error bars represent standard error of the mean (s.e.m.), P -value calculated according to Student's t -test. Arrowheads highlight differences in vacuolar accumulation and plasma membrane abundance of PIN2 protein. White line highlights differences in *DR5rev::3XVENUS-N7* expression in epidermis and lateral root cap tissues. Red fluorescence represents propidium iodide staining. decap, decapitated; unr, untreated; KYN, ι -Kynurenine. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see colour scale). Scale bar = 10 μ m.

back to the plasma membrane versus trafficking to the vacuole, possibly by influencing these trafficking pathways or PIN sorting between them. The WEAK AUXIN RESPONSE1 WXR1/RUS2 protein might play a role in the auxin-mediated decision between PIN recycling and vacuolar targeting since the corresponding mutant shows defects in both transcriptional auxin response and PIN turnover (Ge *et al*, 2010). How the same outcome is achieved by two seemingly opposite signals is unclear. Different sets of proteins transcriptionally regulated by different auxin levels might possibly target different subcellular trafficking processes. Such a notion can be supported by the results of microarray experiment in which transcription profiling was analysed in response to exogenous auxin and in conditional *axr3* auxin

signalling mutant (<http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-3283>). These experimental conditions can be considered as increased and decreased auxin signalling environment, respectively.

Alternatively, different AFB auxin receptors might respond to different auxin levels in various cells and might have opposite effects on the downstream signalling, as recently suggested for AFB4 (Greenham *et al*, 2011). It is possible that the SCF^{TIR1/AFB} signalling pathway induces downstream effectors that post-transcriptionally modify PIN proteins. Similarly to the PIN phosphorylation by the Ser/Thr protein kinase PINOID (PID) that directly affects the PIN polar targeting (Friml *et al*, 2004; Michniewicz *et al*, 2007; Kleine-Vehn *et al*, 2009; Huang *et al*, 2010; Zhang *et al*, 2010),

Figure 7 The effect of genetically reduced transcriptional auxin signalling on PIN2 protein turnover. **(A)** The effect of heat-shock induction on *PIN2* expression in the root apical meristem. $n=4$ biological replicates with 3 technical repetitions for each. **(B)** Total membrane protein fractions isolated from *HS::axr3-1* genetic background were probed with anti-PIN2 antibody. PIN2 protein levels were decreased 5 h after heat-shock induction. PIN2-specific band at ~70 kDa is marked with the cross. **(C, D)** Higher vacuolar PIN2-GFP accumulation in TIR1-mediated auxin signalling-deficient background of the stabilized *IAA17* mutation (induced for 2 h at 37°C) **(D)** than in the same line without an induction **(C)**. Internal panels illustrate the phenotype of *HS::axr3-1* seedlings without and after induction. **(E)** Relative labelling of PIN2-GFP signal at the plasma membrane versus intracellular in *HS::axr3-1* background. $n=3$ independent experiments with at least 5 roots analysed for each assay and 200 cells counted in total. Error bars represent standard error of the mean (s.e.m.), P -value calculated according to Student's t -test. Arrowheads highlight differences in the plasma membrane stability and vacuolar accumulation of PIN proteins. ind, induced; untr, untreated. Signal intensities are coded blue to yellow corresponding to increasing intensity levels (see colour scale). Scale bar = 10 μ m.

other post-transcriptional modifications, such as PIN ubiquitination (Abas *et al*, 2006; Leitner *et al*, 2012), might change the subcellular sorting and trafficking of PIN proteins, leading to their preferential targeting to and degradation in vacuoles. Finally, $SCF^{TIR1/AFB}$ signalling can potentially affect a more general trafficking regulator since not only PIN proteins but also other plasma membrane proteins (although less effectively) are rerouted to the vacuole upon fluctuations in cellular auxin levels. Such a master regulator of vacuolar targeting could be subject to proteasome modifications and in turn direct post-translational

modifications of PINs and other proteins. Such a hypothesis would integrate the involvement of both proteasomal and vacuolar lytic degradation in the regulation of PIN abundance. It would also clarify why PIN degradation is impaired in the presence of proteasome inhibitor (Abas *et al*, 2006) given the fact that the proteasome complex targets mainly soluble and not membrane proteins (Vierstra, 2009). Future work will address which trafficking pathways are targeted by this processes and whether an increase or a decrease in cellular auxin levels would activate a common or distinct pathway.

Auxin differentially regulates PIN2-mediated fluxes during root gravitropic response

Auxin can modify its own transport by regulating PIN transcription (Peer *et al*, 2004; Heisler *et al*, 2005; Vieten *et al*, 2005; Scarpella *et al*, 2006) and inhibiting PIN internalization from the plasma membrane (Paciorek *et al*, 2005; Robert *et al*, 2010; Chen *et al*, 2012; Lin *et al*, 2012). Here, we propose an integration of another auxin-regulated trafficking process, namely PIN turnover as a substantial element of the multilevel control mechanisms by which auxin orchestrates root re-orientation in response to gravity stimulus. Our observations indicate that protein degradation is a significant part of the PIN regulatory network, particularly important during later phases of root gravitropic response.

It has been previously shown that root re-orientation to horizontal position results in auxin transport along the gravity vector leading to an establishment of temporal lateral auxin gradient across the organ (Luschnig *et al*, 1998; Swarup *et al*, 2005). Our study suggests that this gradient does not only involve an increase in the auxin response at the lower side but also its decrease at the upper side of the gravistimulated root. This apparent auxin depletion at the upper side coincides with PIN destabilization at the plasma membrane most likely due to enhanced trafficking to the vacuole for degradation (Abas *et al*, 2006; Kleine-Vehn *et al*, 2008b). As a consequence of this feedback regulation, lowered auxin transport capacity along the upper side of the root leads to decreased cellular auxin levels to 'below optimal'. Fluctuations in auxin level would then trigger changes in rates of cellular elongation (Barbier-Brygoo *et al*, 1991; Ishikawa and Evans, 1993; Evans *et al*, 1994, reviewed in Perrot-Rechenmann, 2010) eventually leading to a differential growth between two sides of the bending root (Zieschang and Sievers, 1991; Ishikawa and Evans, 1993) according to the classical Cholodny-Went hypothesis (Firn *et al*, 2000; Blancaflor and Masson, 2003). Interestingly, in the same developmental context similar cellular output (PIN2 degradation) although separated spatially and shifted temporarily is achieved by elevated auxin levels at lower side of the root. We are speculating that the transient stabilization of PIN2 observed there is the result of inhibitory auxin effect on clathrin-mediated PIN internalization (Paciorek *et al*, 2005; Robert *et al*, 2010; Chen *et al*, 2012; Lin *et al*, 2012). Elevated auxin levels also inhibit expansion of epidermal cells in the elongation zone at the lower side of the root. The subsequent decrease in PIN2 levels could be the result of the promoting effect of prolonged increased auxin levels on PIN2 degradation proceeding with slower kinetics than that of endocytosis (Robert *et al*, 2010). The interplay between these two auxin-mediated effects

running with different kinetics would ultimately lead to re-establishment of the evenly distributed auxin flux on both sides and consequently vertical growth of the root.

The studies presented in this work address specifically a part of events following gravistimulation, namely how auxin influences the turnover of PIN2 thus regulating auxin flow from the place of gravity perception (root tip) to the responsive tissues in the elongation zone. These events follow the initial establishment of auxin asymmetry in the root tip presumably mediated by the gravity-induced relocation of PIN3 and PIN7 in the root columella cells (Friml *et al*, 2002; Harrison and Masson, 2008; Kleine-Vehn *et al*, 2010). Beside PIN action in auxin transport, gravity-induced auxin translocation requires a crucial involvement of auxin influx machinery (Bennett *et al*, 1996; Marchant *et al*, 1999) and ATP-energized auxin transport utilizing ABCB transporters (Geisler *et al*, 2005; Blakeslee *et al*, 2007; Lewis *et al*, 2007; Mravec *et al*, 2008). The model of auxin action on auxin transport activity must be also integrated with other gravity-induced cellular signalling processes; many of which involve signals other than auxin (Evans and Ishikawa, 1997; Moullia and Fournier, 2009). Finally, it is tempting to speculate that the auxin effect on PIN protein degradation besides regulating root gravitropism might contribute to other processes, such as the auxin transport-mediated auxin maxima establishment during *de novo* organ formation (Benková *et al*, 2003; Reinhardt *et al*, 2003; Heisler *et al*, 2005; Vernoux *et al*, 2010) where PIN degradation has been recently shown to play an important role (Marhavý *et al*, 2011).

Regulation of PIN activity at the plasma membrane by different auxin signalling pathways

Auxin has been demonstrated to influence its own efflux in a dual manner by either increasing or decreasing the incidence of PIN auxin transporters at the plasma membrane. These effects are achieved by the inhibition of PIN endocytosis (Paciorek *et al*, 2005; Robert *et al*, 2010) or promotion of PIN degradation (Sieberer *et al*, 2000; Vieten *et al*, 2005; Abas *et al*, 2006; present work), respectively. The auxin inhibitory effect on PIN endocytosis was attributed to the nuclear auxin signalling pathway that depends on the SCF^{TIR1/AFB} auxin receptors (Pan *et al*, 2009). This was, however, most likely an erroneous interpretation as it did not account for the fact that trafficking inhibitor BFA (that was used to indirectly visualize rate of PIN internalization) targets besides PIN recycling to the plasma membrane also its trafficking to the vacuole (Peyroche *et al*, 1999; Sata *et al*, 1999; Geldner *et al*, 2003; Kleine-Vehn *et al*, 2008b; Robert *et al*, 2010). It seems, therefore, that authors unintentionally addressed a process of vacuolar trafficking rather than the effect on endocytosis. Several recent reports strongly support the idea that the auxin effect on endocytosis does not depend on SCF^{TIR1/AFB} machinery but utilizes a direct, non-transcriptional ABP1-mediated signalling pathway that targets a general process of clathrin-mediated endocytosis (Robert *et al*, 2010; Chen *et al*, 2012; Lin *et al*, 2012; Nagawa *et al*, 2012). It has been proposed that ABP1 might sense auxin in the extracellular space where a small portion of the protein was detected (Jones and Herman, 1993; Baulcy *et al*, 2000) and where ABP1 is active in terms of auxin response (Barbier-Bryggo *et al*, 1996; Gehring *et al*, 1998; Steffens *et al*, 2001). Thus, cell surface active ABP1 could activate a rapid signalling

pathways depending on ROP GTPases to inhibit clathrin-mediated endocytosis without involvement of nuclear auxin signalling (Robert *et al*, 2010; Chen *et al*, 2012; Lin *et al*, 2012; Nagawa *et al*, 2012).

In this work, we provide additional data to clarify the involvement of SCF^{TIR1/AFB} pathway in PIN endocytosis versus vacuolar trafficking. We show by independent approaches that targeting of PINs to the vacuole for degradation is controlled by SCF^{TIR1/AFB} mechanism explaining the results of Pan *et al* (2009). Our results support a model, in which auxin regulates its own flux via distinct signalling pathways, which are controlling processes with different kinetics and specificities. This multilevel mechanism for the regulation of PIN-dependent, directional auxin flux presumably contributes to the adaptive plasticity of plant development.

Materials and methods

For the description of reagents, drug application, experimental conditions, image processing, statistical analysis and quantification index, see Supplementary data at <http://www.embojournal.org>.

Plant material and growth conditions

All *Arabidopsis thaliana* mutants and transgenic lines employed in this study are in the Columbia (Col-0) background and have been described previously: *PIN2::PIN2-GFP* (Xu and Scheres, 2005), *DR5rev::3XVENUS-N7* (Heisler *et al*, 2005), *DR5rev::GFP* (Friml *et al*, 2003), *DII-VENUS* (Brunoud *et al*, 2012), *BRI1::BRI1-GFP* (Rusinaova *et al*, 2004), *35S::PIP2-GFP* (Cutler *et al*, 2000), *35S::PIN2-EosFP* (Dhonukshe *et al*, 2007), *tir1afb1*, *tir1afb2*, *tir1afb3*, *tir1afb1,2,3* (Dharmasiri *et al*, 2005b), *HS::axr3-1* (Knox *et al*, 2003), *RPSS::iaaM* (Weijers *et al*, 2005), *abp1-5* (Xu *et al*, 2010) and *tir1-1* (Ruegger *et al*, 1998), ARF promoter::GFP lines (Rademacher *et al*, 2011), *arf2-8* (Ellis *et al*, 2005), *arf6-2* (Nagpal *et al*, 2005), *arf19-1* (Okushima *et al*, 2005), *arf7arf19* (Wilmoth *et al*, 2005), *arf10arf16* (Wang *et al*, 2005). Surface-sterilized seeds were sown on half-strength Murashige and Skoog (0.5 MS) agar plates and stratified for 2 days at 4°C. Plants were grown on vertically oriented plates under continuous light conditions at 22°C for 4–5 days.

Root gravitropism assay

Arabidopsis 4-day-old seedlings grown in continuous light conditions were covered with a layer of solid 0.5 MS medium and placed in Lab-Tek® II Chambered Coverglass (Nalge Nunc International). Chambers were gravistimulated by 90° rotation and transferred to darkness 2 h prior CLSM analysis. Ten Z-sections spaced ~1 and 4.5 µm apart for PIN2 and DR5 promoter analysis, respectively, were collected in the median root section. Single pictures were subsequently combined into the maximum intensity projection. For the specific quantification method used in each experiment, please see Quantification Index.

Trans-activation experiment

RPSS::GAL4 and *UAS::iaaM* (both in wild-type Col-0 background) were used for the cross. F₁ progeny of *RPSS::GAL4* × *UAS::iaaM* was crossed with a homozygous *PIN2::PIN2-GFP* (*eir1-1*). F₁ generation was analysed. F₁ generation of the *PIN2::PIN2-GFP* (*eir1-1*) × Col-0 was used as a control.

Heat-shock induction

All the CLSM analyses using *HS::axr3-1* expressing line were performed after 2 h of heat-shock induction at 37°C followed by 3 h incubation in continuous light at 22°C. In experiment visualized in Supplementary Figure 18, following decapitation, seedlings were subjected to three subsequent heat-shock inductions spaced over a total time of 16 h. Rationale was to maintain reasonable expression of mutated *axr3-1* gene over this period of time.

Immunodetection and microscopy

Whole-mount immunolocalization in *Arabidopsis* roots was done as described previously (Sauer *et al*, 2006). The rabbit anti-PIN1

(Paciorek *et al*, 2005) and rabbit anti-PIN2 (kindly provided by C Luschnig) primary antibodies were used at a dilution of 1:1000 and fluorochrome-conjugated anti-rabbit-Cy3 secondary antibody (Dianova) was diluted 1:600.

Membrane protein extraction and gel blotting analysis

Approximately 15 mg of seeds was germinated vertically on solid 0.5 MS medium. Seven DAG seedlings were subjected to the treatment. Roots were cut at fixed distance from the root tip and collected for analysis. Microsomal membrane fraction was isolated as described previously (Abas and Luschnig, 2010). Equal amount of proteins was separated by 10% SDS-Urea PAGE as described (Abas *et al*, 2006) followed by either Coomassie Brilliant Blue staining (for loading control) or blotting to ECL membranes (GE Healthcare). The membranes were subsequently treated with affinity-purified anti-rabbit PIN2 antibody (overnight at 4°C) and ECLTM-anti-rabbit IgG, horseradish peroxidase (GE Healthcare; 1:10 000) (1 h at RT). Besides the specific band detected for PIN2 (around 70 kDa), other peptides were detected on the western blot by PIN2 antibody. These are most likely conjugates or metabolites of PIN2 detected together with native protein, as reported and commented in Abas *et al* (2006), Figure 2 legend. The immunoreactive signals were detected using the ECL detection system (GE Healthcare).

Quantitative RT-PCR

Total RNA was extracted with the RNeasy kit (Qiagen). Poly(dT) cDNA was prepared from total RNA with Superscript III (Invitrogen). Quantitative RT-PCR was done with LightCycler 480 SYBR Green I Master reagents (Roche Diagnostics) and a LightCycler 480 Real-Time PCR System (Roche Diagnostics). Targets were quantified with specific primer pairs designed with Beacon Designer 4.0 (Premier Biosoft International). Data were analysed with qBASE v1.3.4 (Hellemans *et al*, 2007). Expression

levels were normalized to the non-auxin-responsive genes *CDKA* (At3g48750), *EEF* (At5g60390) and *TUB2* (At5g62690). For the presentation, *TUB2* reference gene was used. For primer sequences, see Supplementary Table 1.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Mark Estelle (University of California, San Diego, CA, USA), Ottoline Leyser (University of Cambridge, Cambridge, UK), Christian Luschnig (University of Applied Life Sciences and Natural Resources, Vienna, Austria), Remko Offringa (Leiden University, Leiden, The Netherlands), Ben Scheres (University of Utrecht, Utrecht, The Netherlands), Chris Somerville (University of California, Berkeley, CA, USA), Sacco de Vries (Wageningen University, Wageningen, The Netherlands) and Dolf Weijers (Wageningen University, Wageningen, The Netherlands) for sharing published materials and Martine De Cock for help in preparing the manuscript. This work was supported by the Odysseus program of the Research Foundation Flanders (to JF), the Vienna Science and Technology fund (WWTF) (to JK-V) and Vetenskapsrådet and VINNOVA (to SR). SV is a Post-doctoral Fellow of the Research Foundation-Flanders.

Author contributions: JK-V, SR and JF designed the research. PB, SR, JK-V, SV, WG and UK performed the experiments. PB analysed the data. JF, SR and PB wrote the article.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Abas L, Benjamins R, Malenica N, Paciorek T, Wiśniewska J, Moulinier-Anzola JC, Sieberer T, Friml J, 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
- Abas L, Luschnig C (2010) Maximum yields of microsomal-type membranes from small amounts of plant material without requiring ultracentrifugation. *Anal Biochem* **401**: 217–227
- Badescu GO, Napier RM (2006) Receptors for auxin: will it all end in TIRs? *Trends Plant Sci* **11**: 217–223
- Band LR, Wells DM, Larrieu A, Sun J, Middleton AM, French AP, Brunoud G, Sato EM, Wilson MH, Péret B, Oliva M, Swarup R, Sairanen I, Parry G, Ljung K, Beeckman T, Garibaldi JM, Estelle M, Owen MR, Vissenberg K *et al* (2012) Root gravitropism is regulated by a transient latera auxin gradient controlled by a tipping-point mechanism. *Proc Natl Acad Sci USA* **109**: 4668–4673
- Barbez E, Kubeš M, Rolčík J, Béziat C, Pěnčík A, Wang B, Rosquete MR, Zhu J, Dobrev PI, Lee Y, Zažímalová E, Petrášek J, Geisler M, Friml J, Kleine-Vehn J (2012) A novel putative auxin carrier family regulates intracellular auxin homeostasis in plants. *Nature* **485**: 119–122
- Barbier-Brygoo H, Ephritikhine G, Klämbt D, Maurel C, Pälme K, Schell J, Guern J (1991) Perception of the auxin signal at the plasma membrane of tobacco mesophyll protoplasts. *Plant J* **1**: 83–93
- Barbier-Brygoo H, Zimmermann S, Thomine S, White IR, Millner P, Guern J (1996) Elementary auxin response chains at the plasma membrane involve external ABP1 and multiple electrogenic ion transport proteins. *Plant Growth Regul* **18**: 23–28
- Bauly JM, Sealy IM, Macdonald H, Brearley J, Dröge S, Hillmer S, Robinson DG, Venis MA, Blatt MR, Lazarus CM, Napier RM (2000) Overexpression of auxin-binding protein enhances the sensitivity of guard cells to auxin. *Plant Physiol* **124**: 1229–1238
- Benjamins R, Scheres B (2008) Auxin: the looping star in plant development. *Annu Rev Plant Biol* **59**: 443–465
- Benková E, Michniewicz M, Sauer M, Teichmann T, Seifertová D, Jürgens G, Friml J (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation. *Cell* **115**: 591–602
- Bennett MJ, Marchant A, Green HG, May ST, Ward SP, Millner PA, Walker AR, Schulz B, Feldmann KA (1996) *Arabidopsis AUX1* gene: a permease-like regulator of root gravitropism. *Science* **273**: 948–950
- Blakeslee JJ, Bandyopadhyay A, Lee OR, Mravec J, Titapiwatanakun B, Sauer M, Makam SN, Cheng Y, Bouchard R, Adamec J, Geisler M, Nagashima A, Sakai T, Martinoia E, Friml J, Peer WA, Murphy AS (2007) Interactions among PIN-FORMED and P-glycoprotein auxin transporters in *Arabidopsis*. *Plant Cell* **19**: 131–147
- Blancaflor EB, Masson PH (2003) Plant gravitropism. Unraveling the ups and downs of a complex process. *Plant Physiol* **133**: 1677–1690
- Brunoud G, Wells DM, Oliva M, Larrieu A, Mirabet V, Burrow AH, Beeckman T, Kepinski S, Traas J, Bennett MJ, Vernoux T (2012) A novel sensor to map auxin response and distribution at high spatio-temporal resolution. *Nature* **482**: 103–106
- Chen X, Naramoto S, Robert S, Tejos R, Löffke C, Lin D, Yang Z, Friml J (2012) ABP1 and ROP6 GTPase signaling regulate clathrin-mediated endocytosis in *Arabidopsis* roots. *Curr Biol* **22**: 1326–1332
- Cheng Y, Dai X, Zhao Y (2006) Auxin biosynthesis by the YUCCA flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*. *Genes Dev* **20**: 1790–1799
- Cheng Y, Dai X, Zhao Y (2007) Auxin synthesized by the YUCCA flavin monooxygenases is essential for embryogenesis and leaf formation in *Arabidopsis*. *Plant Cell* **19**: 2430–2439
- Cho M, Lee SH, Cho HT (2007) P-glycoprotein4 displays auxin efflux transporter-like action in *Arabidopsis* root hair cells and tobacco cells. *Plant Cell* **19**: 3930–3943
- Crane KE, Ross CW (1986) Effects of wounding on cytokinin activity in cucumber cotyledons. *Plant Physiol* **82**: 1151–1152

- Cutler SR, Ehrhardt DW, Griffiths JS, Somerville CR (2000) Random GFP::cDNA fusions enable visualization of subcellular structures in cells of *Arabidopsis* at a high frequency. *Proc Natl Acad Sci USA* **97**: 3718–3723
- Dharmasiri N, Dharmasiri S, Estelle M (2005a) The F-box protein TIR1 is an auxin receptor. *Nature* **435**: 441–445
- Dharmasiri N, Dharmasiri S, Weijers D, Lechner E, Yamada M, Hobbie L, Ehrismann JS, Jürgens G, Estelle M (2005b) Plant development is regulated by a family of auxin receptor F box proteins. *Dev Cell* **9**: 109–119
- Dhonukshe P, Aniento F, Hwang I, Robinson DG, Mravec J, Stierhof YD, Friml J (2007) Clathrin-mediated constitutive endocytosis of PIN auxin efflux carriers in *Arabidopsis*. *Curr Biol* **17**: 520–527
- Ding Z, Demarsy E, Galván Ampudia CS, Kleine-Vehn J, Morita MT, Tasaka M, Offringa R, Fankhauser C, Friml J (2011) Light-mediated polarization of auxin transport for phototropic response in *Arabidopsis*. *Nat Cell Biol* **13**: 447–452
- Ding Z, Wang B, Moreno I, Dupláková N, Simon S, Carraro N, Reemmer J, Pěnčík A, Chen X, Tejos R, Skúpa P, Pollmann S, Mravec J, Petrášek J, Zažímalová E, Honys D, Rolčík J, Murphy A, Orellana A, Geisler M *et al* (2012) ER-localized auxin transporter PIN8 regulates auxin homeostasis and male gametophyte development in *Arabidopsis*. *Nat Commun* **3**: 941
- Ellis CM, Nagpal P, Young JC, Hagen G, Guilfoyle TJ, Reed JW (2005) AUXIN RESPONSE FACTOR1 and AUXIN RESPONSE FACTOR2 regulate senescence and floral organ abscission in *Arabidopsis thaliana*. *Development* **132**: 4563–4574
- Evans ML, Ishikawa H (1997) Cellular specificity of the gravitropic motor response in roots. *Planta* **203**: S115–S122
- Evans ML, Ishikawa H, Estelle MA (1994) Responses of *Arabidopsis* roots to auxin studied with high temporal resolution: comparison of wild type and auxin-response mutants. *Planta* **194**: 215–222
- Firn RD, Wagstaff C, Digby J (2000) The use of mutants to probe models of gravitropism. *J Exp Bot* **51**: 1323–1340
- Friml J, Vieten A, Sauer M, Weijers D, Schwarz H, Hamann T, Offringa R, Jürgens G (2003) Efflux-dependent auxin gradients establish the apical-basal axis of *Arabidopsis*. *Nature* **426**: 147–153
- Friml J, Wiśniewska J, Benková E, Mendgen K, Palme K (2002) Lateral relocation of auxin efflux regulator PIN3 mediates tropism in *Arabidopsis*. *Nature* **415**: 806–809
- Friml J, Yang X, Michniewicz M, Weijers D, Quint A, Tietz O, Benjamins R, Ouwerkerk PBF, Ljung K, Sandberg G, Hooykaas PJJ, Palme K, Offringa R (2004) A PINOID-dependent binary switch in apical-basal PIN polar targeting directs auxin efflux. *Science* **306**: 862–865
- Ge L, Peer W, Robert S, Swarup R, Ye S, Prigge M, Cohen JD, Friml J, Murphy A, Tang D, Estelle M (2010) *Arabidopsis* ROOT UVB SENSITIVE2/WEAK AUXIN RESPONSE1 is required for polar auxin transport. *Plant Cell* **22**: 1749–1761
- Gehring CA, McConchie RM, Venis MA, Parish RW (1998) Auxin-binding-protein antibodies and peptides influence stomatal opening and alter cytoplasmic pH. *Planta* **205**: 581–586
- Geisler M, Blakeslee JJ, Bouchard R, Lee OR, Vincenzetti V, Bandyopadhyay A, Titapiwatanakun B, Peer WA, Bailly A, Richards EL, Ejendal KFK, Smith AP, Baroux C, Grossniklaus U, Müller A, Hrycyna CA, Dudler R, Murphy AS, Martinoia E (2005) Cellular efflux of auxin catalyzed by the *Arabidopsis* MDR/PGP transporter AtPGP1. *Plant J* **44**: 179–194
- Geldner N, Anders N, Wolters H, Keicher J, Kornberger W, Muller P, Delbarre A, Ueda T, Nakano A, Jürgens G (2003) The *Arabidopsis* GNOM ARF-GEF mediates endosomal recycling, auxin transport, and auxin-dependent plant growth. *Cell* **112**: 219–230
- Geldner N, Friml J, Stierhof YD, Jürgens G, Palme K (2001) Auxin transport inhibitors block PIN1 cycling and vesicle trafficking. *Nature* **413**: 425–428
- Greenham K, Santner A, Castillejo C, Mooney S, Sairanen I, Ljung K, Estelle M (2011) The AFB4 auxin receptor is a negative regulator of auxin signaling in seedlings. *Curr Biol* **21**: 520–525
- Grieneisen VA, Xu J, Marée AF, Hogeweg P, Scheres B (2007) Auxin transport is sufficient to generate a maximum and gradient guiding root growth. *Nature* **449**: 1008–1013
- Grunewald W, Friml J (2010) The march of the PINs: developmental plasticity by dynamic polar targeting in plant cells. *EMBO J* **29**: 2700–2714
- Harrison BR, Masson PH (2008) ARL2, ARG1 and PIN3 define a gravity signal transduction pathway in root statocytes. *Plant J* **53**: 380–392
- Hayashi K, Tan X, Zheng N, Hatate T, Kimura Y, Kepinski S, Nozaki H (2008) Small-molecule agonists and antagonists of F-box protein-substrate interactions in auxin perception and signaling. *Proc Natl Acad Sci USA* **105**: 5632–5637
- He W, Brumos J, Li H, Ji Y, Ke M, Gong X, Zeng Q, Li W, Zhang X, An F, Wen X, Li P, Chu J, Sun X, Yan C, Yan N, Xie DY, Raikhel N, Yang Z, Stepanova AN *et al* (2011) A small-molecule screen identifies L-kynurenine as a competitive inhibitor of TAA1/TAR activity in ethylene-directed auxin biosynthesis and root growth in *Arabidopsis*. *Plant Cell* **23**: 3944–3960
- Heisler MG, Ohno C, Das P, Sieber P, Reddy GV, Long JA, Meyerowitz EM (2005) Patterns of auxin transport and gene expression during primordium development revealed by live imaging of the *Arabidopsis* inflorescence meristem. *Curr Biol* **15**: 1899–1911
- Hellemans J, Mortier G, De Paepe A, Speleman F, Vandesompele J (2007) qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol* **8**: R19.1–R19.14
- Huang F, Zago MK, Abas L, van Marion A, Galván-Ampudia CS, Offringa R (2010) Phosphorylation of conserved PIN motifs directs *Arabidopsis* PIN1 polarity and auxin transport. *Plant Cell* **22**: 1129–1142
- Ishikawa H, Evans ML (1993) The role of the distal elongation zone in the response of maize roots to auxin and gravity. *Plant Physiol* **102**: 1203–1210
- Jones AM, Herman EM (1993) KDEL-containing Auxin Binding Protein is secreted to the plasma membrane and cell wall. *Plant Physiol* **101**: 595–606
- Jones AR, Kramer EM, Knox K, Swarup R, Bennett MJ, Lazarus CM, Leyser HMO, Grierson CS (2009) Auxin transport through non-hair cells sustains root-hair development. *Nat Cell Biol* **11**: 78–84
- Kepinski S, Leyser O (2005) The *Arabidopsis* F-box protein TIR1 is an auxin receptor. *Nature* **435**: 446–451
- Kitakura S, Vanneste S, Robert S, Löffke C, Teichmann T, Tanaka H, Friml J (2011) Clathrin mediates endocytosis and polar distribution of PIN auxin transporters in *Arabidopsis*. *Plant Cell* **23**: 1920–1931
- Klee HJ, Horsch RB, Hinchey MA, Hein MB, Hoffmann NL (1987) The effects of overproduction of two *Agrobacterium tumefaciens* T-DNA auxin biosynthetic gene products in transgenic petunia plants. *Genes Dev* **1**: 86–96
- Kleine-Vehn J, Dhonukshe P, Sauer M, Brewer P, Wiśniewska J, Paciorek T, Benková E, Friml J (2008a) ARF GEF-dependent transcytosis and polar delivery of PIN auxin carriers in *Arabidopsis*. *Curr Biol* **18**: 526–531
- Kleine-Vehn J, Ding Z, Jones AR, Tasaka M, Morita MT, Friml J (2010) Gravity-induced PIN transcytosis for polarization of auxin fluxes in gravity-sensing root cells. *Proc Natl Acad Sci USA* **107**: 22344–22349
- Kleine-Vehn J, Friml J (2008) Polar targeting and endocytic recycling in auxin-dependent plant development. *Annu Rev Cell Dev Biol* **24**: 447–473
- Kleine-Vehn J, Huang F, Naramoto S, Zhang J, Michniewicz M, Offringa M, Friml J (2009) PIN auxin efflux carrier polarity is regulated by PINOID kinase-mediated recruitment into GNOM-independent trafficking in *Arabidopsis*. *Plant Cell* **21**: 3839–3849
- Kleine-Vehn J, Leitner J, Zwiewka M, Sauer M, Abas L, Luschig C, Friml J (2008b) Differential degradation of PIN2 auxin efflux carrier by retromer-dependent vacuolar targeting. *Proc Natl Acad Sci USA* **105**: 17812–17817
- Knox K, Grierson CS, Leyser O (2003) AXR3 and SHY2 interact to regulate root hair development. *Development* **130**: 5769–5777
- Laxmi A, Pan J, Morsy M, Chen R (2008) Light plays an essential role in intracellular distribution of auxin efflux carrier PIN2 in *Arabidopsis thaliana*. *PLoS ONE* **3**: e1510
- Lee DJ, Park JW, Lee HW, Kim J (2009) Genome-wide analysis of the auxin-responsive transcriptome downstream of *iaa1* and its expression analysis reveal the diversity and complexity of auxin-regulated gene expression. *J Exp Bot* **60**: 3935–3957
- Leitner J, Petrášek J, Tomanov K, Retzer K, Pařezová M, Korbei B, Bachmair A, Zažímalová E, Luschig C (2012) Lysine63-linked ubiquitylation of PIN2 auxin carrier protein governs hormonally

- controlled adaptation of *Arabidopsis* root growth. *Proc Natl Acad Sci USA* **109**: 8322–8327
- Leitz G, Kang BH, Schoenwaelder MEA, Staehelin LA (2009) Statolith sedimentation kinetics and force transduction to the cortical endoplasmic reticulum in gravity-sensing *Arabidopsis* columella cells. *Plant Cell* **21**: 843–860
- Lewis DR, Miller ND, Splitt BL, Wu G, Spalding EP (2007) Separating the roles of acropetal and basipetal auxin transport on gravitropism with mutations in two *Arabidopsis* multidrug resistance-like ABC transporter genes. *Plant Cell* **19**: 1838–1850
- Lin D, Nagawa S, Chen J, Cao L, Chen X, Xu T, Li H, Dhonukshe P, Yamamuro C, Friml J, Scheres B, Fu Y, Yang Z (2012) A ROP GTPase-dependent auxin signaling pathway regulates the subcellular distribution of PIN2 in *Arabidopsis* roots. *Curr Biol* **22**: 1319–1325
- Ljung K, Bhalerao RP, Sandberg G (2001) Sites and homeostatic control of auxin biosynthesis in *Arabidopsis* during vegetative growth. *Plant J* **28**: 465–474
- Luschnig C, Gaxiola RA, Grisafi P, Fink GR (1998) EIR1, a root-specific protein involved in auxin transport, is required for gravitropism in *Arabidopsis thaliana*. *Genes Dev* **12**: 2175–2187
- Marchant A, Kargul J, May ST, Muller P, Delbarre A, Perrot-Rechenmann C, Bennett MJ (1999) AUX1 regulates root gravitropism in *Arabidopsis* by facilitating auxin uptake within root apical tissues. *EMBO J* **18**: 2066–2073
- Marhavý P, Bielach A, Abas L, Abuzeineh A, Duclercq J, Tanaka H, Pařezová M, Petrášek J, Friml J, Kleine-Vehn J, Benková E (2011) Cytokinin modulates endocytic trafficking of PIN1 auxin efflux carrier to control plant organogenesis. *Dev Cell* **21**: 796–804
- Men S, Boutté Y, Ikeda Y, Li X, Palme K, Stierhof YD, Hartmann MA, Moritz T, Grebe M (2008) Sterol-dependent endocytosis mediates post-cytokinetic acquisition of PIN2 auxin efflux carrier polarity. *Nat Cell Biol* **10**: 237–244
- Michniewicz M, Zago MK, Abas L, Weijers D, Schweighofer A, Meskiane I, Heisler MG, Ohno C, Zhang J, Huang F, Schwab R, Weigel D, Meyerowitz EM, Luschnig C, Offringa R, Friml J (2007) Antagonistic regulation of PIN phosphorylation by PP2A and PINOID directs auxin flux. *Cell* **130**: 1044–1056
- Morita MT (2010) Directional gravity sensing in gravitropism. *Annu Rev Plant Biol* **61**: 705–720
- Moullia B, Fournier M (2009) The power and control of gravitropic movements in plants: a biomechanical and systems biology view. *J Exp Bot* **60**: 461–486
- Mravec J, Kubes M, Bielach A, Gaykova V, Petrášek J, Skůpa P, Chand S, Benková E, Zazimalová E, Friml J (2008) Interaction of PIN and PGP transport mechanisms in auxin distribution-dependent development. *Development* **135**: 3345–3354
- Mravec J, Skůpa P, Bailly A, Hoyerová K, Křeček P, Bielach A, Petrášek J, Zhang J, Gaykova V, Stierhof YD, Dobrev PI, Schwarzerová K, Rolčík J, Seifertová D, Luschnig C, Benková E, Zazimalová E, Geisler M, Friml J (2009) Subcellular homeostasis of phytohormone auxin is mediated by the ER-localized PIN5 transporter. *Nature* **459**: 1136–1140
- Müller A, Guan C, Gälweiler L, Tänzler P, Huijser P, Marchant A, Parry G, Bennett M, Wisman E, Palme K (1998) *AtPIN2* defines a locus of *Arabidopsis* for root gravitropism control. *EMBO J* **17**: 6903–6911
- Nagawa S, Xu T, Lin D, Dhonukshe P, Zhang X, Friml J, Scheres B, Fu Y, Yang Z (2012) ROP GTPase-dependent actin microfilaments promote PIN1 polarization by localized inhibition of clathrin-dependent endocytosis. *PLoS Biol* **10**: e1001299
- Nagpal P, Ellis CM, Weber H, Ploense SE, Barkawi LS, Guilfoyle TJ, Hagen G, Alonso JM, Cohen JD, Farmer EE, Ecker JR, Reed JW (2005) Auxin response factors ARF6 and ARF8 promote jasmonic acid production and flower maturation. *Development* **132**: 4107–4118
- Napier RM, David KM, Perrot-Rechenmann C (2002) A short history of auxin-binding proteins. *Plant Mol Biol* **49**: 339–348
- Nishimura T, Nakano H, Hayashi K, Niwa C, Koshiba T (2009) Differential downward stream of auxin synthesized at the tip has a key role in gravitropic curvature via TIR1/AFBs-mediated auxin signaling pathways. *Plant Cell Physiol* **50**: 1874–1885
- Okushima Y, Overvoorde PJ, Arima K, Alonso JM, Chan A, Chang C, Ecker JR, Hughes B, Lui A, Nguyen D, Onodera C, Quach H, Smith A, Yu G, Theologis A (2005) Functional genomic analysis of the AUXIN RESPONSE FACTOR gene family members in *Arabidopsis thaliana*: unique and overlapping functions of ARF7 and ARF19. *Plant Cell* **17**: 444–463
- Ottenschläger I, Wolff P, Wolvert C, Bhalerao RP, Sandberg G, Ishikawa H, Evans M, Palme K (2003) Gravity-regulated differential auxin transport from columella to lateral root cap cells. *Proc Natl Acad Sci USA* **100**: 2987–2991
- Paciorek T, Zazimalová E, Ruthardt N, Petrášek J, Stierhof YD, Kleine-Vehn J, Morris DA, Emans N, Jürgens G, Geldner N, Friml J (2005) Auxin inhibits endocytosis and promotes its own efflux from cells. *Nature* **435**: 1251–1256
- Pan J, Fujioka S, Peng J, Chen J, Li G, Chen R (2009) The E3 ubiquitin ligase SCFTIR1/AFB and membrane sterols play key roles in auxin regulation of endocytosis, recycling, and plasma membrane accumulation of the auxin efflux transporter PIN2 in *Arabidopsis thaliana*. *Plant Cell* **21**: 568–580
- Peer WA, Bandyopadhyay A, Blakeslee JJ, Makam SN, Chen RJ, Masson PH, Murphy AS (2004) Variation in expression and protein localization of the PIN family of auxin efflux facilitator proteins in flavonoid mutants with altered auxin transport in *Arabidopsis thaliana*. *Plant Cell* **16**: 1898–1911
- Perrot-Rechenmann C (2010) Cellular responses to auxin: division versus expansion. *Cold Spring Harb Perspect Biol* **2**: a001446
- Petrášek J, Mravec J, Bouchard R, Blakeslee JJ, Abas M, Seifertová D, Wiśniewska J, Tadele Z, Kubeš M, Čovanová M, Dhonukshe P, Skůpa P, Benková E, Perry L, Křeček P, Lee OR, Fink GR, Geisler M, Murphy AS, Luschnig C *et al* (2006) PIN proteins perform a rate-limiting function in cellular auxin efflux. *Science* **312**: 914–918
- Peyroche A, Antonny B, Robineau S, Acker J, Cherfils J, Jackson CL (1999) Brefeldin A acts to stabilize an abortive ARF-GDP-Sec7 domain protein complex: involvement of specific residues of the Sec7 domain. *Mol Cell* **3**: 275–285
- Rademacher EH, Möller B, Lokerse AS, Llavata-Peris CI, van den Berg W, Weijers D (2011) A cellular expression map of the *Arabidopsis* AUXIN RESPONSE FACTOR gene family. *Plant J* **68**: 597–606
- Rakusová H, Gallego-Bartolomé J, Vanstraelen M, Robert HS, Alabadi D, Blázquez MA, Benková E, Friml J (2011) Polarization of PIN3-dependent auxin transport for hypocotyl gravitropic response in *Arabidopsis thaliana*. *Plant J* **67**: 817–826
- Rashotte AM, Brady SR, Reed RC, Ante SJ, Muday GK (2000) Basipetal auxin transport is required for gravitropism in roots of *Arabidopsis*. *Plant Physiol* **122**: 481–490
- Reinhardt D, Pesce ER, Stieger P, Mandel T, Baltensperger K, Bennett M, Traas J, Friml J, Kuhlmeier C (2003) Regulation of phyllotaxis by polar auxin transport. *Nature* **426**: 255–260
- Robert S, Kleine-Vehn J, Barbez E, Sauer M, Paciorek T, Baster P, Vanneste S, Zhang J, Simon S, Čovanová M, Hayashi K, Dhonukshe P, Yang Z, Bednarek SY, Jones AM, Luschnig C, Aniento F, Zazimalová E, Friml J (2010) ABP1 mediates auxin inhibition of clathrin-dependent endocytosis in *Arabidopsis*. *Cell* **143**: 111–121
- Romano CP, Robson PRH, Smith H, Estelle M, Klee H (1995) Transgene-mediated auxin overproduction in *Arabidopsis*: hypocotyl elongation phenotype and interactions with the *hy6-1* hypocotyl elongation and *axr1* auxin-resistant mutants. *Plant Mol Biol* **27**: 1071–1083
- Ruegger M, Dewey E, Gray WM, Hobbie L, Turner J, Estelle M (1998) The TIR1 protein of *Arabidopsis* functions in auxin response and is related to human SKP2 and yeast grr1p. *Genes Dev* **12**: 198–207
- Russinova E, Borst JW, Kwakitaal M, Caño-Delgado A, Yin Y, Chory J, de Vries SC (2004) Heterodimerization and endocytosis of *Arabidopsis* brassinosteroid receptors BRI1 and AtSERK3 (BAK1). *Plant Cell* **16**: 3216–3229
- Sata M, Moss J, Vaughan M (1999) Structural basis for the inhibitory effect of brefeldin A on guanine nucleotide-exchange proteins for ADP-ribosylation factors. *Proc Natl Acad Sci USA* **96**: 2752–2757
- Sauer M, Paciorek T, Benková E, Friml J (2006) Immunocytochemical techniques for whole mount *in situ* protein localization in plants. *Nat Protoc* **1**: 98–103
- Scarpella E, Marcos D, Friml J, Berleth T (2006) Control of leaf vascular patterning by polar auxin transport. *Genes Dev* **20**: 1015–1027

- Shin H, Shin HS, Guo Z, Blancaflor EB, Masson PH, Chen R (2005) Complex regulation of *Arabidopsis* AGR1/PIN2-mediated root gravitropic response and basipetal auxin transport by cantharidin-sensitive protein phosphatases. *Plant J* **42**: 188–200
- Shirakawa M, Ueda H, Shimada T, Nishiyama C, Hara-Nishimura I (2009) Vacuolar SNAREs function in the formation of the leaf vascular network by regulating auxin distribution. *Plant Cell Physiol* **50**: 1319–1328
- Sieberer T, Seifert GJ, Hauser MT, Grisafi P, Fink GR, Luschnig C (2000) Post-transcriptional control of the *Arabidopsis* auxin efflux carrier EIR1 requires AXR1. *Curr Biol* **10**: 1595–1598
- Steffens B, Feckler C, Palme K, Christian M, Böttger M, Lüthen H (2001) The auxin signal for protoplast swelling is perceived by extracellular ABP1. *Plant J* **27**: 591–599
- Stepanova AN, Robertson-Hoyt J, Yun J, Benavente LM, Xie DY, Doležal K, Schlereth A, Jürgens G, Alonso JM (2008) TAA1-mediated auxin biosynthesis is essential for hormone crosstalk and plant development. *Cell* **133**: 177–191
- Sun J, Xu Y, Ye S, Jiang H, Chen Q, Liu F, Zhou W, Chen R, Li X, Tietz O, Wu X, Cohen JD, Palme K, Li C (2009) Arabidopsis ASA1 is important for jasmonate-mediated regulation of auxin biosynthesis and transport during lateral root formation. *Plant Cell* **21**: 1495–1511
- Swarup K, Benková E, Swarup R, Casimiro I, Péret B, Yang Y, Parry G, Nielsen E, De Smet I, Vanneste S, Levesque MP, Carrier D, James N, Calvo V, Ljung K, Kramer E, Roberts R, Graham N, Marillonnet S, Patel K *et al* (2008) The auxin influx carrier LAX3 promotes lateral root emergence. *Nat Cell Biol* **10**: 946–954
- Swarup R, Kramer EM, Perry P, Knox K, Leyser HM, Haseloff J, Beemster GT, Bhalerao R, Bennett MJ (2005) Root gravitropism requires lateral root cap and epidermal cells for transport and response to a mobile auxin signal. *Nat Cell Biol* **7**: 1057–1065
- Tamura K, Shimada T, Ono E, Tanaka Y, Nagatani A, Higashi SI, Watanabe M, Nishimura M, Hara-Nishimura I (2003) Why green fluorescent fusion proteins have not been observed in the vacuoles of higher plants. *Plant J* **35**: 545–555
- Tan X, Calderon-Villalobos LIA, Sharon M, Zheng C, Robinson CV, Estelle M, Zheng N (2007) Mechanism of auxin perception by the TIR1 ubiquitin ligase. *Nature* **446**: 640–645
- Tao Y, Ferrer JL, Ljung K, Pojer F, Hong F, Long JA, Li L, Moreno JE, Bowman ME, Ivans LJ, Cheng Y, Lim J, Zhao Y, Ballaré CL, Sandberg G, Noel JP, Chory J (2008) Rapid synthesis of auxin via a new tryptophan-dependent pathway is required for shade avoidance in plants. *Cell* **133**: 164–176
- Ulmasov T, Murfett J, Hagen G, Guilfoyle TJ (1997) Aux/IAA proteins repress expression of reporter genes containing natural and highly active synthetic auxin response elements. *Plant Cell* **9**: 1963–1971
- Vanneste S, Friml J (2009) Auxin: A trigger for change in plant development. *Cell* **136**: 1005–1016
- Vernoux T, Besnard F, Traas J (2010) Auxin at the shoot apical meristem. *Cold Spring Harb Perspect Biol* **2**: a001487
- Vierstra RD (2009) The ubiquitin-26S proteasome system at the nexus of plant biology. *Nat Rev Mol Cell Biol* **10**: 385–397
- Vieten A, Vanneste S, Wiśniewska J, Benková E, Benjamins R, Beeckman T, Luschnig C, Friml J (2005) Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. *Development* **132**: 4521–4531
- Wang JW, Wang LJ, Mao YB, Cai WJ, Xue HW, Chen XY (2005) Control of root cap formation by MicroRNA-targeted auxin response factors in *Arabidopsis*. *Plant Cell* **17**: 2204–2216
- Wasternack C (2007) Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Ann Bot* **100**: 681–697
- Weijers D, Franke-van Dijk M, Vencken RJ, Quint A, Hooykaas P, Offringa R (2001) An *Arabidopsis* Minute-like phenotype caused by a semi-dominant mutation in a RYBOSOMAL PROTEIN S5 gene. *Development* **128**: 4289–4299
- Weijers D, Sauer M, Meurette O, Friml J, Ljung K, Sandberg G, Hooykaas P, Offringa R (2005) Maintenance of embryonic auxin distribution for apical-basal patterning by PIN-FORMED-dependent auxin transport in *Arabidopsis*. *Plant Cell* **17**: 2517–2526
- Wilmoth JC, Wang S, Tiwari SB, Joshi AD, Hagen G, Guilfoyle TJ, Alonso JM, Ecker JR, Reed JW (2005) NPH4/ARF7 and ARF19 promote leaf expansion and auxin-induced lateral root formation. *Plant J* **43**: 118–130
- Wiśniewska J, Xu J, Seifertová D, Brewer PB, Růžicka K, Blilou I, Rouquié D, Benková E, Scheres B, Friml J (2006) Polar PIN localization directs auxin flow in plants. *Science* **312**: 883
- Xu J, Scheres B (2005) Dissection of *Arabidopsis* ADP-RIBOSYLATION FACTOR 1 function in epidermal cell polarity. *Plant Cell* **17**: 525–536
- Xu T, Wen M, Nagawa S, Fu Y, Chen JG, Wu MJ, Perrot-Rechenmann C, Friml J, Jones AM, Yang Z (2010) Cell surface- and Rho GTPase-based auxin signalling controls cellular interdigitation in *Arabidopsis*. *Cell* **143**: 99–110
- Zhang J, Nodzynski T, Pencík A, Rolčík J, Friml J (2010) PIN phosphorylation is sufficient to mediate PIN polarity and direct auxin transport. *Proc Natl Acad Sci USA* **107**: 918–922
- Zieschang HE, Sievers A (1991) Gravitropism and the localization of its initiating cells in roots of *Phleum pratense* L. *Planta* **184**: 468–477