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The auxin influx carriers AUX1 and LAX3 are involved in auxin-ethylene interactions during apical hook development in *Arabidopsis thaliana* seedlings

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SUMMARY

Dark-grown dicotyledonous seedlings form a hook-like structure at the top of the hypocotyl, which is controlled by the hormones auxin and ethylene. Hook formation is dependent on an auxin signal gradient, whereas hook exaggeration is part of the triple response provoked by ethylene in dark-grown *Arabidopsis* seedlings. Several other hormones and light are also known to be involved in hook development, but the molecular mechanisms that lead to the initial installation of an auxin gradient are still poorly understood. In this study, we aimed to unravel the cross-talk between auxin and ethylene in the apical hook. Auxin measurements, the expression pattern of the auxin reporter DR5::GUS and the localization of auxin biosynthesis enzymes and influx carriers collectively indicate the necessity for auxin biosynthesis and efficient auxin translocation from the cotyledons and meristem into the hypocotyl in order to support proper hook development. Auxin accumulation in the meristem and cotyledons and in the hypocotyl is increased ~2-fold upon treatment with ethylene. In addition, a strong ethylene signal leads to enhanced auxin biosynthesis at the inner side of the hook. Finally, mutant analysis demonstrates that the auxin influx carrier LAX3 is indispensable for proper hook formation, whereas the auxin influx carrier AUX1 is involved in the hook exaggeration phenotype induced by ethylene.

KEY WORDS: Apical hook, Auxin biosynthesis, Auxin transport, Ethylene, Photomorphogenesis, Skotomorphogenesis, *Arabidopsis*

INTRODUCTION

Dicotyledonous seedlings that emerge in darkness form an apical hook at the top of the hypocotyl, protecting the apical meristem from damage while growing through soil or mulch (Darwin and Darwin, 1881; Goeschl et al., 1966; Guzman and Ecker, 1990). Together with closed and unexpanded cotyledons, this trait is a hallmark of skotomorphogenic development. When etiolated seedlings are exposed to light, the apical hook opens and cotyledons expand to allow optimal photosynthetic performance. Apart from photomorphogenic control, the maintenance and opening of the hook are dependent on a number of interacting endogenous signals provided by plant hormones, including auxins, ethylene, gibberellins and brassinosteroids (Bleecker et al., 1988; Lehman et al., 1996; De Grauwe et al., 2005; Achard et al., 2003; Li et al., 2004; Vriezen et al., 2004).

Apical hook development has been most extensively connected with auxin. Biosynthesis mutants, such as *yuc1-D* or *sur1* (also known as *alf1* and *rty*), that overproduce auxin have defects in hook development (Boerjan et al., 1995; Celenza et al., 1995; King et al., 1995; Zhao et al., 2001). Likewise, auxin-resistant mutants are hookless, indicating the importance of a proper auxin balance for correct hook formation (Tian and Reed, 1999). In addition, treatments with exogenous auxin or the auxin efflux inhibitor 1-naphthylphthalamic acid (NPA) lead to a hookless phenotype, suggesting that the establishment of an auxin gradient is essential (Schwark and Schierle, 1992; Lehman et al., 1996). From these observations, it has been suggested that an auxin gradient forms in the apical region, resulting in differential growth and, consequently, hook formation. Hook development in *Arabidopsis thaliana* has been shown to be at least in part dependent on the auxin efflux carrier PIN3 (Friml et al., 2002). Apart from this observation, little is known about the mechanisms by which the auxin gradient in the apical hook is formed.

Ethylene is known for its ability to enhance apical hook curvature. Both endogenous and exogenous ethylene can control hook maintenance and exaggeration (Goeschl et al., 1966; Bleecker et al., 1988). Exaggeration of the apical hook is part of the triple response in ethylene-treated etiolated *Arabidopsis* seedlings and has been used to isolate various mutants and dissect ethylene biosynthesis and signaling pathways (De Paepe and Van Der Straeten, 2005; Li and Guo, 2007). Importantly, sensitivity to ethylene is restricted to a time window from 2 to 3 days after germination (Raz and Ecker, 1999).

Several lines of evidence support the existence of cross-talk between auxins and ethylene in hook formation. For example, *hookless 1* (*hls1*) suppresses the constitutive exaggerated hook phenotype of *constitutive triple response 1* (*ctr1-1*). *HLS1* is an ethylene-response gene that encodes an N-acetyltransferase thought to control local auxin concentrations (Lehman et al., 1996). Furthermore, *hls1* and *axr1* mutants lack hook formation upon ethylene treatment. Together, these observations suggest that the auxin signal acts downstream of ethylene signaling. However, AUXIN RESPONSE FACTOR 7 (ARF7; also known as NPH4) is known to be a major regulator of differential growth in aerial tissues (Stowe-Evans et al., 1998; Harper et al., 2000). Hook formation

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defects in *arf7* null mutants can be suppressed by ethylene treatment, and this effect can be blocked by treatment with NPA (Harper et al., 2000).

The possible involvement of the auxin efflux carrier PIN3 in hook development (Friml et al., 2002) points to the participation of auxin transport in this process. Auxin can enter a cell by diffusion or by carrier-mediated uptake and leave a cell through the action of efflux carriers (Kramer and Bennett, 2006). In *Arabidopsis*, both auxin influx and efflux carriers form gene families. The auxin influx carrier family comprises four members: AUX1 and LIKE AUX1 (LAX) 1, 2 and 3 (Parry et al., 2001), whereas the efflux carriers include the PIN family, which consists of eight members (PIN1-8) (Paponov et al., 2005), and a number of ABCB-type transporters [ABCB1 (PGP1), ABCB4 (PGP4), ABCB19 (PGP19)] (Geisler and Murphy, 2006; Lewis et al., 2009). The AUX1 and LAX3 proteins (Yang et al., 2006; Swarup et al., 2008) and some PIN proteins (Petrášek et al., 2006) have been conclusively demonstrated to be functional auxin influx and efflux carriers, respectively.

Here we present evidence that auxin biosynthesis and influx are necessary for apical hook development in *Arabidopsis thaliana*. We demonstrate a dependence on tryptophan (Trp) aminotransferases involved in auxin biosynthesis and on two auxin influx regulators: on LAX3 in the process of hook formation and on AUX1 in hook exaggeration in the presence of ethylene. The accompanying manuscript (Žádníková et al., 2010) illustrates the role of auxin efflux and presents a global model for auxin transport during apical hook development.

MATERIALS AND METHODS

Plant material

Col-0, M0176 of the Jim Haseloff collection and *ein2-1* were purchased at NASC (Nottingham, UK). Mutants *ein4-1*, *etr1-1*, *ein2-5*, *ein3-1* (Roman et al., 1995), *aux1-21*, *aux1-22*, *lax1*, *lax2*, *lax3*, *aux1 lax1*, *aux1 lax2*, *aux1 lax3*, *aux1 lax1 lax2*, *aux1 lax1 lax3*, *aux1 lax2 lax3*, *aux1 lax1 lax2 lax3*, *pAUX1::GUS*, *aux1 pAUX::AUX1-YFP*, *lax3 pLAX3::GUS* and *pLAX::LAX3-YFP* were as described (Marchant and Bennett, 1998; Bainbridge et al., 2008; Swarup et al., 2008). *pSCR::H2B-YFP* was as described (Heidstra et al., 2004).

EBS::GUS1-11 was a kind gift from J. R. Ecker (The Salk Institute, San Diego, CA, USA) (Stepanova et al., 2007). *DR5::GUS* was obtained from T. Guilfoyle (University of Missouri, Columbia, OH, USA) (Ulmasov et al., 1997). *pTAR2::GUS* and *wei8-1 tar2-1* were kind gifts from J. Alonso (North Carolina State University, Raleigh, NC, USA) (Stepanova et al., 2008). *Yuc1-D*, *yuc1*, *4*, *10*, *11* quadruple mutants (homozygous for *yuc1* and *yuc10*, heterozygous for *yuc4* and *yuc11*) and *pYUC1::GUS* were obtained from Y. Zhao (University of California, San Diego, CA, USA) (Zhao et al., 2001; Cheng et al., 2007).

Growth conditions

Seeds were sown on half-strength MS media (Duchefa, Haarlem, The Netherlands) solidified with plant tissue culture agar (LABM, Bury, UK). 1-aminocyclopropane-1-carboxylate (ACC), indole-3-acetic acid (IAA) and 1-naphtoxyacetic acid (1-NOA) were from Sigma (St Louis, MO, USA). 1-naphtylphtalamic acid (NPA) was from Duchefa. After sowing, the seeds were left at 4°C for 48 hours in darkness, then transferred to white light for 6 hours to stimulate germination, and subsequently to darkness and left at 22°C for the desired time.

For investigation of ethylene responses in ethylene mutant lines, a continuous flow of 10 ppm/l of air was delivered to enclosed seedlings. For the studies involving reporter lines, gassing with a continuous flow of ethylene (1 ppm, at a flux of 1 l/hour) or air (at a flux of 1 l/hour) (Air Liquide, Aalter, Belgium) was performed in darkness 48 hours after germination of seedlings grown in 10 ml gas-tight vials (Chrompack, Bergen op Zoom, The Netherlands).

Real-time analysis of apical hook development

Seeds were surface sterilized, planted on plates with agar-containing medium and left for 48 hours at 4°C. Germination was stimulated in white light for 6 hours. The plates were placed in a vertical position in infrared light (880 nm LEDs, Velleman, Gavere, Belgium) at 22°C. Seedlings were photographed every hour for 12 days using a set of Hercules optical glass USB-type CCD cameras without an infrared filter (Guillemot, La Gacilly, France), steered by Active WebCam v.4 software (PY Software, Etobicoke, Canada). Angles of hook curvature were measured using ImageJ (NIH, Bethesda, MD, USA). The angle of curvature of the hook is defined as 180° minus the angle between the tangential of the apical part with the axis of the lower part of the hypocotyl. In the case of hook exaggeration, 180° plus that angle is defined as the angle of hook curvature (see Fig. 1A, inset). The end of the hook formation phase was defined as the point at which the angle reached 95% of its maximum value. The maintenance phase was defined as a plateau in which hook angles differed at most by 2.5% from the mean value. This was followed by the opening phase.

Analysis of reporter lines

Glucuronidase assays were performed as follows. Plants were submerged for 30 minutes in ice-cold 90% acetone, washed in 1 M phosphate buffer (pH 7.2) and transferred to GUS assay buffer containing 2 mM 5-bromo-4-chloro-3-indolyl-glucuronide (X-gluc; Immunosource, Zoersel, Belgium). Staining was for 20 hours at 37°C; thereafter, plants were kept in 75% ethanol. For photography, seedlings were submerged in chloral hydrate (Acros, Geel, Belgium) solution, mounted on a microscope slide and viewed with a Zeiss Axiovert microscope (Carl Zeiss, Zaventem, Belgium) using a 20× Plan Apochromat objective. *Aux1 pAUX::AUX1-YFP* and *lax3 pLAX3::LAX3-YFP* lines (Swarup et al., 2008) were analyzed using a Nikon C1 confocal microscope with a 40× Plan Fluor objective (Nikon Belux, Brussels, Belgium).

Fluorescence recovery after photobleaching (FRAP) analysis of a *pAUX1::AUX1-YFP*-expressing line was performed in dark-grown seedlings at 48-52 hours after germination in the presence or absence of 10 μM ACC (prior to hook exaggeration). A Zeiss LSM 5 confocal microscope (Carl Zeiss, Jena, Germany) with appropriate filter sets for YFP detection (excitation 488 nm, emission 505-550 nm) and a 40× C-Apochromat water-immersion objective (NA=1.2) were used. For bleaching, the region of interest (ROI) of defined size (a rectangle of 40×20 pixels with the membrane in the center) was interactively applied at the transversal plasma membranes of epidermal cells at both concave and convex sides in the middle of the apical hook. Bleaching with maximal laser intensity was followed by 15 minutes tracking of fluorescence recovery (imaging every 20 seconds). For the quantification, another rectangular ROI was applied using Carl Zeiss Image Examiner software to the middle of the ROI used for the bleaching (ROI rectangle 20×10 pixels). In this ROI, the fluorescence after 10 minutes of recovery was subtracted from the fluorescence directly after bleaching and both values were related to the initial fluorescence. The resulting values reflect the rate of fluorescence recovery (i.e. how much relative fluorescence is recovering). The average ratio of these values between concave and convex sides was calculated (n=12 for control and n=7 for ACC-treated seedlings), reflecting the actual difference in the fluorescence recovery rate at both sides.

Analysis of the M0176 line was performed with a Nikon C1 confocal microscope using a 10× Plan objective (Nikon Belux).

Determination of endogenous auxin levels in *Arabidopsis* cotyledons and hypocotyls

Cotyledons (40 pieces) and hypocotyls with apical hooks (20 pieces) of 65-hour-old *Arabidopsis* seedlings grown in darkness were separated under green safety light to prevent light stimulation of photomorphogenesis and collected in 300 μl methanol. After overnight extraction at –20°C, the tissues were separated by centrifugation (10,000 ***g***) and extracts were evaporated to dryness. Dried samples were diluted in 15% (v/v) acetonitrile:water and filtered on Micro-Spin 0.2 μm nylon filters (Alltech, Deerfield, IL, USA). Filtrates were injected into an HPLC machine (Agilent 1200 with UV detector at 270 nm) and precleaned on a C-18 column (Luna 3 μm,

150×4.60 mm) with gradient elution and fractionation on a fraction collector (Gilson 203B). The fraction eluting at 23.05 minutes was collected for 1 minute and dried.

Subsequently, the dried fraction was derivatized with 0.3 ml diazomethane for 15 minutes, then dried and dissolved in 10 µl of acetone. Redissolved sample (8 µl) was injected into a gas chromatography/tandem mass spectrometry (GC-MS/MS) machine (PolarisQ) and analyzed on a DB-5MS column (Agilent Technologies) (injector, PTV in solvent split mode; detector, ion trap in MS/MS scan mode; MS1, full scan 50-300 amu; MS2 IAA, precursor 130.1 amu, product full scan 65-200 amu; MS2 labeled IAA, precursor 136.1 amu, product full scan 70-200 amu). Calibration was performed using an external standard (non-labeled IAA, 99%; Duchefa). Differences in the endogenous free IAA content between the samples are represented by differences in the peak areas. Results represent the mean of four independent repeats for each sample.

RESULTS

Kinematic analysis of ethylene-regulated hook development

It has been reported previously that ethylene sensitivity in hook development is limited to a time window around day 2 after germination (Raz and Ecker, 1999). We employed time-lapse imaging to analyze ethylene-controlled apical hook development. Images were captured at regular intervals and the hook curvature was measured every 4 hours. In the absence of the ethylene precursor ACC, the apical hook forms shortly after germination, until bending reaches a plateau around an angle of 170°, corresponding to the formation phase (Fig. 1A). The plateau consists of hook curvature values that do not differ by more than 2.5% from the mean plateau value. The length of the plateau defines the maintenance phase, which spans a period of 30-60 hours after germination. At the end of the maintenance phase, the hook begins to open (opening phase, 68 hours, from 60-128 hours). Wild-type seedlings treated with ACC started to show exaggeration of the hook (i.e. an extension of the formation phase) at the beginning of day 2 (greater than 30 hours) after germination, equivalent to the point when their non-treated counterparts initiate the maintenance phase (Fig. 1A). Although the maintenance phase in ACC-treated wild-type seedlings was shorter than in control seedlings (12 hours, from 40-52 hours), the opening phase extended to 176 hours (52-228 hours), whereas the kinetics of opening were not dramatically affected. It is noteworthy that opening appears to be under developmental control and not solely regulated by photomorphogenic signals (Liscum and Hangarter, 1993; Wang et al., 2009). Hooks of the ethylene-insensitive mutant *ein2-1* did not enter a maintenance phase and opened before full formation was reached, irrespective of the presence of ACC (Fig. 1; 130° versus 170° in the wild type). This suggests that ethylene is essential for normal hook development.

Exogenous auxin restores the hook in ethylene-insensitive mutants

In addition to its regulation by ethylene, the presence of an apical hook also depends on auxin (Schwark and Schierle, 1992; Lehman et al., 1996). To understand the cross-talk between the auxin and ethylene pathways, ethylene-insensitive mutants were treated with exogenous IAA and compared with the wild type on the third day of growth in darkness (end of maintenance phase). In the absence of exogenous IAA, the ethylene-insensitive mutant *ein2-5* exhibited reduced hook curvature as compared with the wild type (Fig. 1B). However, treatment with 0.1 µM IAA almost fully restored the hook curvature in *ein2-5* seedlings. Higher auxin concentrations, however, caused a reduced hook curvature both in wild-type and *ein2-5* seedlings, suggesting that at high concentrations (above 0.5 µM IAA) auxins interfere with hook development. Similarly, hook curvature was restored in other ethylene-insensitive mutants (*etr1-1*, *ein3-1* and *ein4-1*) when treated with 0.5 µM IAA (Fig. 1C), suggesting that in the ethylene perception and signaling mutants auxin levels are suboptimal. Together, these results suggest that a narrow threshold level of auxin is necessary for normal hook development, and that ethylene signaling is necessary to achieve this threshold.

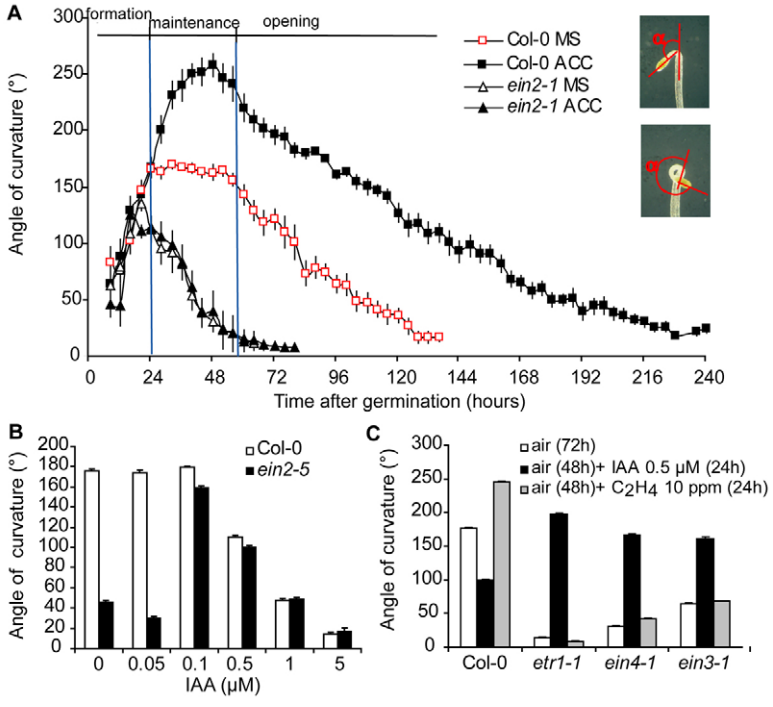

Fig. 1. Ethylene and auxin regulate apical hook development. (**A**) Kinetics of hook development in wild-type *Arabidopsis* seedlings and in the ethylene-insensitive mutant *ein2-1*. Seedlings were photographed and analyzed for hook bending (n=10). Insets depict how the angle of hook curvature was determined. (**B**) Exogenous auxin rescues hook formation in ethylene-insensitive mutants. Seedlings were grown for 3 days in darkness (end of maintenance phase) on the indicated media and apical hook bending was quantified (n>20). (**C**) Seedlings were grown for 2 days in darkness and then treated with auxins (IAA) or ethylene. Hook curvature was evaluated after 24 hours of treatment (n>20). All error bars represent s.e.m.

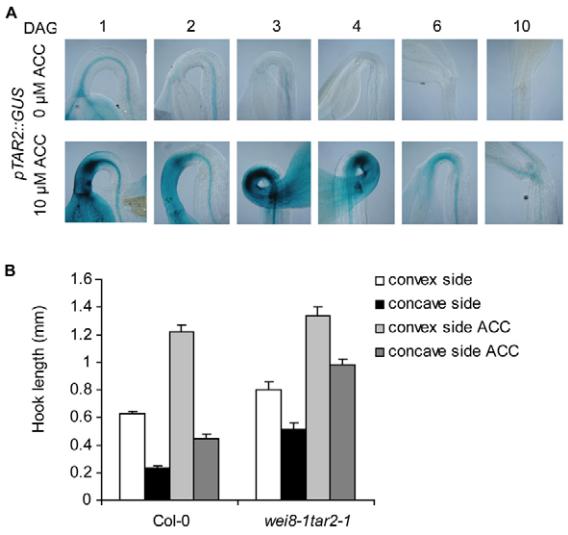

Fig. 2. Auxin biosynthesis genes *TAA1* (*WEI8*) and *TAR2* are necessary for normal hook development. (**A**) *pTAR2::GUS* expression during apical hook development in the presence and absence of ACC. DAG, day after germination. (**B**) Effect of ACC on the length of the concave and convex sides of the hook in the *wei8-1 tar2-1* double mutant and wild type (Col-0) at the end of the maintenance phase.

Ethylene regulates auxin biosynthesis in the hook

We next addressed how the threshold auxin concentration for hook bending is reached and the role that ethylene plays in its accumulation. The *yucca1* dominant mutant (*yuc1-D*) has an elevated level of free IAA and is hookless (Zhao et al., 2001). We investigated whether the expression of the *YUC1* gene is correlated with hook development. Analysis of *pYUC1::GUS* revealed rather uniform expression in the hook during the formation and maintenance phases (see Fig. S1A in the supplementary material, days 1-3). However, during hook opening, *YUC1* expression was only visible on the convex side (see Fig. S1A in the supplementary material, day 5). No ethylene regulation of *pYUC1::GUS* expression was observed, except for during the opening stage, when staining extended towards the basal end of the hypocotyl. A *yuc1 yuc10* double-null mutant background exhibited no obvious defects during hook development, suggesting that other auxin biosynthesis and/or transport genes are involved in hook development (see Fig. S1B in the supplementary material).

It has recently been shown that genes coding for the tryptophan (Trp) aminotransferases TAA1 (WEI8) and its closely related family member TAR2 are expressed in the apical hook and that ethylene can induce *TAR2* expression (Stepanova et al., 2008). We followed the expression of the latter gene with time, and found elevated expression in the presence of 10 µM ACC from the first day after germination. *TAR2* expression was homogenous over the apical part of the hook during the first 2 days after germination, but was enhanced on the concave (inner) side of the hook from day 3. This differential expression pattern was maintained as long as the hook showed an exaggerated curvature (Fig. 2A). The *taa1* (*wei8-1*) single mutant had a normal hook phenotype both in the absence and presence of ACC, but hook formation was almost abolished in the *wei8-1 tar2-1* double mutant (Stepanova et al., 2008) owing to a longer concave side, as compared with the wild type (Fig. 2B). These data suggest that the elevation of auxin levels by Trp aminotransferase activity is required to reach a threshold necessary for the eventual differential elongation in the hook, and that the persistence of this activity during ethylene-induced hook exaggeration on the concave side inhibits elongation.

The ethylene signal in the hook is non-differential

Our data suggest that stimulation of the ethylene pathway can enhance auxin biosynthesis at the concave side of the hook, thus contributing to an auxin gradient. An auxin maximum at the concave side has been shown to occur in the absence of a strong ethylene signal, at the transition between the formation and maintenance phases (our data) (Friml et al., 2002). To investigate whether the differential auxin signal is caused by a differential ethylene response, reporter lines for both signaling pathways were grown in parallel, in darkness and in the absence or presence of ethylene or its precursor ACC. The primary ethylene response reporter *EBS::GUS* (in which the *GUS* gene is driven by a promoter based on the response element for EIN3) (Stepanova et al., 2007) exhibited uniform staining in the apical hook in the presence of ethylene (see Fig. S2 in the supplementary material) or its precursor ACC (Fig. 3A), but showed only a very faint signal in the absence of these signaling molecules. By contrast, the auxin response as visualized by the *DR5::GUS* reporter was restricted to the concave side of the hook. This differential auxin maximum gradually disappeared during the opening phase (Fig. 3A and see Fig. S2 in the supplementary material). However, in the presence of a strong ethylene signal, the auxin maximum was both displaced towards the basal end of the hypocotyl and restricted to fewer cells. On the third day after germination, seedlings grown on MS media had an auxin response that reached from the hook region (cells 11-21 in the hypocotyl epidermis on the concave side of the hook) to the lower hypocotyl (not part of the hook; cells 6-10) (Fig. 3B,C). Both in control and in ACC-treated seedlings, *DR5::GUS* staining was virtually absent in the basal part of the hypocotyl (cells 1-5). However, in ACC-treated seedlings, *DR5::GUS* staining rarely occurred at the apical end (the top five cells, 17-21) (Fig. 3B,C). Thus, ethylene treatment results in the restriction of the auxin maximum to cells 6-16 and has the additional effect of displacing the auxin maximum towards the basal end of the hypocotyl, as seen by a reduction in staining of the top five cells (Fig. 3C). The ethylene signal appeared between 30 and 42 hours after germination (Fig. 3A). This matches the maintenance/exaggeration phase of the kinematic analysis (Fig. 1A).

Since the ethylene signal is either absent or diffuse throughout the hypocotyl (Fig. 3A), these observations indicate that the auxin response maximum does not simply induce a higher ethylene production or signal to cause local inhibition of cell elongation, and that the radial auxin gradient is probably not caused by a differential ethylene signal. Likewise, the diffuse ethylene signal does not induce an overall increase in the auxin response signal.

The auxin response maximum on the concave side is essential for correct hook development

In order to assess whether the auxin response maximum at the concave side of the hook (Fig. 3A) is essential for hook formation, auxin signaling was disrupted by expression of the dominant auxin-insensitive mutant *axr3-1* in a spatially specific manner. In roots, expression of *axr3-1* results in auxin resistance, which confers reduced inhibition of root growth by exogenous auxins (Dharmasiri et al., 2006). Specific expression of *axr3-1* on the concave side of the hook was achieved by a transactivation approach (Swarup et al., 2005). The *UAS::axr3* effector line does not display a hook phenotype (Fig. 3D,G). The M0176 activator line (J. Haseloff, NASC) drives

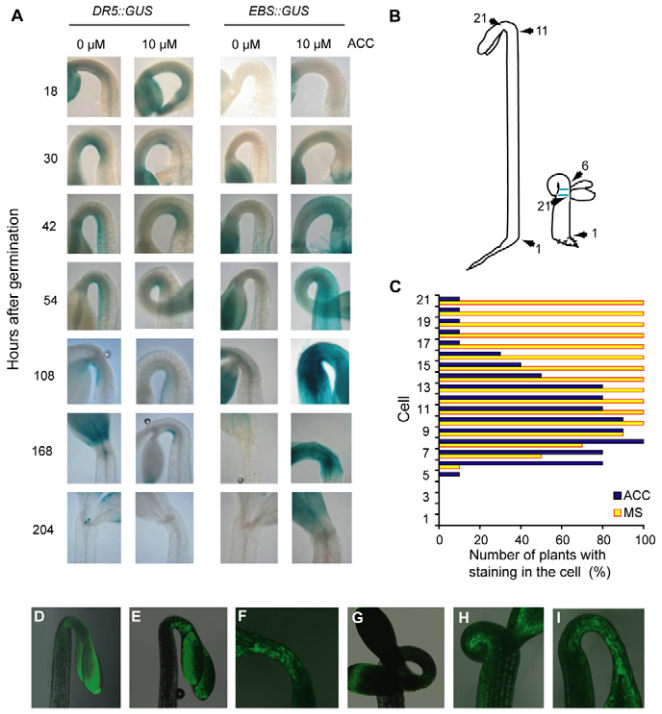

Fig. 3. Uniform ethylene-driven gene expression triggers directional auxin-driven gene expression. (**A**) Auxin and ethylene signal maxima in the apical hook region, as assessed by reporters *DR5::GUS* and *EBS::GUS*. Seeds were sown in the presence or absence of the ethylene precursor ACC and seedlings harvested at the times indicated. (**B**) A 3-day-old dark-grown *Arabidopsis* seedling (at the end of the maintenance phase). Left, untreated control; right, ACC- or ethylene-treated seedling. Numbers indicate the position of cells, counting from the root-shoot junction. Typically, ten cells are present in the hook of control plants grown on MS medium, whereas 15 cells are present in the hook of ACC-grown plants. (**C**) The auxin maximum, as represented by DR5::GUS staining, is displaced in ACC-treated seedlings. Seedlings (n>20) were analyzed for staining in the epidermal layer all along the hypocotyl, in cells that are present on the concave side of the hook at day 3 after germination. The frequency of stained cells is indicated according to their position along the epidermal cell layer. The auxin maximum in ACC-treated seedlings peaks from cells 6-16, whereas it stretches from cells 7 to 21 in control seedlings. (**D-I**) Localized overexpression of the mutant *axr3* gene interferes with apical hook development. Confocal laser scanning microscopy images of apical hooks of plants expressing GFP under the control of the M0176 driver. Seedlings were analyzed at the end of the maintenance phase, on day 3 after germination and growth in darkness. (D) Autofluorescence of *UAS::axr3*. (E) The GFP expression pattern in the M0176 driver line. (F) Phenotype of F1 plants from crosses of M0176 and *UAS::axr3*. (G) As D, but in the presence of 10 μM ACC. (H) As E, but in the presence of 10 μM ACC. (I) As F, but in the presence of 10 μM ACC.

GFP expression under GAL4 control in the hook (Fig. 3E,H). Upon crossing activator and effector, a strong attenuation of the hook phenotype was observed in plants grown on medium with or without ACC (Fig. 3F,I). This indicates that the auxin signal at the concave side of the hook is essential for the characteristic architecture of the shoot apical region of etiolated seedlings. In fact, several components of the auxin signaling pathway [e.g. SHY2 (IAA3)] are expressed in the apical hook and are essential for its development [see figure 1E,H in Žádníková et al. (Žádníková et al., 2010)].

Auxin transport inhibitors affect hook development

Apart from the spatial regulation of auxin biosynthesis, auxin transport can also cause local auxin accumulation. To investigate the impact of auxin influx on the auxin maximum in seedling shoots, the *DR5::GUS* reporter line was grown on media containing the auxin influx inhibitor 1-NOA, in the presence or absence of ACC. 1-NOA restricted the auxin maximum to the upper part of the hypocotyl, the meristem and the cotyledons (Fig. 4A).

In order to assess the importance of auxin influx during apical hook development, we performed a kinematic analysis on wild-type seedlings treated simultaneously with 1-NOA and ACC. On 1-NOA, seedlings exhibited a reduced rate of hook formation and less bending, revealing the importance of auxin influx during the early phases of hook development. In addition, the rate of opening, as indicated by the slope of the descending part of the curve, was lower than in control seedlings, supporting a role for auxin influx in the opening phase (Fig. 4C). ACC increased the amplitude of hook bending in the presence of 1-NOA, albeit to a limited extent as compared with seedlings without 1-NOA (Fig. 4C). By contrast, the auxin efflux inhibitor NPA fully blocked the formation of the hook from germination onwards, resulting in the absence of a hook structure regardless of the presence or absence of ACC (Žádníková et al., 2010) (see Fig. S3 in the supplementary material). This demonstrates the dependence of hook formation and exaggeration on auxin efflux. Likewise, treatment of the constitutive ethylene response mutant *ctr1-1* with NPA abolishes the hook, confirming the necessity for auxin efflux in the ethylene response (Lehman et al., 1996).

Together, these data indicate the importance of both auxin influx and efflux in apical hook development.

AUX1 and LAX3 are involved in apical hook development

Mutations in the AUX1 influx carrier have been suggested to influence the extent of apical hook development (Roman et al., 1995; Stepanova et al., 2007). The apical hook phenotype of *aux1-21* is very similar to that of the strong *aux1* alleles *aux1-7* and *aux1-22* (data not shown). However, their hook phenotype is mild compared with wild-type plants grown on the auxin influx blocker 1-NOA (compare Fig. 4A with 4B), suggesting the involvement of other auxin influx carriers. In order to determine which other influx carriers might be involved in apical hook development, single and multiple auxin influx carrier mutants were grown for 3 days in continuous darkness. From all combinations tested, only those with *lax3* displayed a partial hookless phenotype (Fig. 4B). In the presence of an enhanced ethylene signal (10 μM ACC), not only did *lax3* lack an exaggerated hook, but also *aux1*. As is typical for ACC-treated *aux1* mutants, the hypocotyl displayed bending above the 'bottle neck' (the region where the hypocotyl becomes thinner) and an upper part of the hypocotyl without further bending towards the cotyledons (Fig. 4B). Combining *lax3* and *aux1* mutations had an additive effect, resulting in a phenotype reminiscent of 1-NOA treatments, both in the presence and absence of ACC. By contrast, mutations in *LAX1* or *LAX2* did not have a significant effect (P<0.05) on hook architecture (Fig. 4B; see Fig. S4 in the supplementary material).

In order to discover which phases of hook development are regulated by *AUX1* and *LAX3*, the single and double mutants were grown in the presence and absence of ACC and compared with wild-type plants over time (Fig. 4D-F). On MS medium, *aux1* single mutants exhibited hook establishment and maintenance phases similar

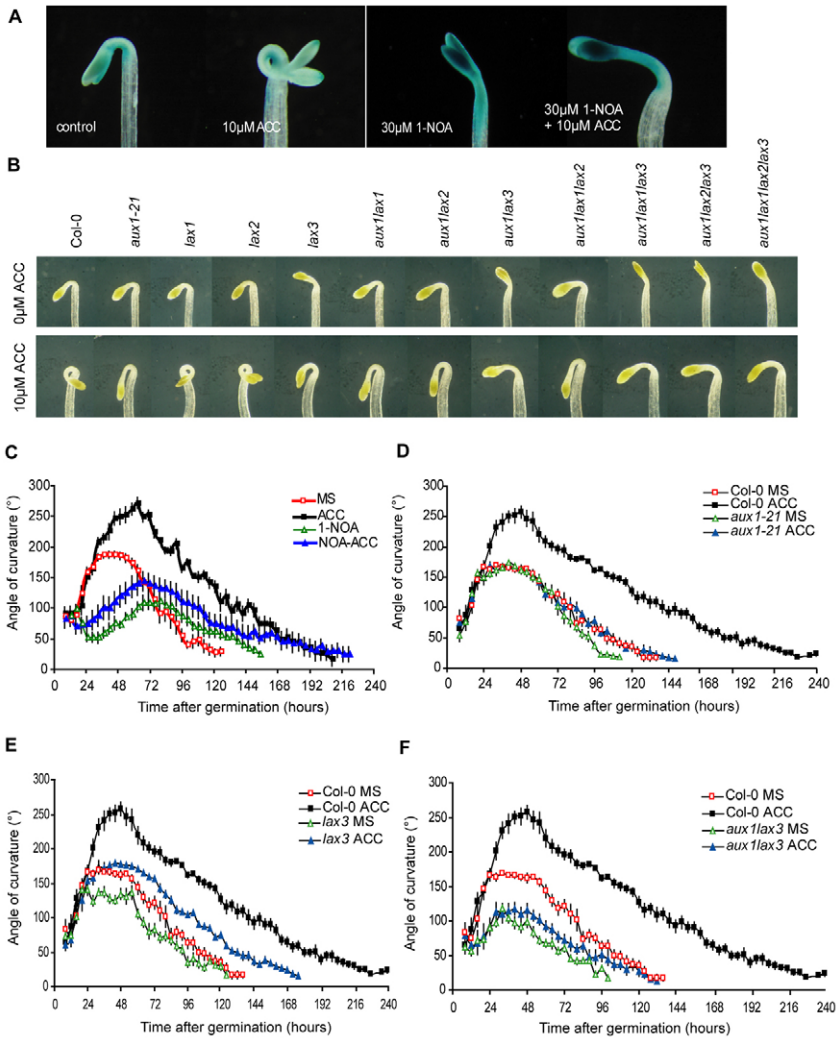

Fig. 4. Auxin influx is necessary for hook development. (**A**) Dark-grown *DR5::GUS* seedlings in the presence or absence of ACC and/or 30 μM 1-NOA, an auxin influx inhibitor, at the end of the maintenance phase on day 3 after germination. (**B**) The hook region of the wild type and of various auxin influx carrier mutants after 3 days of etiolated growth in the presence or absence of 10 μM ACC. (**C**) Effect of 1-NOA on the kinetics of apical hook development in wild-type *Arabidopsis* seedlings in the presence or absence of 10 μM ACC. (**D**) Kinetics of hook development in *aux1-21* mutants. (**E**) Kinetics of hook development in *lax3* mutants. (**F**) Kinetics of hook development in *aux1 lax3* double mutants. In C-F, error bars represent s.e.m. and *n*=10.

to the wild type, but slightly faster hook opening. However, in the presence of ACC, the opening of the hook in *aux1* single mutants did not differ from that of the untreated wild type, with the exception of at a few time points between 72 and 96 hours after germination, nor did the amplitude of curvature differ (Fig. 4D). The *lax3* single mutant displayed a normal rate of hook formation, but a lower amplitude of curvature than the wild type. This was the case both in the absence and presence of exogenous ACC (Fig. 4E). However, in contrast to what was seen for *aux1* seedlings, ACC had a clear effect on the amplitude of bending of *lax3* mutants (Fig. 4E). The *aux1 lax3* double mutant exhibited slower hook formation and attained a smaller amplitude than the *lax3* single mutant (Fig. 4F). An effect of ACC on the *aux1 lax3* double mutant was visible as a reduction in the velocity of hook opening, and hence a longer maintenance phase.

The development of the apical hook is thus dependent on the presence of the AUX1 and LAX3 auxin influx carriers. LAX3 is of major importance in developmental hook establishment, being assisted by AUX1 in this process, and AUX1 appears to play a prominent role in ethylene-regulated hook exaggeration.

Hooking defects are associated with impaired differential growth

Defects in hook development or bending are generally caused by impaired differential growth. This can occur by alterations in growth on the concave and/or convex side. In order to determine which side is most affected, the lengths of the concave and convex sides of hooks of 3-day-old etiolated wild-type, *aux1* and *lax3* seedlings were measured. *aux1* mutants on ACC did not display an exaggerated hook. This was mainly due to a longer concave side of the hook, as compared with the wild type (Fig. 5). Close examination revealed that the most apical part of the hook, close to the cotyledons, is straight (Fig. 4B; see Fig. S5 in the supplementary material). The lack of differential growth in this area is correlated with epidermal cell size. In *aux1* mutants, the epidermal cells on the concave and convex sides of the apical region of the hook were much more similar in size than in the wild type, in which cells on the convex side were clearly larger than those on the concave side (see Fig. S5 in the supplementary material). The hook lengths on the convex and concave sides of *lax3* mutants were not significantly different from those in the wild type on control media. However, in the presence of ACC, cells on the convex side of *lax3* lacked sufficient elongation for normal hook development (Fig. 4B, Fig. 5). This defective elongation correlated with epidermal cell size, which was consistently shorter than in the wild type (see Fig. S5 in the supplementary material).

AUX1 and *LAX3* are expressed in the hypocotyl

In order to determine how AUX1 and LAX3 control the development of the hook, their expression was followed in dark-grown plants by means of reporter constructs. *pAUX1::GUS* is

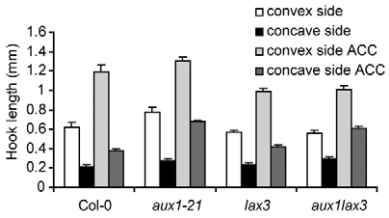

Fig. 5. Differential elongation in the hook region. The length of the hook on the concave and convex sides in wild-type, *aux1* and/or *lax3* mutant seedlings at the end of the maintenance phase, grown in the presence or absence of ACC. Error bars represent s.e.m. (*n*=10).

expressed in the apical region and is induced in the presence of a strong ethylene signal. Intense *pAUX1::GUS* reporter activity was visible on the concave side of the hook on day 3 after germination (Fig. 6A). The AUX1-YFP fusion protein localizes to the plasma membrane, with a strong signal in the epidermal cells (Fig. 6D). AUX1-YFP did not show readily apparent differences in relative fluorescence on the transversal and lateral cell membranes on the concave and convex sides of the hook, suggesting an absence of asymmetry in protein deposition (see Fig. S6 in the supplementary material); this contrasts with what is found for the auxin efflux carrier PIN3 (Žádníková et al., 2010). However, FRAP analysis of the epidermal AUX1-YFP fusion protein (in the middle of the concave and convex sides of the hook after 48-52 hours of etiolated growth) revealed a higher number of seedlings with faster fluorescence recovery on the concave side of the hook upon ACC treatment (Fig. 6F). On average, the recovery of AUX1-YFP fluorescence in plants exposed to ACC was twice as fast on the concave versus the convex side of the hook (ratio 2.05) as in the control (ratio 0.88) (see Fig. S7 and Table S1 in the supplementary material). This suggests faster AUX1 turnover on the concave side of the hook in the presence of an ethylene signal.

However, LAX3 was also expressed in the stele tissues of the hook, irrespective of the presence of ACC, and remained expressed until 6 days after germination on ACC (Fig. 6B). The LAX3-YFP signal in the stele of the hypocotyl was not specifically localized to the membrane. This pattern is atypical for an auxin influx carrier and is reminiscent of the localization of LAX3-YFP in the root stele, where its expression cannot rescue the *lax3* phenotype (Swarup et al., 2008). Therefore, it is likely that the presence of LAX3 in the stele does not influence the apical hook phenotype. However, *pLAX3::GUS* was also expressed outside the vasculature in the basal part of the hypocotyls, but was never expressed outside the stele in the hook region (Fig. 6C). In this basal part of the hypocotyl, the LAX3-YFP fusion protein does appear in the plasma membrane (Fig. 6E).

Transient gene expression patterns during hypocotyl development, such as those of *AUX1* and *LAX3*, are possibly linked with tissue maturation and organization of the hypocotyl (Busse and Evert, 1999). Expression of *pSCR::H2B-YFP*, an endodermal/starch sheath marker, is associated with vascular development (Wysocka-Diller et al., 2000; Peer et al., 2009). Only at the end of the first day after germination (hook formation phase) did the expression of *pSCR::H2B-YFP* appear in the most basal part of the hook (see Fig. S8 in the supplementary material). From day 2 after germination (maintenance phase), *pSCR::H2B-YFP* was present throughout the seedling. Hence, there is no direct correlation between the process of vascular differentiation/maturation and progress through the developmental phases of the apical hook. Moreover, the addition of

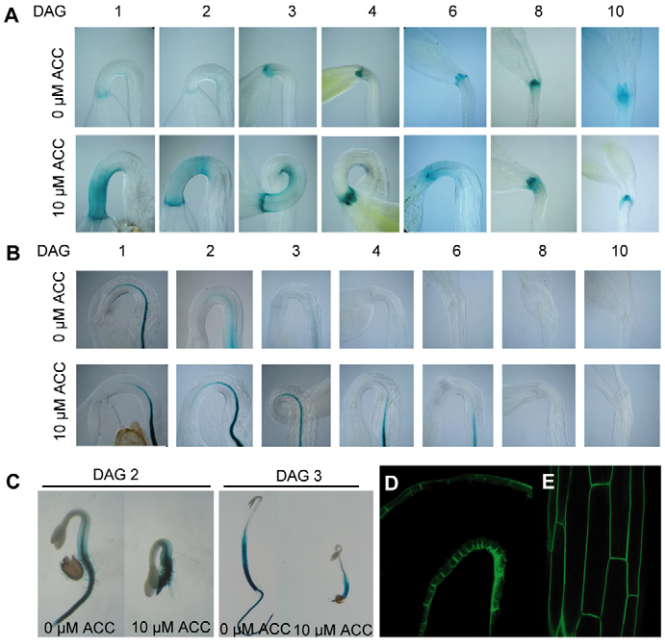

Fig. 6. Expression patterns of *AUX1* and *LAX3*. (**A**,**B**) Time line for *pAUX1::GUS* (A) and *pLAX3::GUS* (B) expression in the apical hook region in the presence and absence of 10 µM ACC. (**C**) Overview of *pLAX3::GUS* staining in 2- and 3-day-old seedlings (maintenance/exaggeration phase). (**D**) Membrane localization of AUX1-YFP in the hook. (**E**) Membrane localization of LAX3-YFP in the lower part of the hypocotyl.

ACC did not alter the expression pattern of *pSCR::H2B-YFP*; however, it did enhance the expression of *pAUX1::GUS* (Fig. 6A; see Fig. S8 in the supplementary material). LAX3 expression only changed at the opening phase and did not change significantly during the formation and maintenance phases. Therefore, a strict correlation between the AUX1 and LAX3 expression patterns and vascular maturation is absent.

The distribution of free IAA in hypocotyls is affected by auxin influx carrier mutations

The staining pattern of the auxin reporter *DR5::GUS* indicates an auxin maximum in the cotyledons when auxin influx is blocked by 1-NOA (Fig. 4A). This suggests an active auxin influx from cotyledons and meristems towards the hypocotyls. We aimed to verify whether the auxin response maximum on 1-NOA is indeed caused by an increase in auxin content in the cotyledons, and to determine whether the AUX1 and LAX3 auxin influx carriers are involved in regulating influx from the meristem and cotyledons into the hypocotyls. The auxin content was measured in cotyledons together with meristems and in hypocotyls of dark-grown 65-hour-old *Arabidopsis* seedlings. Wild-type seedlings had about twice as much auxin in the hypocotyls than in meristems and cotyledons (Fig. 7). This distribution was preserved upon an enhanced ethylene signal (10 µM ACC), the absolute values being almost doubled. This increase in auxin levels is probably due to the effect on the *TAR2* biosynthesis gene product (Fig. 2). However, when 1-NOA was present, or in the auxin influx carrier double mutant *aux1 lax3*, the ratio was reversed, with more auxin in the cotyledons and meristem than in the hypocotyls. On ACC, this effect was blocked, with no significant difference in auxin levels between the hypocotyls and the meristems and cotyledons.

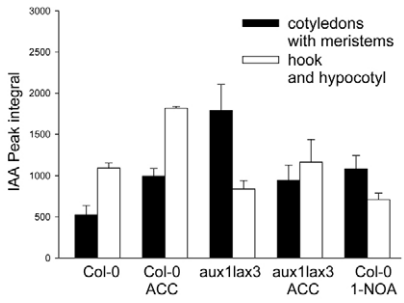

Fig. 7. Endogenous IAA content in cotyledons and hypocotyls treated with ACC or 1-NOA. IAA content is shown for Col-0, Col-0 treated with 10 μM ACC, *aux1 lax3*, *aux1 lax3* treated with 10 μM ACC and Col-0 treated with 30 μM 1-NOA. GC-MS/MS determination in 65-hour-old etiolated seedlings. Error bars indicate s.e.m. (*n*=4).

DISCUSSION

Local auxin levels regulate apical hook development

The plant hormone auxin is implicated in many processes that involve differential growth. These include tropisms, nastic movements and development of the apical hook in dicotyledonous seedlings. Unequal distribution of auxin leads to uneven elongation and hence to uneven movement or hook development. As is the case for roots, the control of elongation in the hypocotyl depends on the auxin concentration, with growth inhibition at supra-optimal concentrations, i.e. when concentrations surpass a threshold that defines the optimum (Evans et al., 1994; Vandenbussche et al., 2003). Supra-optimal levels of auxin are believed to block elongation via an ethylene effect. Indeed, many ACC synthase genes are upregulated by auxins (Abel et al., 1995; Tsuchisaka and Theologis, 2004). Yet, recent studies have also indicated that exogenous auxin can inhibit elongation independently of ethylene signaling (Stepanova et al., 2005; Stepanova et al., 2007).

During root gravitropism, auxin accumulates on the inner side of the curving root, causing inhibition of elongation (Friml et al., 2002; Ottenschläger et al., 2003; Swarup et al., 2005). In this respect, the situation in the apical hook is reminiscent of that in the roots, the auxin maximum being visible as DR5::GUS activity at the concave, inner side of the hook. A threshold of auxin is necessary to block cell elongation at the concave side of the hook. This threshold arises from the coordinate action of auxin biosynthesis and transport mechanisms (Figs 1-3). The occurrence of an auxin maximum on the concave side of the apical hook contrasts with the maximum that occurs on the non-illuminated (outer, convex) side of the hypocotyls in the region below the apical hook upon phototropic stimulus, and might indicate cell-type and developmentally specific fine-tuning of the auxin signal (Friml et al., 2002). Indeed, the hypocotyl can be divided into a 'hook zone' and a 'below-hook zone' based on auxin-related gene expression: particular genes can either be expressed only in the hook or below the hook. Examples of the former are *AUX1*, *IAA13*, *ARF2* and particular PIN genes, whereas likely examples of the latter are *SAUR-AC1*, *LAX3* and *PIN7* (Fig. 6) (Lehman et al., 1996; Li et al., 2004; Žádníková et al., 2010). Since the maximum auxin signal is located on the long side or the short side of the differentially elongating tissue depending on the conditions, it is likely that other signals modulate the auxin effect. Hormones, such as gibberellins and brassinosteroids, are known to influence hook development and hypocotyl elongation and are therefore likely candidates for the fine-tuning of auxin responses (De Grauwe et al., 2005; Vriezen et al., 2004). Moreover, interplay between the signaling pathways of these hormones has been demonstrated (Fu and Harberd, 2003; Vert et al., 2008).

Functional specification of auxin influx carriers

Genetic redundancy in the four-membered AUX-LAX family of *Arabidopsis* auxin influx carriers appears to be limited. Instead, auxin influx carriers adopt distinct developmental functions, as reflected in their expression patterns. AUX1, LAX1 and LAX2 proteins direct phyllotaxis, with the first two being the main players (Bainbridge et al., 2008), whereas LAX3 is not important in this process. By contrast, AUX1 and LAX3 proteins have additive functions in lateral root development, at least in part owing to their distinct spatial expression (Swarup et al., 2008). Our data indicate that AUX1 and LAX3 are the most important auxin influx carriers during apical hook development. Both AUX1-YFP and LAX3-YFP fusion proteins locate to cell membranes, consistent with their influx carrier function (Fig. 6D,E). The hook phenotype of loss-of-function mutants in these genes is additive, whereas they are expressed in different hypocotyl cells (Fig. 4B). Both proteins contribute to the correct kinetics of hook development in the wild type (Fig. 4D-F). LAX3 regulates the amplitude of hook bending during the maintenance phase irrespective of the presence of ethylene (Fig. 4). The expression of *LAX3* in the lower hypocotyl suggests a role in draining auxin from the hook towards the root, thus lowering auxin content in the hook and allowing differential auxin distribution to direct apical hook development. By contrast, AUX1 activity is strongly associated with an elevated ethylene signal. This corresponds to their differential expression in response to an enhanced ethylene signal, supporting their functional specification (Fig. 6A,B). Contrary to *LAX3*, *AUX1* expression is induced by ethylene until the hook opens. However, there is a synergy between these two influx carriers during the hook formation phase, as *aux1 lax3* double mutants display a reduced rate of hook formation, whereas single mutants do not (Fig. 4).

A variety of auxin control mechanisms are regulated by ethylene

There are numerous examples of how auxin and ethylene signals interact during *Arabidopsis* development (Lehman et al., 1996; Vandenbussche et al., 2003; De Grauwe et al., 2005; Ruzicka et al., 2007; Swarup et al., 2007; Stepanova et al., 2007). Depending on the developmental state and tissue type, each hormone reciprocally influences the biosynthesis of the other (Tsuchisaka and Theologis, 2004; Stepanova et al., 2005; Stepanova et al., 2008). In roots, ethylene positively controls the expression of two anthranilate synthases, which leads to inhibition of root elongation (Stepanova et al., 2005). Similarly, the importance of Trp aminotransferases in ethylene-related processes has been proven (Stepanova et al., 2008). A local increase in auxin content can also be achieved by regulating auxin transport. Ethylene has been shown to enhance the expression of several PIN genes and of *AUX1* in the root elongation zone (Ruzicka et al., 2007).

We found that ethylene can enhance the level of auxin in the upper part of the hypocotyl in three ways: (1) by local and differential induction of the Trp aminotransferase gene *TAR2*; (2) by induction of the flavin monooxygenase *YUC1* in the opening phase; and (3) by regulation of *AUX1*. The latter could enhance the influx of auxin from the meristem and cotyledons into the hypocotyl. In the apical hook region, ethylene induces *AUX1* promoter activity (Fig. 6A) and affects AUX1 protein distribution (see Table S1 in the supplementary material). Differential FRAP rates at the concave and

convex sides of the hook in the presence of ACC suggest a faster turnover of AUX1 on the concave side. This presents a remarkable parallel with the regulated turnover of the auxin efflux-regulating PIN proteins (Abas et al., 2006; Kleine-Vehn et al., 2008). Cells in the concave region of the hook need to refrain from elongation, and a higher turnover of AUX1 could help to transport auxin from the cotyledons to the concave side of the hook, where the auxin maximum occurs in the presence of an ethylene signal, as seen in *DR5::GUS* seedlings (Fig. 3).

Thus, basal auxin production in the hook resulting from TAA1 activity, and additional accumulation caused by TAR2 and AUX1 activities, shape the apical hook upon an enhanced ethylene signal. Hook opening might be controlled by the differential synthesis of auxins, at least in part controlled by YUC1 at the convex side (see Fig. S1 in the supplementary material).

Auxin distribution during apical hook development

Based on auxin measurements (Fig. 7) and on the localization of the auxin maximum, we propose a model for auxin flow during hook development (see Fig. S9 in the supplementary material). Although the importance or extent of diffusive auxin influx cannot be estimated, the auxin flow is clearly dependent on carrier-mediated influx. The expression patterns of AUX1 and LAX3 (Fig. 6), and the auxin distribution in the double mutant (Fig. 7), support the following model (see Fig. S9 in the supplementary material): AUX1 helps to move auxin from cotyledons and meristem into the hook and LAX3 contributes to the movement out of the hook and towards the root. Consequently, the functional disruption of both proteins leads to an accumulation of auxins in the cotyledons and upper hypocotyl.

Auxin influx is not the only transport mechanism involved, as auxin requires PIN proteins to efficiently exit the cells. Indeed, blocking auxin efflux leads to a stronger phenotype than blocking active influx, which indirectly supports the involvement of diffusion in addition to active influx (Žádníková et al., 2010) (see Fig. S3 in the supplementary material). Passive diffusion into the cells might be responsible for the milder hook phenotype that is seen when active auxin influx is blocked as compared with the full reversal of the phenotype upon treatment with auxin efflux inhibitors. The efflux carriers that are responsible for directing auxin flow in the apical hook have been characterized, among which PIN3 plays a predominant role (Friml et al., 2002; Žádníková et al., 2010), although additional PIN proteins also appear to be involved (Žádníková et al., 2010).

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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Table S1. Quantification of FRAP analysis of AUX1-YFP fluorescence at the concave and convex sides of the hook

Control			10 μM ACC		
Concave	Convex	Concave/convex	Concave	Convex	Concave/convex
25.42	14.96	1.70	22.33	3.63	6.16
11.21	14.37	0.78	14.42	11.34	1.27
17.25	24.75	0.70	32.08	23.99	1.34
26.12	27.66	0.94	44.09	20.53	2.15
17.41	33.49	0.52	30.25	12.61	2.40
38.86	36.01	1.08	20.91	19.05	1.10
9.78	22.48	0.43	29.18	8.20	3.56
			31.18	24.07	1.30
			30.84	18.44	1.67
			21.48	28.29	0.76
			28.87	18.77	1.54
			24.06	17.32	1.39

	Number of hooks with faster fluorescence recovery at concave side (%)	Number of hooks with faster fluorescence recovery at convex side (%)
Control	28.6	71.4
10 μM ACC	91.6	8.4

Relative fluorescence values after 10 minutes of FRAP subtracted from the fluorescence directly after bleaching (percentage of the initial fluorescence) in control and ACC-treated seedlings. These values reflect the rate of fluorescence recovery (i.e. how much of the fluorescence recovers). Ratios of these values between the concave and convex sides (reflecting the difference in fluorescence recovery rates) were calculated for each individual seedling. On average, the fluorescence recovery of AUX1-YFP fluorescence after exposure to ACC was twice as fast on the concave side of the hook (ratio 2.05) in comparison with the control (ratio 0.88). The test of equality of variances indicated that the variance of control and ACC-treated seedlings were significantly different ($F=12.24$, $P=0.003$). Therefore, a two-sample *t*-test that does not assume equal variances was performed. The mean ratio for ACC-treated seedling (M=2.05; s.d.=1.43; $n=12$) was significantly higher than the mean ratio of control plants (M=0.88; s.d.=0.39; $n=7$) using the two-sample *t*-test for unequal variances [$t(14)=2.56$, $P\leq0.005$].