Development 137, 607-617 (2010) doi:10.1242/dev.041277

Role of PIN-mediated auxin efflux in apical hook development of *Arabidopsis thaliana*

Petra Žádníková[1,2], Jan Petrášek[3,4], Peter Marhavý[1], Vered Raz[5], Filip Vandenbussche[4], Zhaojun Ding[1], Kateřina Schwarzerová[6], Miyo T. Morita[7], Masao Tasaka[7], Jan Hejátko[2], Dominique Van Der Straeten[4], Jiří Friml[1,2] and Eva Benková[1,*]

SUMMARY

The apical hook of dark-grown *Arabidopsis* seedlings is a simple structure that develops soon after germination to protect the meristem tissues during emergence through the soil and that opens upon exposure to light. Differential growth at the apical hook proceeds in three sequential steps that are regulated by multiple hormones, principally auxin and ethylene. We show that the progress of the apical hook through these developmental phases depends on the dynamic, asymmetric distribution of auxin, which is regulated by auxin efflux carriers of the PIN family. Several PIN proteins exhibited specific, partially overlapping spatial and temporal expression patterns, and their subcellular localization suggested auxin fluxes during hook development. Genetic manipulation of individual PIN activities interfered with different stages of hook development, implying that specific combinations of PIN genes are required for progress of the apical hook through the developmental phases. Furthermore, ethylene might modulate apical hook development by prolonging the formation phase and strongly suppressing the maintenance phase. This ethylene effect is in part mediated by regulation of PIN-dependent auxin efflux and auxin signaling.

KEY WORDS: PIN3, Apical hook, Auxin, *Arabidopsis*

INTRODUCTION

In dicotyledonous plants, the apical hook protects the delicate shoot meristem as the seedling makes its way through the soil towards the surface (Darwin and Darwin, 1881). The apical hook develops soon after germination, is maintained until seedling emergence from the soil, and opens upon exposure to light. The formation of the apical hook is coordinated by localized cell division and differential cell elongation on each side of the hook (Raz and Koornneef, 2001). The maintenance phase depends on adjusted differential elongation rates (Silk and Erickson, 1978) that retain the hook curvature (Raz and Ecker, 1999), whereas gradual equilibration of cell lengths on the concave and convex sides of the hook results in its opening.

To ensure proper differential growth coordination, this process must be tightly regulated. Plant hormones have been identified as important regulators of cell division and differentiation, including cell elongation. Typically, the output of one hormonal pathway is modulated by interactions with other hormones, thus creating a hormonal network that represents an important developmental regulatory system (Coenen and Lomax, 1997; Pitts et al., 1998; Collett et al., 2000; Vandenbussche and Van Der Straeten, 2004).

[1]Department of Plant Systems Biology, Flanders Institute for Biotechnology (VIB) and Department of Plant Biotechnology and Genetics, Ghent University, 9052 Gent, Belgium. [2]Laboratory of Molecular Plant Physiology, Department of Functional Genomics and Proteomics, Institute of Experimental Biology, Masaryk University, 62500 Brno, Czech Republic. [3]Institute of Experimental Botany, 16502 Prague, Czech Republic. [4]Unit Plant Hormone Signaling and Bio-imaging, Department of Physiology, Ghent University, 9000 Gent, Belgium. [5]Department of Human Genetics, Leiden University Medical Center, 2300 RC Leiden, The Netherlands. [6]Department of Plant Physiology, Charles University, 12844 Prague, Czech Republic. [7]Graduate School of Biological Sciences, Nara Institute of Science and Technology, 630-0101 Ikoma, Japan.

*Author for correspondence (eva.benkova@psb.vib-ugent.be)

Accepted 10 December 2009

The plant hormones ethylene and auxin have been implicated as regulators of the differential growth in the apical hook (reviewed by Lomax, 1997; Lehman et al., 1996). Ethylene enhances hook curvature (reviewed by Ecker, 1995), as observed in the *constitutive triple response1* (*ctr1*) (Kieber et al., 1993) and the *ethylene overproducer* (*eto*) (Woeste et al., 1999) mutants, or by application of exogenous ethylene (Guzmán and Ecker, 1990). However, the mechanism by which ethylene triggers differential growth is unclear.

Differential auxin responses during apical hook development have been reported (Friml et al., 2002b; Li et al., 2004). Mutants in auxin perception genes, such as *TRANSPORT INHIBITOR RESPONSE 1* (*TIR1*) and AFB members (Dharmasiri et al., 2005a), and in the auxin signaling pathway, such as *auxin resistant1* (*axr1*) (Leyser et al., 1993), *nonphototropic hypocotyl4* (*nph4*; also known as *arf7*) (Harper et al., 2000) or *superroot1* (*sur1*; also known as *alf1* and *hls3*), that accumulates high levels of free auxin (Boerjan et al., 1995; Lehman et al., 1996), exhibit reduced hook curvature. Exogenous application of auxin and polar auxin transport (PAT) inhibitors affects hook curvature (Schwark and Schierle, 1992; Lehman et al., 1996), suggesting that PAT is required for proper differential growth in the apical hook. PAT is regulated by the AUX/LAX family of auxin influx carriers (Bennett et al., 1996; Yang et al., 2006), the PIN family of auxin efflux carriers (Luschnig et al., 1998; Petrášek at al., 2006) and by the multiple drug resistance/P-glycoprotein (MDR/PGP) family of ABC transporters (Noh et al., 2001; Blakeslee et al., 2007). Mutants in genes regulating PAT, such as *roots curl in NPA* (*rcn1*) (Garbers et al., 1996) and *pin3* (Friml et al., 2002b), have impaired apical hook development, and expression of the *MDR1* (*ABCB19*) transporter was shown to be increased in etiolated hypocotyls (Blakeslee et al., 2007).

As both auxin and ethylene are involved in the regulation of apical hook development, their activities are mutually coordinated. Ethylene has been shown to enhance apical hook bending by

activating transcription of *HOOKLESS1* (*HLS1*), which inhibits *AUXIN RESPONSE FACTOR2* (*ARF2*) (Lehman et al., 1996; Li et al., 2004). A stimulatory effect of ethylene on the auxin biosynthetic pathway (Swarup et al., 2007) points to another mode of interaction at the hormone metabolism level. Several genes in the auxin biosynthesis pathway are under the transcriptional control of ethylene, including *ANTHRANILATE SYNTHASE α1* (*ASA1*) and *ANTHRANILATE SYNTHASE β1* (*ASB1*) (Stepanova et al., 2005), or are under the control of a small family of tryptophan aminotransferase-encoding genes, *TAA1* and *TAR2*, which regulate the indole-3-pyruvic acid branch of the auxin biosynthetic pathway (Stepanova et al., 2008; Tao et al., 2008).

Here, we show that apical hook development depends on the auxin transport that controls the temporal and spatial distribution of auxin. Several auxin efflux carrier genes of the PIN family were found to exhibit partially overlapping expression patterns in the apical hook, and elimination of their activities by mutation interfered with particular phases of hook development. Ethylene-induced enhancement of apical hook formation and subsequent hook curvature exaggeration were accompanied by changes in auxin distribution and in the expression of several PIN genes. This suggests that part of the ethylene effect might be mediated by auxin and through regulation of the auxin efflux.

MATERIALS AND METHODS

Plant material

The transgenic *Arabidopsis thaliana* (L.) Heynh. lines have been described elsewhere: *DR5::GUS* (Sabatini et al., 1999); *PIN1,2,3,4::GUS; PIN1::PIN1-GFP* (Benková et al., 2003); *PIN7::GUS* (Vieten et al., 2005); *PIN4::PIN4-GFP; PIN7::PIN7-GFP* (Blilou et al., 2005); *nph4-1*; *arf19-1*; *nph4-1 arf19-1* double mutant; *ARF7::GUS* (Okushima et al., 2005); *pSHY2::GUS*; *pIAA12::GUS*; *pIAA13::GUS*; *pBDL::bdl*; *pSHY2::shy2-2*; *pIAA13::iaa13*; *pSHY2::shy2/DR5::GUS* (Weijers et al., 2005); *tir1-1* (Ruegger et al., 1998); *pin1-3* (Okada et al., 1991); and *pin4-1* (Friml et al., 2002a). The mutant lines *pin7-2*, *pin3-4* and *pin3-5* with sequence-indexed insertions were identified in the Signal Insertion Mutant Library (http://signal.salk.edu/cgi-bin/tdnaexpress/). The double mutants *pin4 pin7* and *pin3 pin4* have been described previously (Friml et al., 2003b). The *PIN3::PIN3-GFP* and *PIN3::PIN3-YFP* plants were obtained as follow: a 12.5 kb *PIN3* genomic fragment generated from BAC F15H11 and digested with *Ngo*M IV was cloned into the *Xma*I site of the pBIN19 binary vector. The *GFP* or *YFP* fragment was inserted at the end of the first exon of *PIN3* using the Counter-Selection BAC Modification Kit (Gene Bridges). *pin3-4* was crossed with *DR5::GUS* and *PIN3::PIN3-GFP* lines. The *pin1* phenotype was analyzed on the heterozygous population. Apical hook development was recorded, seedlings were numbered and the *pin1* homozygotes were identified based on pin-like shoot phenotypes at the adult stage.

Growth conditions

Seeds were plated on half-strength Murashige and Skoog (MS) medium (Duchefa) with 1% sucrose, 1% agar (pH 5.7), or supplemented with 5 μM 1-aminocyclopropane-1-carboxylate (ACC; Sigma-Aldrich) or 5 μM *N*-(1-naphtyl)phtalamic acid (NPA; Duchefa), vernalized for 2 days at 4°C, exposed to light for 3 hours at 18°C, and cultivated in the dark at 20°C. Seedlings were processed 12, 24, 36, 48, 60 and 72 hours after germination or used for real-time phenotype analysis.

Real-time analysis and statistics of apical hook development

Development of seedlings was recorded at 1-hour intervals for 5 days at 24°C with an infrared light source (880 nm LED; Velleman, Belgium) by a spectrum-enhanced camera (EOS035 Canon Rebel Xti, 400DH) with built-in clear wideband-multicoated filter and standard accessories (Canon) and operated by the EOS utility software. Angles between the hypocotyl axis and cotyledons were measured by ImageJ (NIH; http://rsb.info.nih.gov/ij). Fifteen seedlings with synchronized germination start were processed.

GUS analysis

Histochemical β-glucuronidase (GUS) staining was performed as described (Friml et al., 2003a) for 24 hours at 37°C. Seedlings mounted in chloral hydrate (Fluka) were analyzed with an automatic virtual slide-scanner microscope frame (dotSlide BX51 microscope, Olympus) and 10× UPLSAPO objective equipped with a digital CCD camera (2/3″ CCD camera, 6.45×6.45 μm pixel size, high sensitivity, high resolution, Peltier cooled, dynamic range of 3×12 bit). Images were processed in Adobe Photoshop.

Localization of PIN::PIN-GFP reporter proteins and PIN3-GFP quantitative analysis

Confocal laser-scanning micrographs were obtained with a Leica TCS SP2 AOBS with a 488-nm argon laser line for excitation of GFP fluorescence. Emissions were detected between 505 and 580 nm. Using a 20× water-immersion objective (NA=1.25, pinhole), confocal scans were performed with the pinhole at 1 Airy unit.

PIN3-GFP localization was examined by confocal *z*-sectioning and 3D reconstruction. Each image represents either a single focal plane or a projection of individual images taken as a *z*-series. *z*-stacking was performed in a 1024-scan format, collecting at least ten images through cortex and epidermal layers. Full *z*-stack confocal images were 3D-projected using the Leica confocal software.

The fluorescence intensity of the membrane PIN3-GFP signal was quantified with ImageJ. At least ten seedlings were analyzed per treatment. Images were processed in Adobe Illustrator.

Localization of PIN3-YFP on transverse sections

PIN3::PIN3-YFP seedlings 24 hours old were fixed for 2 hours in 3.7% paraformaldehyde (Serva) in MTSB (50 mM PIPES, 5 mM EGTA, 1 mM $MgSO_4$, pH 6.8) and immobilized in 5% (w/v) low melting point agarose (Sigma) in water. The agarose blocks were mounted with agarose onto the stage of a motorized Advance Vibroslice (World Precision Instruments, Stevenage, UK) and 25 μm transverse sections through the apical hook were observed with Zeiss LSM 510 confocal microscope (Carl Zeiss, Jena, Germany).

RESULTS

Real-time analysis of apical hook development

To follow apical hook development in real time, we performed continuous monitoring of seedling growth in the dark (for details, see Materials and methods). Hook growth was recorded continuously from germination and the angle of curvature was measured as a parameter that reflects the stage of apical hook development (see Fig. S1A in the supplementary material). Typically, the hook underwent three developmental phases: (1) formation, which is the period from seed germination until the hook angle reaches 180°; (2) maintenance, during which the hook remains fully closed; and (3) opening, when it gradually opens to reach an angle of 0° (Fig. 1A and see Fig. S1B in the supplementary material) (Raz and Ecker, 1999). Real-time analysis enabled us to precisely monitor the duration and kinetics of hook development through the particular phases. Under our experimental conditions, the formation phase lasted 26 hours (from germination until 95% of the seedlings exhibited a closed hook angle of 177.75±2.50°); the maintenance phase was typically 25 hours (at least 95% of the seedlings had a fully closed apical hook); and the opening phase lasted ~88 hours (from the start of opening, when less than 95% of the seedlings had a closed hook angle of 177.75±2.25°, until 95% of the seedlings exhibited an open hook angle of 8±5.77°). These observations confirm previous reports (Raz and Ecker, 1999).

The advantage of our approach when compared with previous analyses is that the analysis in real-time minimizes inaccuracies caused by variability in germination time and enables an accurate

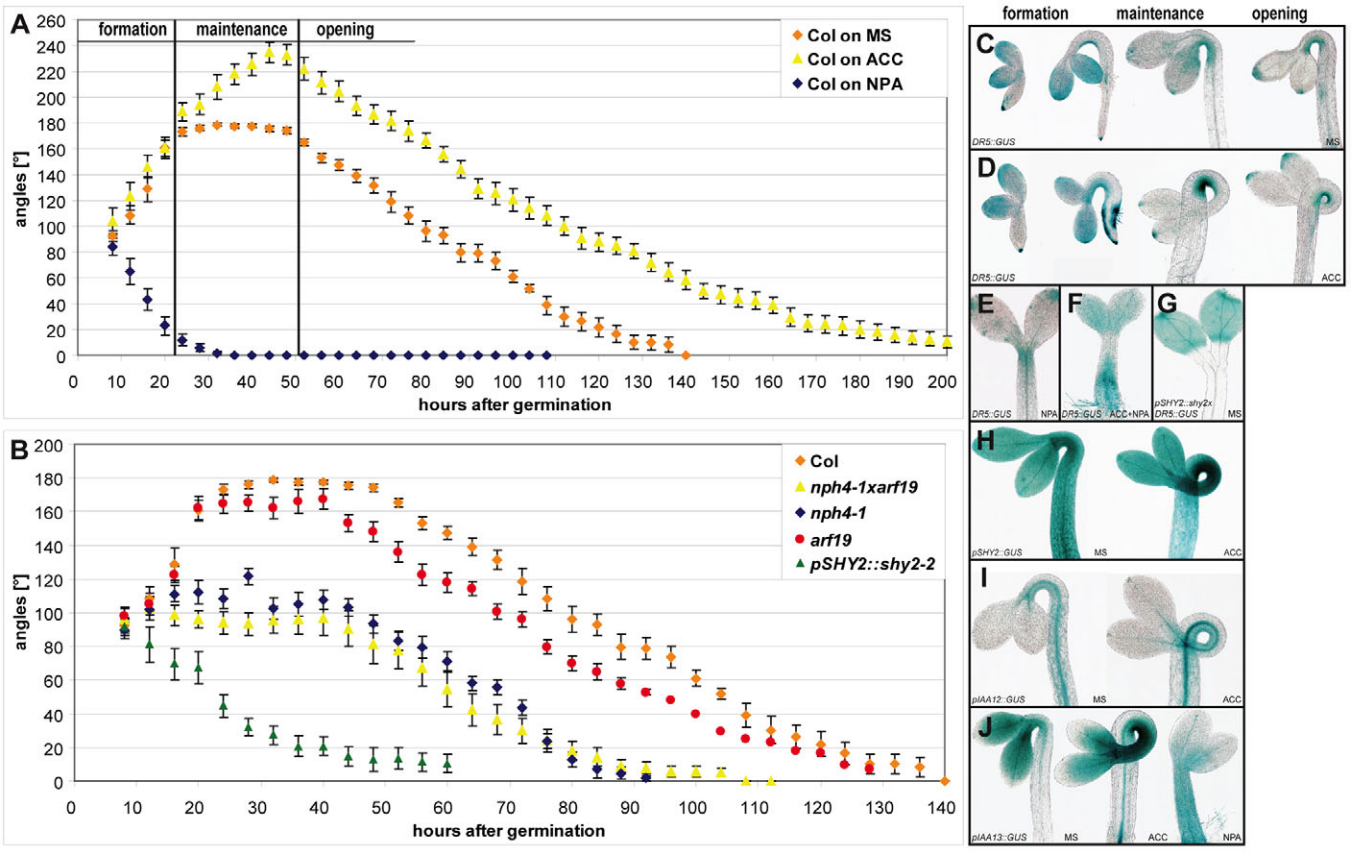

Fig. 1. Auxin distribution and response during apical hook development in *Arabidopsis*. (**A**) Kinetics of apical hook development in Col seedlings on MS medium, upon NPA and ethylene treatment. (**B**) Apical hook development in *shy2-2*, *nph4-1*, *arf19* single mutants and *nph4-1 arf19* double mutants. (**C**,**D**) *DR5::GUS* expression through apical hook development in MS- (C) and ethylene-treated (D) seedlings. (**E**,**F**) *DR5::GUS* expression upon NPA (E) and NPA plus ethylene (F) treatment. (**G**) *DR5::GUS* expression in the apical hook of *shy2-2* mutants. (**H-J**) *IAA3::GUS* (H), *IAA12::GUS* (I) and *IAA13::GUS* (J) expression in untreated, ethylene- and NPA-treated apical hooks. ACC was used as a precursor of ethylene biosynthesis. MS, Murashige and Skoog medium only; Col, control Columbia seedlings. Error bars represent s.e.m.

monitoring of all growth phases. This phenotype analysis is a powerful approach to identify the developmental effects of specific mutations.

Dynamics of auxin distribution and response during apical hook development

To examine auxin distribution during apical hook development, we monitored the expression of the auxin response reporters *DR5::GUS* and *DR5rev::GFP* (Ulmasov et al., 1997; Sabatini et al., 1999; Friml et al., 2003b); their activity has been shown to correlate well with the auxin distribution in embryogenesis and root development (Benková et al., 2003; Friml et al., 2003b). Shortly upon germination, *DR5* reporter activity was detected in cotyledons and at the boundary zone between the cotyledons and hypocotyl; subsequently, during hook formation, increased *DR5* expression was localized in the cortex and epidermal cells at the concave (inner) side of the hook (Fig. 1C). This pattern of *DR5::GUS* activity was retained throughout the maintenance phase. Hook opening was accompanied by a more diffuse *DR5* signal in the zone beneath the hook (Fig. 1C) (Vandenbussche et al., 2010).

Developmental responses to auxin are mediated by the TIR/AFB auxin receptors of the F-box protein family (Dharmasiri et al., 2005a), which direct the negative regulators of the Aux/IAA family for degradation (Overvoorde et al., 2005) in an auxin-sensitive manner. ARF transcription factors (Okushima et al., 2005) are released from inhibition by the Aux/IAAs and activate the expression of auxin response genes (Dharmasiri et al., 2005b; Kepinski and Leyser, 2005). To determine how asymmetry in the auxin distribution is transmitted by downstream factors, we examined the expression and function of genes of the auxin signaling pathway. *IAA3*, *IAA12* and *IAA13* exhibited an asymmetric expression pattern similar to that of *DR5*, suggesting that the auxin signaling pathway is differentially active on the concave and convex sides of the apical hook (Fig. 1H-J). Genetic inhibition of the auxin response by expression of the stabilized repressors in *shy2-2*, *bdl* or *iaa13* mutants prevented hook formation (Fig. 1B; see Fig. S3A,B in the supplementary material) and strongly reduced *DR5* expression in the hook of the *shy2-2* mutant (Fig. 1G). Mutations in genes encoding ARF response regulators [*nph4* (*arf7*) and *arf19*] resulted in defective apical hook development. Mild defects in hook formation and maintenance were exhibited by the single *arf19* mutant, in contrast to severe defects in the *nph4-1* mutant (Fig. 1B) (Harper et al., 2000). Lack of both NPH4 and ARF19 functions led to an additive apical hook phenotype (Fig. 1B). *ARF7::GUS* expression was detected in the apical hook zone but did not exhibit a pronounced asymmetry (see Fig. S3C in the supplementary material).

In summary, the auxin distribution during apical hook development is very dynamic. An auxin maximum on the concave side is gradually established during hook formation, is retained

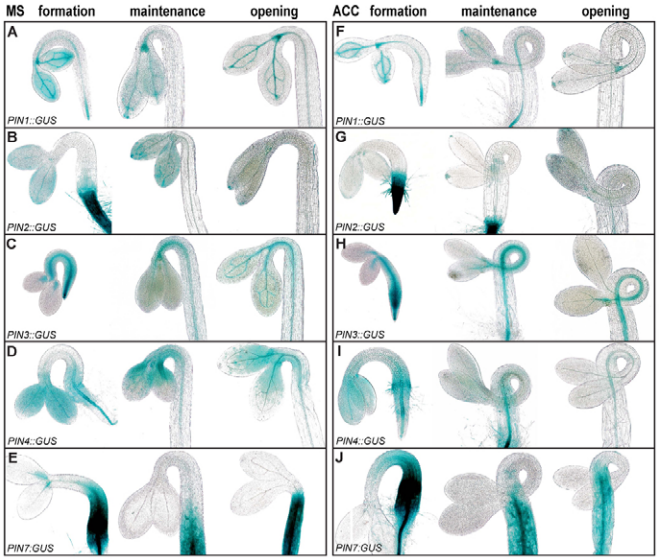

Fig. 2. Differentially expressed auxin efflux carriers in control and ethylene-treated apical hooks. (**A-J**) Expression patterns of *PIN1::GUS* (A,F), *PIN2::GUS* (B,G), *PIN3::GUS* (C,H), *PIN4::GUS* (D,I) and *PIN7::GUS* (E,J) during the formation, maintenance and early opening phases of apical hook development in MS-treated seedlings (A-E) and upon ethylene treatment (F-J). ACC was used as precursor of ethylene biosynthesis. The formation, maintenance and opening phases corresponded to 12, 48 and 72 hours after germination, respectively.

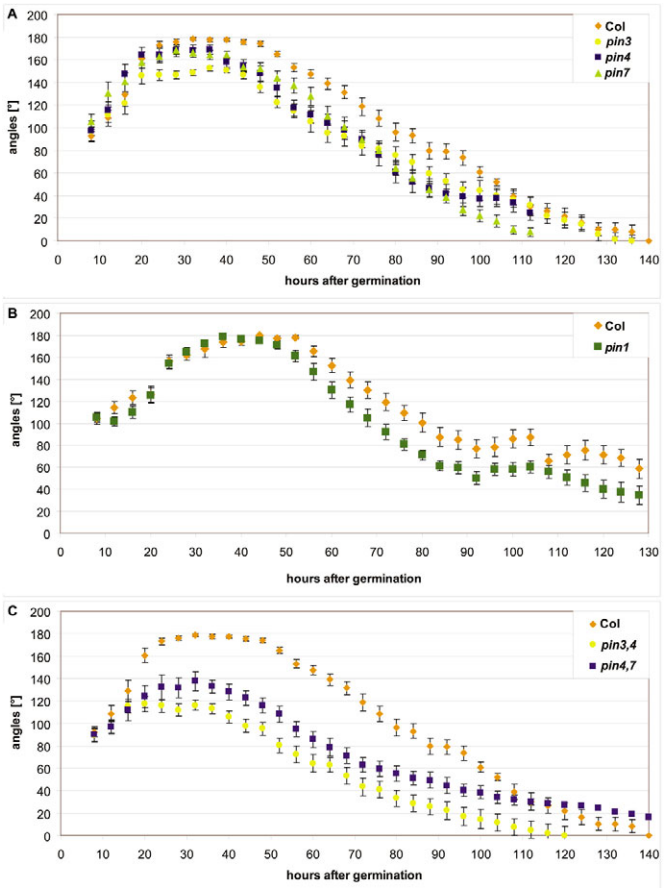

Fig. 3. Apical hook development in auxin efflux carrier mutants. (**A-C**) Apical hook development in *pin3*, *pin4* and *pin7* (A), *pin1* (B) single mutants and in *pin3 pin4* and *pin4 pin7* double mutants (C) in comparison with the wild type. MS, Murashige and Skoog medium only; Col, control Columbia seedlings. Error bars represent s.e.m.

during the maintenance phase, and is lost by the opening of the hook. Asymmetry in the auxin distribution is interpreted by the downstream auxin signaling pathway, involving the Aux/IAA and ARF genes.

Auxin efflux carriers are differentially expressed during apical hook development

The differential *DR5* expression pattern during apical hook development might result from asymmetry either in local auxin biosynthesis or in PAT activity. For the tryptophan aminotransferase *TAA1* (*WEI8*), its close family member *TAR2* and the flavin monooxygenase *YUC1*, tissue-specific expression in the apical hook has been demonstrated (Stepanova et al., 2008; Vandenbussche et al., 2010). We tested the importance of auxin efflux for apical hook development with the auxin efflux inhibitor NPA (Morris, 2000). NPA abolished apical hook formation (Fig. 1A) and the asymmetric expression of *DR5::GUS* (Fig. 1E) and *IAA13::GUS* (Fig. 1J) in the 'hook zone', and caused a modest increase in *ARF7::GUS* expression (see Fig. S3D in the supplementary material). Transient NPA application at the moment of maximal hook bending resulted in enhanced hook opening, suggesting that auxin efflux is required for the hook opening process as well (see Fig. S2 in the supplementary material).

To study the spatial and temporal expression patterns of the auxin efflux components during apical hook development, *PIN::GUS* reporter lines were used. We focused on the *PIN1*, *PIN2*, *PIN3*, *PIN4* and *PIN7* subfamily of plasma membrane-localized PIN proteins (Mravec et al., 2009).

During hook formation, *PIN1* expression was restricted to the central cylinder (Fig. 2A), whereas *PIN3* and *PIN4* were additionally expressed in the epidermis and cortex along the hypocotyls. The expression zones of *PIN3* and *PIN4* partially overlapped, but the zone of maximal curvature was almost free of *PIN4::GUS* signal (Fig. 2C,D). During the maintenance phase, *PIN4* expression changed with an increased signal in the hook, preferentially at its concave side (Fig. 2D). In the opening phase, a general reduction in the expression of most of the PIN genes occurred (Fig. 2A,C,D), except for *PIN7*. *PIN7* expression was eliminated from the hook and restricted to the hypocotyl zone beneath during the formation and maintenance phases and spread during the opening phase upwards towards the cotyledons (Fig. 2E). *PIN2* was not expressed in the apical hook (Fig. 2B).

In conclusion, during apical hook development a complex spatiotemporal regulation of PIN gene expression occurs, suggesting that specific combinations of PIN genes might control the progress of the apical hook through particular developmental phases.

Auxin efflux carriers are involved in regulation of apical hook development

To examine the function of individual auxin efflux carriers, hook development in *pin1*, *pin3*, *pin4* and *pin7* loss-of-function mutants was analyzed in real time. The most severe defects were found in the *pin3* mutant: apical hooks of *pin3* never reached the fully closed stage and were unable to maintain the bent structure. Having reached the maximal angle of curvature, *pin3* hooks gradually progressed through the opening phase (Fig. 3A). The kinetics of the opening

phase in the *pin3* mutant did not significantly differ from that of the control. The *pin3* mutant exhibited strongly reduced *DR5* reporter expression in the bending zone, which was below the detection limit throughout the whole of hook development (see Fig. S4A in the supplementary material). By contrast, an enhanced *DR5* signal was detected in *pin3* cotyledons. This implies that cotyledons might play a role as a source of auxin in apical hook development, similar to the results of Vandenbussche et al. (Vandenbussche et al., 2010). Additional inhibition of auxin efflux by NPA in the *pin3* mutant enhanced the *DR5* signal in the cotyledons, but also at the most apical part of hypocotyls (see Fig. S4B in the supplementary material). Induction of *DR5::GUS* expression upon NPA treatment, which was below the detection limit in MS-treated *pin3* hooks, points to the existence of additional, NPA-sensitive transporters in these tissues.

When compared with *pin3*, the *pin4* mutant exhibited less severe defects. The *pin4* apical hooks reached only slightly less than 180° (Fig. 3A) and were able to remain in the closed state, although the duration of the maintenance phase was shorter than that of the controls (Fig. 3A). No dramatic changes were observed in the kinetics of hook opening. Analysis of the *pin4* mutant suggests that *PIN4* might be primarily involved in the control of the maintenance phase or the transition between the maintenance and opening phases. Because of their partially overlapping expression, we examined apical hook development in *pin3 pin4* double mutants. The *pin3 pin4* mutant showed an additive phenotype and both hook formation and maintenance were more severely affected than in either single mutant (compare Fig. 3C with 3A). Development in the *pin7* mutant was comparable to that in *pin4*, suggesting that *PIN7* and *PIN4* might act redundantly (Fig. 3A). Indeed, lack of both PIN4 and PIN7 in the *pin4 pin7* double mutant affected apical hook development more severely than in either single mutant. The *pin4 pin7* mutant was unable to form closed hooks, the maintenance phase was reduced, and the opening of their hooks was delayed (Fig. 3C). The *pin1* mutant exhibited a phenotype that was similar to that of *pin4* and *pin7*, characterized by a reduced maintenance phase and premature transition to the opening phase (Fig. 3B).

Altogether, our analysis of the PIN gene mutants revealed that several auxin efflux carriers participate in the regulation of apical hook development. We identified *PIN3* as the key regulator of the formation and maintenance phases, assisted by *PIN4* and *PIN7*. In addition, *PIN1*, *PIN4* and *PIN7* seem to be involved in the regulation of the transition between the maintenance and opening phases.

Membrane localization of PIN3-GFP suggests transport routes of auxin during apical hook development

Expression and mutant phenotype analyses denote PIN3 as the major auxin efflux carrier during hook formation and maintenance. As the direction of PAT has been shown to be determined by the membrane localization of auxin efflux carriers (Wiśniewska et al., 2006), we analyzed the membrane localization of PIN3 protein with the *PIN3::PIN3-GFP* and *PIN3::PIN3-YFP* reporter lines. The functionality of both constructs was tested using real-time analysis. *PIN3::PIN3-GFP* rescued the *pin3* phenotype, but in both the *pin3/PIN3::PIN3-GFP* and Columbia wild-type (wt) wt/*PIN3::PIN3-GFP* backgrounds, formation of the hook was delayed, possibly because of a slight overexpression of *PIN3::PIN3-GFP* (see Fig. S5A in the supplementary material). Indeed, no effect on apical hook development was detected in the Columbia wild type when the weakly expressing *PIN3::PIN3-YFP* line was assayed (see Fig. S5B in the supplementary material).

PIN3 membrane localization in whole-mount and transverse sections of fixed apical hooks was analyzed with a combination of *z*-stacks and three-dimensional projection. Both *PIN3::PIN3-GFP* and *PIN3::PIN3-YFP* lines exhibited the same expression pattern and membrane localization. On longitudinal confocal sections, the presence of PIN3 in the cortex and epidermis was confirmed as previously demonstrated by *PIN3::GUS* (Fig. 4A; see Movie 1 in the supplementary material). Additionally, transverse sections revealed the PIN3-YFP signal in endodermis (Fig. 4D; see Movie 2 in the supplementary material). However, whereas *PIN3::GUS* expression was not obviously different between the concave and convex sides of the hook, the PIN3-GFP signal was stronger on the membranes at the convex side. Fluorescence quantification showed a reduction in the PIN3-GFP signal by 21% (n=10) on the transverse membranes and, similarly, by 28% (n=10) on the lateral membranes on the concave compared with the convex side (see Fig. S6A in the supplementary material).

Membrane localization of PIN3 varied depending on the tissue type and cell position along the bending of the apical hook. In endodermis, the PIN3-YFP signal was often found at the outer and transverse plasma membranes (Fig. 4D; see Movie 2 in the supplementary material). In the cortex, PIN3-GFP was localized on the lateral membranes pointing towards the outer epidermal tissues and on the transverse membranes preferentially at the basal side of cells (Fig. 4A; see Movie 1 in the supplementary material). Along the apical-basal axis, in the epidermal cells closer to the hypocotyl-root junction, PIN3-GFP was detected predominantly on the basal side, whereas near the cotyledons it was predominantly on the apical side of cells (Fig. 4A; see Movie 1 in the supplementary material).

Next, we analyzed the impact of inhibited auxin efflux on *PIN3* expression and protein localization. Quantification of the PIN3-GFP signal revealed that upon NPA treatment, PIN3 localization did not differ significantly between the convex and concave sides, neither on the transverse membranes nor on the lateral membranes of cortex cells (Fig. 4C; see Fig. S6A,B in the supplementary material). Furthermore, proportionally more PIN3-GFP was localized to transverse than to lateral membranes (see Fig. S6A in the supplementary material).

Our studies suggest that PIN3 participates in regulating auxin distribution during apical hook development by transporting auxin in a lateral direction through the endodermis, cortex and epidermis and basipetally towards roots. In the epidermal tissues, PIN3 seems to regulate the auxin redistribution in both apical (towards shoot) and basal (towards root) directions. Asymmetry of PIN3, with reduced signal on the concave side of the hook, might cause differential transport rates on the two sides of the bending hypocotyl during the formation phase, resulting in enhanced accumulation of auxin on the concave side of the apical hook. Loss of PIN3-GFP asymmetry and reduction of lateral in favor of transverse membrane localization upon NPA treatment hint at an additional involvement of post-transcriptional regulatory mechanisms.

PIN1, PIN4 and PIN7 participate in auxin redistribution during hook development

Phenotype analysis of *pin1*, *pin4* and *pin7* single and multiple loss-of-function mutants suggested the involvement of particular combinations of PIN proteins in apical hook development. To reveal the auxin redistribution directed by these PIN family members, their localization was studied using translational GFP-fusion reporter lines.

In agreement with the *PIN4::GUS* results, only weak *PIN4::PIN4-GFP* expression was detected during early hook formation in the cortex and epidermal cells at the base of hypocotyls

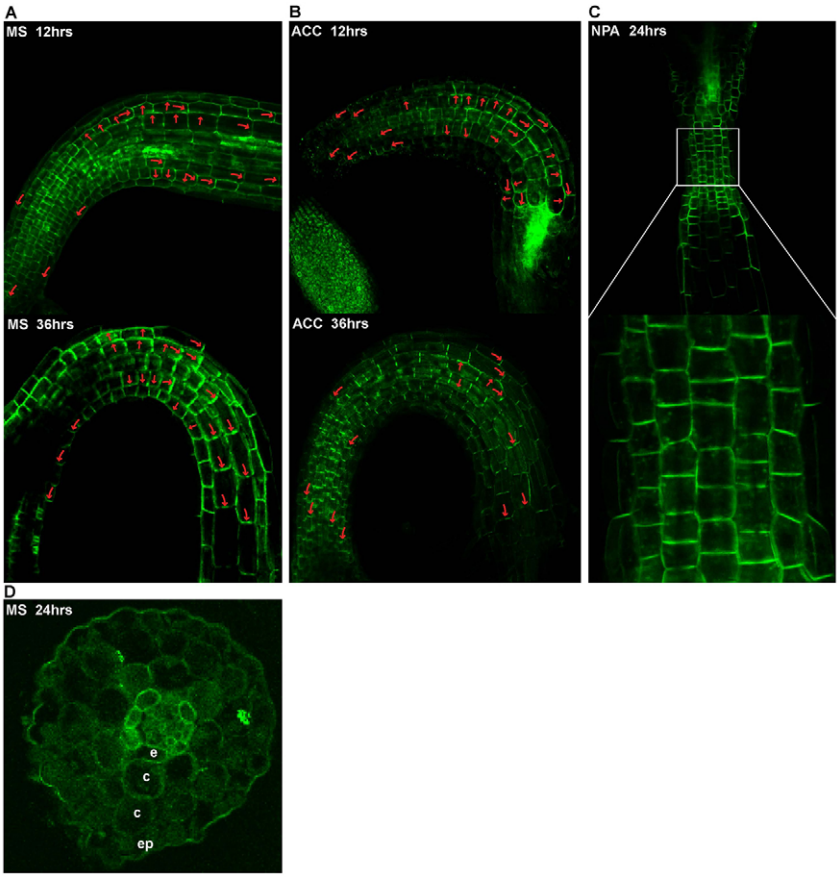

Fig. 4. Analysis of PIN3 membrane localization. (**A-C**) Subcellular localization of PIN3-GFP (green) on MS (A), upon ethylene (B) and NPA (C) treatment. (**D**) Transverse section through apical hook expressing *PIN3::PIN3-YFP* (green). ACC was used as a precursor of ethylene biosynthesis. Shown are longitudinal confocal sections in vivo (A-C) and transverse sections of fixed (D) apical hooks. Arrows point to the membranes with prevalent localization of PIN3-GFP. e, endodermis; c, cortex; ep, epidermis.

(data not shown). With progress of the formation phase and in the maintenance phase, the PIN4-GFP signal appeared in the hook curvature. However, in contrast to *PIN4::GUS* expression, which was enhanced on the concave side, the PIN4-GFP signal was stronger on the convex side of the hook (Fig. 2D compared with Fig. 5A; see Movies 3 and 4 in the supplementary material). PIN4-GFP was predominantly located at the basal and partially also at the lateral membranes heading towards the epidermis. Notably, the PIN4-GFP signal was stronger in the cortex than epidermis, suggesting a prevalent role for PIN4 in auxin distribution through cortex tissue. Inhibition of hook development by NPA resulted in a symmetric PIN4-GFP signal in the cortex and epidermis and enhanced localization at the basal sides of cells (Fig. 5A).

A weak PIN7-GFP signal was detected during the formation phase in the cortex and epidermal cells of the hook curvature, which gradually increased in the zone beneath, similarly to *PIN7::GUS*. Later developmental stages were accompanied with a gradual enhancement of the PIN7-GFP signal in the apical hooks (Fig. 5B; see Movie 5 in the supplementary material). No signs of preferential cell membrane polarity or asymmetric expression between the convex and concave sides were observed. In contrast to PIN4-GFP, the PIN7-GFP signal was significantly stronger in the epidermis than cortex. In hypocotyls treated with NPA, PIN7-GFP was localized in a non-polar manner in epidermal cells and a weaker signal was also detected in the cortex cells (Fig. 5B).

During hook formation, *PIN1::PIN1-GFP* was expressed in neither cortex nor epidermis. Only a few, random, isolated cells in the apical hooks were found to express PIN1-GFP (data not shown). At first, during the maintenance phase, PIN1-GFP appeared in the epidermal cells, exclusively on the concave side of the hook (Fig. 5C; see Movie 6 in the supplementary material), as similarly reported (Blakeslee et al., 2007). This localized expression of *PIN1* was not revealed by *PIN1::GUS*, probably because it was below the detection limit. In NPA-treated seedlings, PIN1-GFP was detected in all epidermal cells of the 'hook zone' and preferentially at their transverse membranes (Fig. 5C).

In summary, auxin redistribution during apical hook development involves the coordinated action of several auxin efflux carriers. PIN4, along with PIN3, seems to facilitate transport of auxin in cortex and epidermal cells at the convex side of the hook. PIN7 appears to be an important regulator of the auxin efflux in the epidermis, and PIN1, besides its role in the central cylinder, seems to be involved in the control of auxin levels at the position of the auxin maxima during the maintenance and opening phases.

Ethylene postpones the transition between the formation and maintenance phases by interaction with the PAT machinery

Besides auxin, another major regulator of apical hook development is the phytohormone ethylene. To investigate the possible mechanisms involved in ethylene-regulated apical hook development, we analyzed the kinetics of hook development and the presumed interaction of ethylene with the auxin transport machinery.

To increase the ethylene levels, we used ACC, a precursor of ethylene biosynthesis (Yang and Hoffman, 1984). Ethylene did not affect the hook formation kinetics, but seemed primarily to prolong the formation phase leading to an exaggeration of the hook curvature, with the final hook angle significantly exceeding 180° (Fig. 1A). The exaggerated apical hooks tended not to remain in a state of maximal bending but, after having reached the peak, gradually progressed through the opening phase. The kinetics of opening was not markedly affected by ethylene (Fig. 1A) (Vandenbussche et al., 2010).

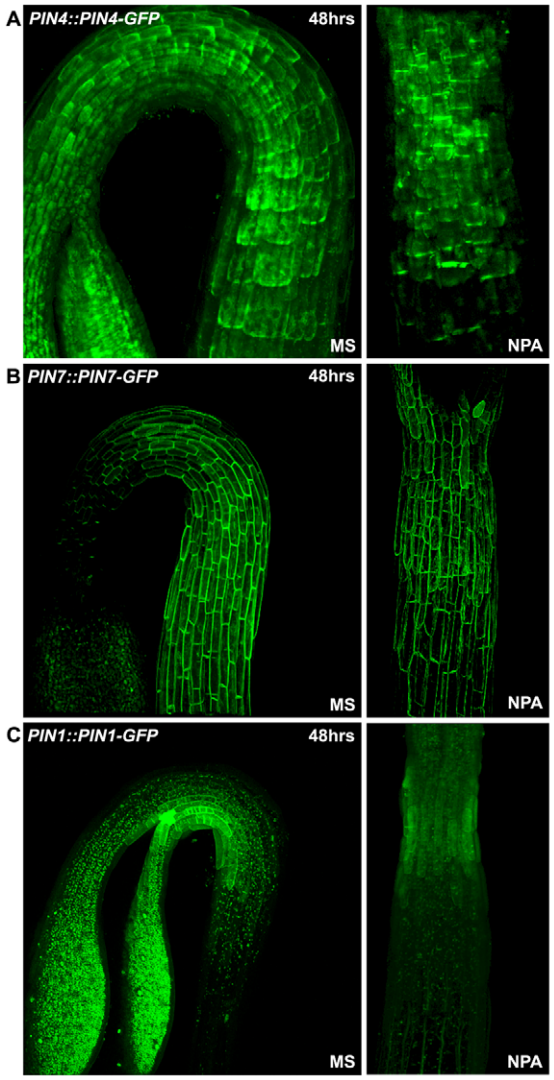

Fig. 5. Analysis of PIN1, PIN4 and PIN7 membrane localization. (**A-C**) Subcellular localization of PIN4-GFP (A), PIN7-GFP (B) and PIN1-GFP (C) on MS and NPA medium. Localization of PIN-GFP signal (green) was examined by longitudinal confocal sections through whole-mount apical hooks.

To correlate changes in hook development with auxin distribution, *DR5::GUS* expression was monitored. Typically, at the beginning of the hook formation phase, the *DR5* expression pattern did not significantly differ in ACC-treated and untreated hooks (Fig. 1D). Differences were most apparent at the stage of maximal curvature. In the exaggerated apical hooks, *DR5::GUS* expression was restricted to a few cells on the concave side (Fig. 1D) (Vandenbussche et al., 2010), suggesting that the mechanisms regulating asymmetric auxin accumulation in cells on the concave side remain active for longer. During the opening phase, the asymmetric *DR5::GUS* expression gradually disappeared in a manner similar to that in control seedlings (Fig. 1D). Accordingly, enhanced asymmetry in the expression of regulators of the auxin signaling pathway (*IAA3::GUS*, *IAA12::GUS* and *IAA13::GUS*) was observed upon ethylene treatment (Fig. 1H-J). No obvious changes were detected in the expression of *ARF7* upon ACC treatment when compared with control seedlings (Fig. S3E in the supplementary material).

Modulation of *DR5::GUS* expression by ethylene might point to the interaction of ethylene with PAT. Our observation that NPA completely blocks the ethylene-induced formation of exaggerated apical hooks in seedlings simultaneously treated with ACC and NPA supports the hypothesis that mechanisms regulating auxin distribution might act downstream of ethylene (Fig. 1F) (Vandenbussche et al., 2010).

Analysis of several PIN genes revealed that their expression was modulated in ethylene-treated apical hooks. Ethylene downregulated *PIN1::GUS* and *PIN4::GUS* transcription (Fig. 2F,I compared with 2A,D). *PIN3::GUS* expression was sustained at an elevated level at the time when reduced *PIN3* signal was observed in the untreated hooks (Fig. 2H compared with 2C). Expression of *PIN7::GUS* was absent in the hook at the time when in untreated seedlings it progressed to the hook zone (Fig. 2J compared with 2E). *PIN2::GUS* expression in the apical hook was not changed by ethylene treatment (Fig. 2B compared with 2G).

Because of its dominant role, we closely examined hook development in the *pin3* mutant under increased ethylene levels. *pin3* seedlings exhibited reduced sensitivity to ethylene and never formed exaggerated apical hooks (see Fig. S4D in the supplementary material). Accordingly, *DR5* expression was drastically reduced in the apical hooks of *pin3* seedlings, but was enhanced in cotyledons (Fig. S4C in the supplementary material).

Next, the effects of ethylene on *PIN3::PIN3-GFP* expression and membrane localization were studied. Quantification revealed increased PIN3-GFP accumulation on the cell membranes at the convex side of the apical hook, similar to control seedlings (Fig. 4B; see Fig. S6A in the supplementary material). However, in contrast to control seedlings, the asymmetry between the convex and concave sides was enhanced for PIN3-GFP at the lateral membranes, and was reduced at the transverse membranes (see Fig. S6 in the supplementary material). The apical localization of PIN3-GFP in the epidermal cells in the proximity of the cotyledons was more pronounced in ethylene-treated hooks (Fig. 4B).

In conclusion, our data show that ethylene prolongs the formation phase, resulting in an exaggeration of hook curvature. Ethylene-regulated apical hook development might involve modulation of the activity of the PIN-dependent auxin efflux machinery by transcriptional and post-transcriptional mechanisms.

DISCUSSION

Dynamic auxin distribution during apical hook development

Development of the apical hook depends on the tightly regulated differential growth of cells. The hook curvature is a consequence of differential growth, resulting in shorter cells on the concave side than on the convex side (Raz and Ecker, 1999; Vandenbussche and Van Der Straeten, 2004; Vriezen et al., 2004). This asymmetry must be maintained during the maintenance phase, whereas during the opening phase mechanisms that gradually equilibrate cell sizes on both sides of the hook have to be activated. Although, mechanistically, the process of apical hook development is well described, knowledge of the regulatory molecular mechanisms involved is limited.

The plant hormone auxin has been shown to play an important role in the regulation of differential growth that determines gravitropic or phototropic responses of plant organs and, in all cases, the differential growth of cells correlated with asymmetric auxin distribution (Friml et al., 2002b; Abas et al., 2006). Analysis of auxin response factor distribution during apical hook development, using the *DR5* reporter (Ulmasov et al., 1997), revealed a dynamic

modulation of auxin levels through particular developmental phases. Shortly after germination, the auxin distribution in hypocotyls is not asymmetric. During hook formation, auxin maxima are gradually established in the cortex and epidermal cells on the concave side of the hook. Later, during transition to the opening phase, this differential auxin distribution is gradually lost. Hence, the apical hook progresses through developmental phases that might require mechanisms for: (1) the establishment of asymmetric auxin levels, leading to differential growth rates of cells; (2) the regulation of the formation-to-maintenance phase transition and stabilization of the asymmetric auxin distribution; (3) the control of the maintenance-to-opening phase transition; and (4) management of the opening process, accompanied with gradual loss of auxin maxima.

PIN-dependent auxin efflux is involved in the regulation of auxin distribution during apical hook development

The dynamic modulation of auxin levels during hook development might result from the coordinated action of local, tissue-specific auxin metabolism and auxin transport. Recently, genes involved in auxin biosynthesis have been identified (Stepanova et al., 2008; Zhao et al., 2001) and their role in apical hook development demonstrated (Stepanova et al., 2008; Vandenbussche et al., 2010). Here we show that PIN-mediated auxin efflux (Petrášek et al., 2006) is an important mechanism participating in the dynamic distribution of auxin during apical hook development. Inhibition of auxin efflux by NPA interferes with the asymmetric auxin distribution and prevents formation of the apical hook structure. Several auxin efflux carrier genes of the PIN family are differentially expressed during apical hook development. *PIN1*, *PIN3* and *PIN4* are expressed primarily during hook formation and maintenance, whereas *PIN1* and *PIN7* might additionally modulate auxin transport during the transition between the maintenance and opening phases.

Elimination of PIN genes led to specific defects in apical hook development. The phenotype of the *pin3* mutant supports a central role for *PIN3* in the formation and maintenance phases, whereas *PIN1*, *PIN4* and *PIN7* seem to control the maintenance phase or the maintenance-to-opening phase transition. The analysis of multiple *pin3*, *pin4* and *pin4 pin7* mutants confirmed that *PIN3* is assisted by *PIN4* and *PIN7* during hook formation.

Establishment of auxin maxima during hook formation

The increased accumulation of auxin in a few cells at the concave side of the hook suggests that the formation of the auxin maxima might require a redistribution in different directions: lateral, from the central cylinder towards the cortex and epidermal cells, but also between the cortex and epidermal cells; and longitudinal, along the hypocotyl axis. Analysis of the membrane localization suggests that PIN3 might coordinate auxin redistribution laterally from endodermis through the cortex and epidermis layers. Along the main axis in cortex and epidermis, auxin seems to be transported primarily in a basipetal direction towards the root. Auxin transport in the cortex cells appears to be regulated by PIN3, along with PIN4, whereas in the epidermis it is mainly regulated by the combined action of PIN3 and PIN7. In addition, auxin might be transported partly in an acropetal direction, as suggested by the apical location of PIN3 in epidermal cells in the proximity of the cotyledons.

An important question is how the asymmetry in the auxin distribution is achieved between the convex and concave sides. As shown by the quantification of the PIN3-GFP signal, asymmetry in favor of cells on the convex side can be detected very early during the hook formation phase. Slightly later, the PIN4 protein is also observed at the convex, but not concave, side of the hook. We hypothesize that higher levels of PIN3 and PIN4 might increase the auxin transport rate at the outer side of the hook and thereby contribute to the asymmetry in distribution that results in auxin accumulation at the inner side.

In the early phases, establishment and maintenance of the auxin maxima are crucial, whereas later, loss of the auxin maxima takes place to coordinate hook opening. The asymmetric character of *YUC1* expression, with a maximum on the convex side of the apical hook (Vandenbussche et al., 2010), points to the role of local auxin biosynthesis at this developmental phase. This probably illustrates a mechanism of equilibration of auxin levels on the opposing sides of the hook curvature. However, as also shown by Vandenbussche et al., differentiation/maturation of the vasculature in hypocotyls does not seem to be involved in the timing of hook opening (Vandenbussche et al., 2010). Transient inhibition of auxin transport by NPA induces apical hook opening, demonstrating that PAT needs to be functional also at this developmental phase. The PIN1-GFP signal observed on the concave side of the hook, as also shown by Blakeslee et al. (Blakeslee et al., 2007), suggests that PIN1 might be an important co-regulator of auxin levels directly at the auxin maxima. The *pin1*, as well as *pin4* and *pin7*, loss-of-function mutants exhibit shortening of the maintenance phase, but the relatively weak phenotypic defect of these single mutants suggests a redundancy of PIN genes in the regulation of hook maintenance and transition to opening.

Although auxin metabolism and transport represent important machineries mediating apical hook opening, the crucial question remains of how exogenous stimuli, such as light, are integrated into the initiation and regulation of the opening process.

Ethylene interaction with the PAT machinery during apical hook development

Although ethylene-induced exaggeration of the apical hook is a well-known phenomenon (Raz and Ecker, 1999), knowledge of the underlying mechanisms is limited. Our real-time analysis revealed that ethylene primarily affects the transition from the formation to the maintenance phase. Ethylene-treated hooks continue to bend at the time when control hooks have already passed to the maintenance phase. This leads to an absence of the maintenance phase and a direct transition from hook formation to opening.

Ethylene stimulates the expression of *TAR2*, which is involved in auxin biosynthesis and increases the auxin level in cotyledons and hypocotyl tissues (Stepanova et al., 2008; Vandenbussche et al., 2010). These observations point to an important role for ethylene-regulated local auxin biosynthesis in apical hook development, similar to that shown for ethylene-regulated root elongation (Růžička et al., 2007; Stepanova et al., 2007; Swarup et al., 2007). Moreover, rescue of the apical hook phenotype in the ethylene perception mutants by exogenous auxin hints at auxin acting downstream of the ethylene pathway (Vandenbussche et al., 2010). NPA, as well as a lack of PIN3 activity, counteract the ethylene effect on hook development, revealing that modulations in the auxin level in responsive tissues are not only mediated through ethylene-regulated local auxin biosynthesis, but also through auxin transport.

Ethylene restricts expression of the DR5 reporter to several cells on the concave side of the hook (Vandenbussche et al., 2010) and enhances the expression of the Aux/IAA regulators *IAA3*, *IAA12* and *IAA13*, as also recently demonstrated in the tomato apical hook (Chaabouni et al., 2009). There are specificities in the ethylene-modulated expression of PIN genes in the apical hook as compared

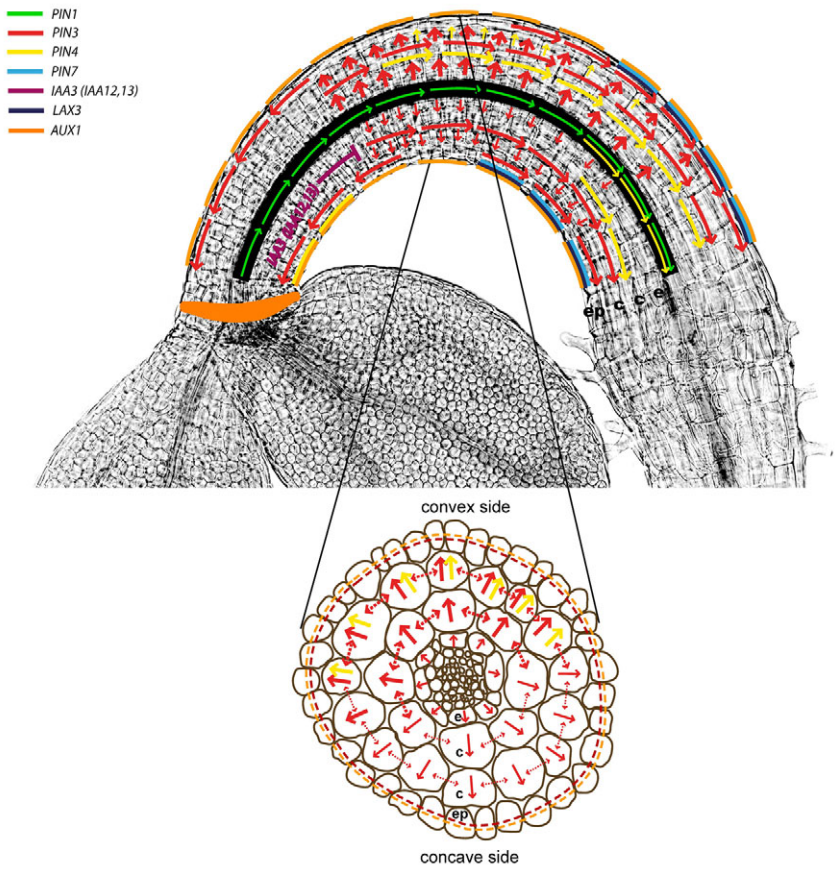

Fig. 6. Model of auxin distribution during apical hook formation. Auxin synthesized in cotyledons, shoot apical meristem and in the apical zone of the hypocotyl is transported in a basipetal direction towards the root. This basipetal movement is enhanced by the auxin influx carrier (AUX1) that controls the auxin flow from the cotyledons and shoot apical meristem and by the efflux carriers (PIN1 and PIN3) that act in the central cylinder. Lateral redistribution of auxin through endodermis is promoted by PIN3 and further through the cortex and the epidermis by AUX1, PIN3 and PIN4. In the cortex, auxin is basipetally directed by PIN3 and PIN4 and in the epidermis by PIN3, PIN7, AUX1 and LAX3. In the proximity of cotyledons, part of the auxin might be transported in an apical direction by PIN3 at apical membranes in the epidermis. Increased PIN3 and PIN4 localization at the cell membranes on the convex side might enhance the auxin transport rate, resulting in a more efficient draining of auxin from the outer cortex and epidermal layers of the hook. The asymmetric expression of Aux/IAA genes with maxima on the concave side might be involved in a regulatory feedback loop that reinforces the asymmetric auxin redistribution. e, endodermis; c, cortex; ep, epidermis.

with in roots. In the root meristem, *PIN1*, *PIN2* and *PIN4* are upregulated (Růžička et al., 2007), and this stimulatory effect of ethylene was also confirmed in the roots of etiolated seedlings (data not shown). By contrast, during apical hook development, *PIN1* and *PIN4* seem to be downregulated, whereas *PIN2* expression is not affected in the presence of ethylene.

On the protein level, ethylene enhanced the asymmetry in the PIN3-GFP signal in favor of the lateral localization of PIN3 in the cortex cell membranes on the convex side.

In summary, our data suggest that ethylene suppresses mechanisms involved in the transition from the formation to the maintenance phase. This might include control over auxin activity through: (1) interaction with the auxin biosynthesis pathway; (2) auxin response factors; (3) the regulation of PIN gene expression; and (4) modulation of the polar localization of PIN proteins.

Model of auxin distribution during apical hook formation

We propose a model to explain the auxin distribution during apical hook development in the absence of an enhanced ethylene signal (Fig. 6). Auxin, synthesized in the cotyledons, the shoot apical meristem and the apical zone of the hypocotyl is transported basipetally towards the root (Vandenbussche et al., 2010). This movement is regulated by the coordinated activities of the auxin influx carrier AUX1, which controls the auxin flow from the apical meristem and cotyledons towards the hypocotyl (Vandenbussche et al., 2010), and efflux carriers (PIN1 and PIN3) acting in the central cylinder. Lateral redistribution of auxin from the endodermis towards the cortex is promoted by PIN3 and further through the cortex and epidermis by AUX1 (Vandenbussche et al., 2010), PIN3 and PIN4. In both cortex and epidermal cells, auxin might be basipetally directed by PIN3 and PIN4 acting predominantly in the cortex and by PIN3, PIN7, AUX1 and LAX3 (Vandenbussche et al., 2010) in the epidermis. As suggested by the membrane localization of PIN3 in the epidermis, some auxin might be redirected acropetally towards the cotyledons.

The increased localization of PIN3 and PIN4 on the convex side of the hook might contribute to the lateral asymmetry in auxin distribution, probably by enhancing the auxin transport rate resulting in more efficient auxin draining from the outer (epidermal and cortex) hook layers. Later, the transition to the opening phase is accompanied by *PIN1* expression on the concave side of the hook and by the simultaneous progress of PIN7 into the unfolding hook.

In addition, the enhanced asymmetrical expression of the Aux/IAA genes [*IAA3* (*SHY2*), *IAA12* (*BDL*) and *IAA13*] on the concave side of the hook points to the existence of a negative-feedback regulatory loop. Increased accumulation of IAA3 and other homologs might contribute to the regulation of PIN gene expression (Vieten et al., 2005; Dello Ioio et al., 2008) and PIN polarity (Sauer et al., 2006), thus reinforcing the imbalance in the auxin levels at the convex and concave sides of the apical hook. Recently, proteolytic turnover of PIN proteins was reported as an important mechanism in the regulation of differential PIN-mediated PAT (Abas et al., 2006; Kleine-Vehn et al., 2008). Although there is as yet no direct evidence, discrepancy between *PIN3::GUS* and *PIN3::PIN3-GFP* expression or between *PIN4::GUS* and *PIN4::PIN4-GFP* expression, might hint at this type of regulation during apical hook development. Resolution of this question requires more detailed examination.

Acknowledgements
We thank Herman Höfte, Gerrit S. T. Beemster and Karel Spruyt for help with the establishment of the real-time analysis; Stéphanie Robert and Jürgen Kleine-Vehn for helpful comments; and Martine De Cock for help in preparing the manuscript. This work was supported by grants from the European Research Council (Starting Independent Research Grant ERC-2007-Stg-207362-HCPO to E.B. and P. M.), the Ministry of Education, Youth and Sports of the Czech Republic (LC06034 and MSM0021622415 to P.Z., J.H and J.F.), The Grant Agency of the Academy of Sciences of the Czech Republic (IAA601630703 to P.Z., J.H. and J.F.), Ghent University (Bijzonder Onderzoeksfonds, Bilateral Cooperation, BILA-06), and the Interuniversity Attraction Poles Programme (IUAP VI/33), initiated by the Belgian State Science Policy Office. F.V. is a postdoctoral fellow of the Research Foundation-Flanders.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.041277/-/DC1

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