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Light triggers PILS-dependent reduction in nuclear auxin signalling for growth transition

Chloé Béziat^{1,2}, Elke Barbez^{1,2}, Mugurel I. Feraru¹, Doris Lucyshyn¹, and Jürgen Kleine-Vehn^{1,2,*}

¹Department of Applied Genetics and Cell Biology, University of Natural Resources and Life Sciences (BOKU), Muthgasse 18, 1190 Vienna, Austria

²Department of Plant Systems Biology, VIB and Department of Plant Biotechnology and Bioinformatics, Ghent University, Technologiepark 927, 9052 Gent, Belgium

Abstract

The phytohormone auxin induces or represses growth depending on its concentration and the underlying tissue type. However, it remains unknown how auxin signalling is modulated to allow tissues transiting between repression and promotion of growth. Here we used apical hook development as a model for growth transitions in plants. A PIN-FORMED (PIN)-dependent intercellular auxin transport module defines an auxin maximum that is causal for growth repression during the formation of the apical hook. Our data illustrates that growth transition for apical hook opening is largely independent of this PIN module, but requires the PIN-LIKES (PILS) putative auxin carriers at the endoplasmic reticulum. PILS proteins reduce nuclear auxin signalling in the apical hook, leading to the de-repression of growth and the onset of hook opening. We also show that the phytochrome (phy) B-reliant light-signalling pathway directly regulates PILS gene activity, thereby enabling light perception to repress nuclear auxin signalling and to control growth. We propose a novel mechanism, in which PILS proteins allow external signals to alter tissue sensitivity to auxin, defining differential growth rates.

Keywords

Auxin; growth; apical hook; PIN-LIKES; light perception; phyB

The phytohormone auxin has a central role in plant growth regulation. Most auxin responses require its nuclear receptors TIR1/AFBs, which interact with the co-receptor Aux/IAAs in

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*Correspondence should be addressed to Jürgen Kleine-Vehn (juergen.kleine-vehn@boku.ac.at).

Contributions

C.B. carried out most of the experiments; J.K.V. and C.B. interpreted the results and designed experiments. E.B. cloned most constructs and contributed to the statistical analysis. M.F. genotyped *pils* loss of function mutant and crosses. D.L. and C.B. performed ChIP and qPCR experiments. J.K.V. and C.B. wrote the manuscript. All authors saw and commented on the manuscript.

Competing interests

The authors declare no competing financial interests.

Data availability

The underlying data of this study is available upon request.

an auxin-dependent manner. The subsequent degradation of Aux/IAA releases ARF transcription factors, initiating auxin-dependent gene regulation 1,2. Auxin exerts seemingly distinct responses in plant tissues, preferentially promoting growth in aerial parts, but repressing root organ growth 3. Exogenous application of auxin supported a bi-phasic role of auxin, proposing that low auxin levels stimulate, whereas high doses repress tissue expansion 4. As such, auxin is central in most if not all differential growth process.

The apical hook is a dark-grown, protective structure crucial for a seedling's emergence from the soil, and is an ideal model for studying differential growth regulation. The apical hook initially displays an auxin-dependent asymmetric growth repression in the inner (concave) side, leading to its form (formation phase). Afterwards growth in the hook is halted (maintenance phase) until a subsequent, light sensitive growth-promotion machinery in the very same tissue initiates the opening phase 5–7. It has been demonstrated that polar, PIN-FORMED (PIN)-dependent intercellular auxin transport focuses auxin at the inner site of the hook, leading to an auxin signalling maximum and differential growth repression 7,8. It is currently unknown how the induction of growth during apical hook opening is molecularly controlled, but high auxin levels seem to inhibit apical hook opening 9–11. Accordingly, the apical hook is certainly an interesting model to study how tissues transit from repression to promotion of growth.

Besides the rather detailed insights into auxin metabolism, signalling and intercellular transport 1,2,12–18, little is known about intracellular auxin transport and its developmental importance. We previously identified the PIN-LIKES (PILS) putative auxin-carrier family in *Arabidopsis thaliana* 19. PILS2 and PILS5 reside at the endoplasmic reticulum (ER) where they increase intracellular accumulation of auxin in plant cells and heterologous in yeast cells. PILS action negatively impacts nuclear auxin signalling, presumably by limiting auxin diffusion into the nucleus 19,20. Previous work has led to the paradigm that intercellular auxin transport forms auxin maxima to control differential growth 12. Here we show, using the apical hook, that growth transition from repression to promotion is accompanied by a PILS-dependent reduction in nuclear auxin signalling. Moreover, we show that *PILS* expression is under the control of the photoreceptor phytochrome B (phyB), allowing light to dynamically repress auxin signalling for growth regulation.

Results

Intercellular auxin transport machinery preferentially impacts on apical hook formation

PIN-dependent intercellular, polar auxin transport provides a common module for differential accumulation of auxin and seems central to differential growth regulation 12. Previous work has revealed that PIN-dependent auxin transport also forms an asymmetric auxin signalling maximum in the inner side of apical hooks, initiating their formation 7,8. Following the formation of the apical hook, a light-sensitive pathway is able to de-repress growth, leading to apical hook opening 21. PIN1, PIN3, PIN4 and PIN7 redundantly control the apical hook development and the respective single *pin* mutants are all defective in the formation of the apical hook. It remains however unknown whether the PIN intercellular transport module is also developmentally important for the growth transition during apical hook opening. To address this question, we used time-lapse imaging of dark grown *pin*

mutants and exposed them to a light stimulus to induce the onset of opening. We focused on *pin3/pin4* single and *pin3 pin4* double mutants, because their impact on hook development was most severe 7. Even though these mutants show defects in apical hook formation 7, light-induced opening kinetics were largely indistinguishable from wild types (WT) (Supplementary Fig. 1a-b; see also Supplementary Fig. 2a). This finding suggests that PIN3 and PIN4 proteins are required for the formation of the apical hook, but do not play a primary role in light-induced apical hook opening. Accordingly, we assume that apical hook formation and opening are molecularly distinct.

***PILS2* and *PILS5* show asymmetric expression during apical hook development**

To assess whether intracellular auxin transport processes may function in differential growth control, we initially assessed the promoter activity of *PILS* genes in apical hooks, using transcriptional fusions of *PILS* promoter sequences (*pPILS*) with a β -glucuronidase (GUS) and green fluorescent protein (GFP) reporter fusion. A nuclear localisation signal (NLS) was additionally added to the GUS-GFP fusion to prevent intercellular diffusion (through plasmodesmata) of the reporter. When kept in the dark, the apical hook undergoes the defined formation, maintenance and opening phases 6,7. We detected the expression of *pPILS2:GUS-GFP*, *pPILS3:GUS-GFP*, and *pPILS5:GUS-GFP* during these three phases of the apical hook development (Fig. 1a). *PILS2* promoter activity was visibly asymmetric, showing stronger expression in the inner side of the hook (Fig. 1a, c; Supplementary Fig. 3a). Notably, it showed relatively low expression during the formation phase and increased expression towards the opening phase (Fig. 1b). *PILS5* reporter displayed an asymmetrical expression pattern in dark-grown hypocotyls (Supplementary Fig. 3b), but the *PILS5* expression domain shifted shoot-wards into the apical hook region only during the opening phase (Fig. 1d, e). *pPILS3:GUS-GFP* showed strongest expression in inner cell layers of apical hooks (Fig. 1a) and also showed increased expression towards the opening phase (Fig. 1b). This data suggests a possible involvement of PILS putative intracellular auxin transporters in differential responses during apical hook development.

As mentioned above, PIN-dependent, intercellular auxin transport controls the formation of the apical hook. Intercellular auxin transport inhibitor 1-N-Naphthylphthalamic acid (NPA) severely blocks the formation of the apical hook, phenocopying multiple *pin* loss-of-function mutants 7. Notably, NPA treatments does not interfere with cellular PILS activity 20, suggesting indeed a specificity for intercellular auxin transport. Therefore, we used NPA to test whether intercellular auxin transport provides positional information for *PILS* expression. NPA application strongly reduced the expression of *PILS3*, whereas the *PILS5* expression domain even extended shoot-wards into the presumptive hook region (Supplementary Fig. 4a,b). This suggests that either auxin and/or hook curvature distinctly affect *PILS3* and *PILS5* expression. In contrast, *PILS2* promoter remained active in the presumptive site of apical hook formation in the presence of NPA (Supplementary Fig. 4a,b), suggesting that *PILS2*-decorated apical hook identity is in part independent of intercellular auxin transport. Notably, *PILS2* expression lost its asymmetry upon NPA application (Supplementary Fig. 4a, b), suggesting that intercellular auxin transport provides positional cues to establish asymmetric expression of *PILS2*. Based on this data, we assume that the

regulation of *PILS2*, *PILS3* and *PILS5* expression is partially diverged, with only *PILS2* being a stable marker for apical hook identity.

PILS proteins impact on apical hook development

To assess the functional importance of PILS proteins for apical hook development, we used infrared-based imaging 6,7 which enabled us to record the growth of seedlings in the dark. As mentioned before, dark conditions induce the temporally-defined formation, maintenance and (slow) opening phases of apical hooks 6,7. Compared to WT seedlings, *pils2* knockdown mutants 19 showed a slightly delayed onset of growth for hook opening, but later opening kinetics were not distinguishable from wild types (Supplementary Fig. 5a; see also Supplementary Fig. 2b). The *pils5* knockout mutants on the other hand were not defective in apical hook development (Supplementary Fig. 5a). In contrast, the *pils2 pils5* double mutants showed a strongly (9-12 hours) delayed onset of apical hook opening (Fig. 2a, b; Supplementary Fig. 5a). This data confirms the functional redundancy of PILS2 and PILS5 in plant development 19 and indicate a role of PILS proteins in inducing growth during apical hook opening. To also address whether PILS3 affects apical hook development, we analysed a hypomorphic *pils3-1* mutant allele (Supplementary Fig. 6a-c). *pils3* mutants also displayed a prolonged apical hook maintenance phase (Supplementary Fig. 7a, b), which was complemented by *pPILS3:PILS3-GFP* (Supplementary Fig. 6d). Accordingly, both *pils3* and *pils2 pils5* similarly impact on apical hook development, suggesting further functional redundancy. However, *pils2 pils3 pils5* triple mutants did not show enhancement of the apical hook opening phenotypes (Supplementary Fig. 5b). This could either hint at partially diverged function of distinct PILS proteins during apical hook development or reflect additional functional redundancy in the pathway.

These overall findings reveal that PILS proteins are required for apical hook development. We subsequently assessed PILS overexpression and its impact on the apical hook development. Representative *p35S:PILS3-GFP* and *p35S:PILS5-GFP* expressing lines showed a dramatically faster opening of apical hooks, suggesting a faster transition to growth promotion (Fig. 2b; Supplementary Fig 7b). Notably, *PILS2* overexpression seems to be less tolerated compared to *PILS3* and *PILS5* in *Arabidopsis* seedlings, because we obtained only lines that weakly express *p35S:PILS2-GFP*. In agreement, these lines induced only a weak apical hook opening phenotype in the dark (Supplementary Fig. 8a, b). Despite some possible divergence (e.g. gene regulation or protein abundance), PILS2, PILS3 and PILS5 appear to fulfil a similar function for inducing growth in apical hooks.

Next we assessed whether PILS-dependent apical hook development could be due to an indirect impact on intercellular auxin transport. We did neither observe an exaggeration of the apical hook nor any other defects in the formation phase of the apical hook in *pils* loss-of-function lines. This finding implies that intercellular auxin transport is not notably affected in *pils* mutants, because this stage is sensitive to interference with intercellular auxin transport 7. *PILS5* overexpression on the other hand did not affect the initial formation kinetic of apical hooks, but prevented its full closure, triggering a premature opening in most seedlings. To further assess whether PILS indirectly affect intercellular auxin transport and thereby growth, we used ethylene, because its exogenous application prolongs the formation

phase of apical hooks in a PIN-dependent manner 7,8. Ethylene still prolonged the formation phase in *p35S:PILS5-GFP* and also in *p35S:PILS3-GFP* expressing seedlings (Supplementary Fig. 9a-c), confirming that intercellular transport is not compromised in these lines. Even though we did not directly assess PIN protein activity in *pils* mutants, our data collectively suggests that PILS proteins have a specific role in guiding the growth induction for apical hook opening.

PILS proteins determine the depletion of nuclear auxin signalling during apical hook opening

An auxin-signalling maximum is progressively established at the concave (inner) side of forming apical hooks 7. We subsequently addressed the impact of PILS activity on nuclear auxin signalling during apical hook development, using the nuclear auxin response marker *pDR5:GFP22*. Compared to dark grown WT seedlings, *pils2 pils5* double mutants displayed higher nuclear auxin signalling during apical hook maintenance and opening phases (Fig. 2c, d). Similar phenotypes were observed with *pils3* mutants, also displaying elevated auxin signalling in the apical hook (Supplementary Fig. 7c, d). Conversely, *PILS3* and *PILS5* overexpression reduced auxin signalling during apical hook maintenance and opening when kept in the dark (Fig. 2e, f; Supplementary Fig. 7e, f). These findings reveal that high and low auxin signalling correlate with delayed and faster hook opening in dark grown seedlings, respectively.

Light induced PILS expression reduces nuclear auxin signalling

Here we show that a reduction in auxin signalling coincides with growth promotion in dark grown apical hooks. We hence hypothesize that PILS negatively impacts nuclear auxin signalling, which presumably triggers a growth transition and apical hook opening. As mentioned before, apical hook opening is triggered by light exposure 21, which also accelerates the actual opening speed (Fig. 3a). Hence, we subsequently used light to further investigate whether the reduction in auxin signalling is causal for growth induction. Accordingly, we tested whether light impacts on *PILS* genes and observed light-induced expression of *PILS2* and to a lower extent also of *PILS3* in apical hooks (Fig. 3b, c). In contrast, light exposure caused repression of *PILS5* in the hypocotyl, but *PILS5* expression was specifically maintained in the apical hook region (Supplementary Fig. 10). Moreover, light did not only induce *PILS2* and *PILS3* expression, but also repressed DR5-based, nuclear auxin signalling in the apical hook (Fig. 3b, c). To assess whether PILS activity is required for light-induced changes in auxin signalling, we exposed dark-grown, *pDR5:GFP*-expressing WT and *pils* mutants to a light stimulus. The light-induced repression of nuclear auxin signalling was reduced in *pils2 pils5* double mutants (Fig. 4a, b) and enhanced in the *PILS5* overexpressing lines (Fig. 4c, d). Consequently, we conclude that the regulation of *PILS* expression implements the light-induced repression of auxin signalling.

Light signalling utilizes PILS proteins to induce growth in apical hooks

Our data suggests that light signalling utilizes *PILS* genes to repress nuclear auxin signalling for apical hook opening. Next we assessed whether light perception indeed exploits this PILS-dependent mechanism to promote differential growth. Accordingly, we exposed dark-grown seedlings, displaying fully closed apical hooks (early maintenance phase), to a

constant light stimulus. As expected, light triggered the rapid opening of the apical hook in WT seedlings (Fig. 4e,f), but this response was strongly delayed in *pils2 pils5* (Fig. 4f) and *pils3* (Supplementary Fig. 11a,b). Next we assessed the effect of *PILS5* overexpression on apical hook opening. To directly compare the opening kinetics to wild type, we only considered individual *PILS5* overexpressing seedlings showing a closed apical hook during early stages of apical hook maintenance. In agreement with a faster decay in auxin signalling, the light response was strongly accelerated in constitutive *PILS5* lines (Fig. 4e,f). *PILS2* and *PILS3* activity also clearly affected apical hook opening response to light (Supplementary Fig. 8c; Supplementary Fig. 11a, b). These findings suggest that a reduction in auxin signalling results in growth induction during apical hook opening.

Phytochrome B pathway utilizes PILS proteins to initiate growth in apical hooks

Our data indicates that light-triggered PILS expression initiates a reduction in auxin signalling and consequently a growth transition during apical hook development. Consequently, we turned our attention to the molecular mechanism by which light can affect *PILS* gene activity. Phytochrome B (phyB)-dependent light perception has been implicated in apical hook opening 21. Upon light stimulation, phyB represses a subgroup of basic helix-loop-helix (bHLH) transcription factors, termed Phy-Interacting-Factors (PIFs), that suppress photomorphogenic responses 23. Accordingly, we assumed that this pathway might also contribute to light-controlled PILS activity. To test this, we analyzed *PILS2* and *PILS3*, because they showed the most pronounced light-induced expression. In accordance with our assumptions, PIF5 overexpression caused transcriptional repression of *PILS2* and *PILS3* (Fig. 5a-d). *PILS2* and *PILS3* genes are potential candidates to function directly downstream of PIFs, since their promoters display *in silico* predicted, putative PIF binding sites (Supplementary Fig. 12a). Correspondingly, chromatin immunoprecipitation (ChIP) of PIF5:HA revealed the enrichment of PIF5 binding at the *PILS2* and *PILS3* promoters (Fig. 5e). This suggests that PIF5 is a direct repressor of *PILS2* and *PILS3* transcription.

To further consolidate the role of PILS proteins in photomorphogenesis, we addressed their genetic interaction with the phyB/PIF5-signalling pathway. Single time point analysis of *phyB* mutants suggested a delay in apical hook opening 21, which was also phenocopied by PIF5 overexpression 9. Intriguingly, apical hook growth kinetics of *phyB* (Fig. 6a) and PIF5^{OX} (Fig. 6b,c) were reminiscent of reduced PILS activity. Conversely, the *pif5* loss-of-function exhibited an earlier opening of dark-grown apical hooks 9 (Fig. 6d). That being the case, we tested whether de-repression of *PILS* affects the prolonged hook maintenance phenotype in *phyB* and PIF5^{OX}. The constitutive expression of *PILS3* or *PILS5* induced an earlier onset of apical hook opening in *phyB* and PIF5^{OX} (Fig. 6a-c). This finding suggests that the repression of *PILS* could be causal for growth repression in *phyB* and PIF5^{OX} in apical hooks. In agreement, *pils3 pif5* double mutants showed prolonged apical hook maintenance compared to *pif5* single mutants (Fig. 6d). This finding indicates that *PILS* indeed function downstream of PIF5.

We noted, however, that the initial opening, but not the later opening kinetic itself was strongly influenced by PILS activity. We hence assume that the initial onset of light-induced differential growth is modulated by PILS activity. However, the subsequent phyB/PIF-

dependent opening speed for apical hooks (Fig. 6a-d) appears to be modulated by additional factors, suggesting at least two distinctly controlled phases of apical hook opening.

Our data reveals that light-induced release of PIF5 triggers *PILS* expression, which presumably increases its auxin carrier activity at the ER. Consequently, PILS activity reduces nuclear auxin signalling rates, initiating growth for the onset of apical hook opening (Fig. 6e).

Discussion

PILS proteins directly impact on nuclear auxin signalling rates

Auxin is known to be a positive and negative regulator of growth, depending on its concentration and the respective tissue type 3. However, it remains enigmatic how tissues transit from auxin-dependent repression to promotion of growth. We used apical hook development to assess such fundamental questions. During apical hook formation the PIN-dependent intercellular auxin transport machinery focuses auxin at the inner side, leading to an auxin-signalling maximum required for growth repression 7,8. *pin3* and *pin4* mutants show a strong impact on apical hook development, but still respond to light induced apical hook opening. This data suggests that PINs do not play a major role in apical hook opening. However, the reduction in clathrin-dependent internalisation of PIN3 from the plasma membrane enhances the auxin maximum during apical hook formation and also causes a delay in hook opening 11. Hence, other conditions may modulate intercellular auxin transport to temporally prolong the auxin signalling maxima and consequently the maintenance of the apical hooks. On the other hand, our data indicates that PILS putative intracellular auxin carriers actively reduce the rate of auxin signalling, which is the actual trigger for hook opening. This implies that PILS proteins counterbalance the PIN-dependent auxin maxima to induce growth. Relatively little is known about intracellular auxin transport processes and how it impacts plant development. We have previously shown that PILS putative auxin carriers reside at the endoplasmic reticulum, where they induce intracellular accumulation of auxin 19. PILS proteins restrict nuclear auxin signalling 19,20, presumably by retaining auxin in the ER and consequently limiting its diffusion into the nucleus. Our novel data enforces a role of PILS proteins beyond its impact on auxin homeostasis 19, revealing a developmentally important function in differential growth regulation in *Arabidopsis*. This PILS-dependent growth mechanism integrates environmental signals, such as light, to trigger morphogenetic responses. Using light-induced apical hook opening we could show that PILS putative intracellular auxin carriers indeed directly impact on nuclear auxin signalling. During the formation of the apical hook, the asymmetric increase in auxin signalling triggers the inhibition of growth. Subsequently, PILS-induced reduction of nuclear auxin signalling leads to the de-repression of growth, initiating apical hook opening. This model is also in agreement with previous assumptions that an auxin-like molecule inhibits light induced apical hook opening in beans 10.

Besides the overall role of PILS2, PILS3 and PILS5 in promoting growth for apical hook opening, we observed deviation in tissue distribution and overall gene regulation between *PILS2*, *PILS3*, and *PILS5*. Such deviations could theoretically imply distinct developmental functions for these genes. In this scenario, it is noteworthy that *pils2 pils3 pils5* triple

mutants did not enhance the *pils2 pils5* phenotype. However, it remains to be addressed whether this is due to the roles of these genes in apical hook development being partly independent. On the other hand, there could be a more complex functional redundancy among PILS genes, which has been for example reported for multiple *pin* mutants 24. Here the loss of certain *PINs* leads to an ectopic upregulation of other family members. It is of course also still conceivable that other pathways could compensate for the loss of multiple *PILS* genes.

phyB/PIF-dependent light perception utilizes PILS genes to repress auxin signalling

Our data suggests that the phyB/PIF5 light perception module directly impacts on *PILS* transcription and utilizes its activity to alter nuclear auxin signalling. The phyB/PIF signalling circuit is well known to trigger auxin-reliant growth during shade avoidance responses (reviewed in 25). Shade conditions trigger PIF-dependent transcription of *YUCCA* 26, *TAA1* and *CYP79B2* 27 auxin biosynthesis genes, leading to high auxin levels. Concomitantly, PIN-dependent auxin transport is increased, which leads to the induction of growth in hypocotyls 26,28. Hence, the phyB/PIF-dependent shade avoidance response is mechanistically distinct from the light induced opening of apical hooks, where a PILS-dependent decrease in auxin signalling initiates tissue expansion. Here we suggest that PIF5 functions as a transcriptional repressor on *PILS* genes. Accordingly, light-dependent activity of phyB (and subsequent repression of PIFs) seems decisive for both low and high auxin signalling rates by its inverse control of *PILS* (this study) and auxin biosynthesis genes 27 26, respectively. Accordingly, it is tempting to speculate that PILS genes, besides their role in apical hook development, could have a more general function in phyB responses. Recently, phyB signalling has been shown to contribute to shoot branching by reducing auxin signalling 29. Even though it is not known how phyB defines auxin sensitivity during shoot branching, this finding suggests that possibly tissue-specific, downstream components have distinct effects on auxin signalling 29. Hence, it is now an emerging question whether PILS proteins also contribute for example to shoot branching in a phyB-dependent manner or whether de-repression of PILS genes would affect shade avoidance responses.

Besides the important light-dependent transcriptional control of *PILS2* and *PILS3*, there could be also an additional level of regulation. We noticed that *PILS* overexpressing lines showed accelerated hook opening in the light. This was particularly striking in *PILS2* overexpressing lines, which displayed a much stronger opening phenotype in light compared to dark-grown seedlings. This aspect certainly requires further investigation, but it is tempting to speculate that light could also affect PILS activity in a post-transcriptional manner. Strikingly, the protein kinase WAG2 functions also downstream of PIF5 and is defective in apical hook opening⁹. In contrast to PILS proteins, WAG2 is a negative regulator of hook opening and it seems to function in maintaining high auxin signalling levels. It would be interesting to investigate whether WAG2 and PILS proteins interact during apical hook opening.

PILS genes as a versatile switch for tissue sensitivity to auxin

It is largely unknown how plant tissues switch between opposing growth responses. Here, we propose a novel regulatory mechanism in plants; one that appears to impact on growth

transition by modifying the cellular sensitivity to auxin. Our previous data indicates that PILS proteins affect intracellular accumulation of auxin at the ER, where, we presume, they are well positioned to restrict auxin diffusion into the nucleus 16,17. Accordingly, the presence of PILS proteins would reduce the probability of an auxin molecule meeting its nuclear receptor, ultimately reducing nuclear auxin signalling rates. Exogenous application of auxin suggests that low auxin levels induce, whereas higher auxin concentration to repress, tissue expansion in a cell type-dependent manner 4,30. Our data illustrate that PILS-induced reduction in nuclear auxin signalling allows cells to progress from auxin-triggered repression (relatively high signalling) to promotion (relatively low signalling rates) of growth (Fig. 6e). Accordingly, we conclude that PILS-dependent intracellular auxin transport not only contributes to cellular auxin homeostasis 19, but also initiates alterations in auxin signalling rates for differential growth control. We moreover suggest that external signals, such as light, utilize this cellular mechanism, switching tissue sensitivity to auxin for plant growth regulation. This machinery guides growth for apical hook opening, but we consider it could have additional, widespread importance for plant development, allowing the tissue specific formation of auxin signalling minima.

Material and methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia 0 (Col-0 or WT) was used for all experiments. The following lines and constructs have been described previously: *pif5* 9, *p35S::PIF5:HA* 23, *phyB* 31, *pils2-2* (SALK_125391), *pils5-2* (SALK_072996) 19, *pils2-2 pils5-2* 19, *pDR5rev::GFP*; *pDR5rev::GUS* 22, *pDR5rev::mRFP1er* 32, *pils2pils5 pDR5rev::GFP*, *p35S::PILS5:GFP pDR5rev::mRFP1er* 19, *pin3-5*, *pin4-3*, *pin3-5 pin4-3* 24. The following mutant line was obtained from the Nottingham *Arabidopsis* Stock Centre (NASC): *pils3-1* (SALK_049515). *T-DNA* insertion site was verified, homozygous line selected and the decrease of the *respective PILS3* transcript was shown by semi quantitative and quantitative RT-PCR. Gateway cloning was used to construct *pPILS1-7::GFP:GUS-NLS* (primer sequences in Supplementary table 1), *p35S::GFP:PILS3*, *p35S::GFP:PILS2* and *pPILS3::PILS3:GFP* as described 19. Multiple mutants and marker lines have been generated by crossing. Plants were grown under long-day (16 h light/8 h dark) conditions at 20 °C. Seedlings were grown on ½ Murashige and Skoog (MS) *in-vitro* plates supplemented with 1% sucrose and 1% agar. For the depicted treatments, the MS medium was supplemented with 5 µM 1-aminocyclopropane-1-carboxylate (ACC) or 5 µM N-(1-naphthyl)phtalamic acid (NPA). Control plates were supplemented with water (for ACC treatment) or solvent DMSO (NPA treatment). The seeds were stratified at 4 °C for 2 days in the dark and then were exposed to 8h of light (22°C) and subsequently grown vertically in the dark (20°C) until respective time points. For light-induced apical hook opening experiments, 3 days dark-grown seedlings (in apical hook maintenance phase) were exposed to constant low light (4 µmoles/m²/s) for a duration of time indicated in figure legends.

GUS staining analysis and quantification

Dark-grown seedlings were collected during the formation, maintenance and opening phases of apical hook development. Apical hooks in maintenance phase were exposed to light for

indicated time points. Fixation step, histochemical GUS staining, clearing and rehydration steps were performed as indicated 33. The rehydrated seedlings were mounted in chloralhydrate for analysis by light microscopy (Leica DM 5500) equipped with a DFC 300 FX camera (Leica). The intensity of the staining was quantified as described in 33 in a region of interest (ROI) kept constant or depicted along a line. Number of analysed seedlings per line and condition are given in the figure legends; experiments were repeated at least 3 times and a representative experiment is shown.

Real time analysis of apical hook development

Seedlings were grown in a light-sealed box equipped with an infrared light source (880 nm LED) and a spectrum-enhanced camera (EOS035 Canon Rebel T3i) modified by Hutech technologies with a built-in, clear, wideband-multicoated filter. The camera was operated by EOS utility software. For light exposure experiments, 40 hours after germination (hooks in maintenance phase) the box was opened and a light source (4 $\mu\text{moles/m}^2/\text{s}$) was placed in front of the plate. Angles between the cotyledons and the hypocotyl axis were measured every 3 hours in dark and every hour during light exposure until complete opening using ImageJ (<http://rsb.info.nih.gov/ij/>) software. The outer angle of the apical hook is reported in the graphs. Representative experiments are shown. At least 3 independent experiments were performed and sample size is indicated in the figure legends.

Confocal imaging analysis

Dark-grown *pDR5::GFP/RFP* seedlings in WT and different mutant backgrounds were imaged during the formation phase (15h), maintenance phase (40h), opening phase (60h) as well as after 1, 2 and 3 hours of light exposure (on apical hook in maintenance phase (40h)) using a SP5 Leica DM6000 confocal laser scanning microscope equipped with a 20.0x1.25 WATER objective. GFP (excitation 488 nm and emission 500 nm - 546 nm) and RFP (excitation 561 nm and emission 574 nm – 618 nm) were quantified on 3D maximum projection obtained from a Z-stack series of pictures taken in the inner side of the apical hook using the Leica software LAS AF 3.1. The same region of interest (ROI) was defined for each individual seedling. Sample size is given in the figure legends and the data was confirmed in 3 independent experiments. Representative experiments are shown.

Chromatin immunoprecipitation and quantitative real time PCR (qPCR)

Chromatin immunoprecipitation (ChIP) was performed as described 34 with minor modifications. WT and *p35S::PIF5:HA* seedlings were grown in the dark for 6 days at 20 °C. 3 grams of dark grown hypocotyls were harvested and crosslinked with a solution of 1% formaldehyde. DNA sonication was performed using a bioruptor (Diagenode) to achieve a fragmentation of approximately 0.3-0.8 kb. For each sample an input sample was kept and not used for ChIP. ChIP was done using HA-tag antibody ab9110 from Abcam (1 μl for 25 μg of sonicated chromatin) and magnetic beads (Dynabeads Novex ThermoFisher scientific) coated with Protein A. The binding of the antibody to the beads was done at 4 °C for 4 hours and the chromatin binding to the antibody overnight at 4 °C.

After several washes, the complexes were eluted with a mixture 1/3 of Tris1M pH9 and Glycine Buffer (100mM Glycine-HCl pH 2.8, 500mM NaCl, 0.05% Tween 20). Release of

crosslinking was performed by adding Proteinase K overnight at 37 °C followed by a heating step at 65 °C for 8 hours. DNA was purified by phenol-chloroform extraction and recovered by ethanol precipitation in the presence of 1 µg of glycogen. Precipitated DNA was analysed by qPCR. qPCR analysis was performed using a C1000 Touch Thermal Cycler equipped with the CFX 96 Real Time System (BioRad) and with the iQ SYBR Green Supermix (BioRad). qPCR was carried out in 96-well reaction plates heated for 3 min to 95 °C to activate hot-start *Taq* DNA polymerase, followed by 40 cycles of denaturation for 10 s at 95 °C, annealing for 30 s at 55 °C and extension for 30 s at 72 °C. Sequences of primers used for amplifying the G boxes of *PILS2* and *PILS3* promoters (detailed promoters analysis in Supplementary Fig. 12a), the *WAG2* positive control 9 and the negative *FT* control 35 can be found in Supplementary table 1. To evaluate the relative enrichment, the percentage of input was calculated by making a Ct difference in between the input and the respective ChIP sample (Ct) and then according to the method 2^{-Ct} 36. 3 independent experiments were performed.

Statistical analysis

One-way ANOVA followed by Tukey's test was performed in case of multiple columns comparisons procedure. Two-way ANOVA followed by Bonferroni post-hoc test was carried out to compare 2 different genotypes at different time points or treatments. Apical hook kinetics have been statistically analysed by non-linear regression and a subsequent Extra Sum of squares F test. (More information can be found in Supplementary Fig. 2). All statistical analysis were performed with GraphPad (<https://www.graphpad.com/scientific-software/prism/>).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *PILS* expression during apical hook development.

a, Expression patterns of *pPILS1-7::GUS* during the formation, maintenance and opening phases. Scale bars, 100 μ m. **b**, Quantification of GUS expression of *pPILS2::GUS-GFP* (left panel) and *pPILS3::GUS-GFP* (right panel) expressing seedlings during formation, maintenance and opening phases (n=10). The region of quantification considered was the apical hook zone (inner and outer sides). **c**, Confocal imaging of asymmetric *pPILS2::GUS-GFP* signal in apical hooks in maintenance phase. Scale bar: 100 μ m. **d**, Confocal imaging of *pPILS5::GUS-GFP* during the 3 phases. The white rectangle delimits the region of

interest (ROI) used for quantification which depicts spreading of the signal into the apical hooks during opening. **e**, Quantification of mean grey values of *pPIL55* activity in the depicted ROI. The formation, maintenance and opening phases correspond to 15, 40 and 80 hours after germination, respectively. Error bars represent standard error of the mean. Statistical significance was evaluated by one-way ANOVA followed by multiple comparisons Tukey's test. Distinct letters indicate statistically significant differences ($p < 0,05$).

Figure 2. PILS2 and PILS5 requirements for apical hook development.

a, Representative pictures of apical hooks in dark-grown wild type (WT), *PILS5^{ox}* and *pils2 pils5* seedlings during formation (15 hours), maintenance (40 hours) and early opening (60 hours). Scale bars, 300 μ m. **b**, Kinetics of apical hook development in WT, *PILS5^{ox}* and *pils2 pils5* dark-grown seedlings (n = 12). Dashed lines represent the duration of the different phases. F: Formation M: Maintenance O: Opening. Statistical significance was evaluated by non-linear regression and a subsequent Extra Sum of squares F test. End of maintenance phase (X_0) and speed of opening (K) were compared to WT (see Supplementary Fig. 2b).

*** denotes $p < 0.0001$ for both parameters. **c,e**, DR5 promoter activity in *pils2pils5* (**c**) and in *PILS5^{OX}* (**e**) seedlings compared to WT seedlings at 15, 40 and 60 hours. Colour code (black to green or red) depicts low to high signal intensity of *pDR5rev::GFP* and *pDR5rev::RFP1er*. Scale bars, 100 μ m. **d,f**, Quantification of mean grey values of the DR5 promoter activity in *pils2pils5* (**d**) as well as *PILS5^{OX}* (**f**) compared to WT seedlings at 15, 40 and 60 hours (n = 5). The region of interest (ROI) was delimited in the inner side of the apical hook quantifying the auxin maxima. Error bars represent standard error of the mean. Statistical significance was evaluated with two-way ANOVA followed by Bonferroni post-hoc test comparing the 2 genotypes of interest at the respective phases; the p value is indicated by * $p < 0.05$, ** $p < 0.001$ *** $p < 0.0001$; ns: non significant; *PILS5^{OX}*: *p35S::PILS5::GFP*.

Figure 3. Light-induced apical hook opening correlates with elevated *PILS* expression.

a, Kinetics of apical hook opening in WT in the dark or after light exposure (n=25).

Statistical significance was evaluated by non-linear regression and a subsequent Extra Sum of squares F test. Speed of opening (K) of WT in dark was compared to WT in light (see Supplementary Fig. 2a). *** denotes $p < 0.0001$. **b**, Expression patterns of *pPILS2::GUS*, *pPILS3::GUS* and *pDR5::GUS* in apical hook maintenance phase of dark-grown seedlings or after 1, 2 or 3 hours of light exposure. Scale bars, 100 μ m. **c**, GUS quantification of the respective lines and treatments (n = 8). The region of quantification was the apical hook

region. Error bars represent standard error of the mean. Statistical significance was evaluated by one-way ANOVA followed by multiple comparisons Tukey's test. Distinct letters indicate statistically significant differences ($p < 0,05$). Each transgenic line was analysed independently (indicated by dashed lines).

Figure 4. Light-induced apical hook opening is PILS-dependent manner.
a,c, DR5 promoter activity kinetics in *pils2 pils5* (a) and *PILS5^{ox}* seedlings (c) compared to WT seedlings in the dark and after 1 to 3 hours of light exposure. Colour code (black to green or red) depicts low to high signal intensity of *pDR5rev::GFP/RFP*. Scale bars, 100 μ m. **b,d**, Quantification of mean grey values of the DR5 promoter activity in *pils2pils5* (b) as well as *PILS5^{ox}* (d) compared to WT seedlings in the dark, and after 1 to 3 hours of light exposure (n = 5). The region of interest (ROI) was delimited in the inner side of the hooks. Error bars represent standard error of the mean. Statistical significance was evaluated by

one-way ANOVA followed by multiple comparisons Tukey's test. Distinct letters indicate statistically significant differences ($p < 0.05$). **e**, Representative pictures of apical hooks dark-grown or after being exposed 5 hours to light in wild type (WT), *pils2 pils5*, *PILS5^{OX}* seedlings. Scale bars, 150 μm **f**, Kinetics of apical hook opening during light exposure in *pils2 pils5* and *PILS5^{OX}* dark-grown seedling compared to WT ($n=15$). Error bars represent standard error of the mean. Statistical significance was evaluated by non-linear regression and a subsequent Extra Sum of squares F test. Speed of opening (K) was compared to WT (see Supplementary Fig. 2a). *** denotes $p < 0.0001$. *PILS5^{OX}: p35S::PILS5:GFP*.

Figure 5. Phytochrome interacting factor 5 represses *PILS* expression.

a,c, Expression patterns of *pPILS2::GUS* (**a**) or *pPILS3::GUS* (**c**) in WT and *PIF5^{OX}* dark grown seedlings at 40 and 72 hours after germination. Scale bars, 100 μ m. **b,d**, Quantification of GUS expression of *pPILS2::GUS* (**b**) and *pPILS3::GUS* (**d**) in WT and *PIF5^{OX}* dark grown seedlings at 40 and 72 hours after germination (n=10). **e**, ChIP analysis of PIF5-HA binding to *PILS2* and *PILS3* promoters in *PIF5^{OX}*-HA as compared to WT. G-box of *WAG2* (*pWAG2*) and 3'UTR of *FT* were used as a positive and negative control, respectively. The data presented are from 3 independent biological ChIP experiments (left

panel). Mode of action of PIF5-HA on *PILS2* and *PILS3* promoters (right panel). Error bars represent standard error of the mean. Statistical significance was evaluated with two-way ANOVA followed by Bonferroni post-hoc test; the p values are indicated by * $p < 0.05$, ** $p < 0.001$ *** $p < 0.0001$. *PIF5^{OX}*: *p35S::PIF5:HA*

Figure 6. phyB/PIF5 pathway regulates PILS dependent growth transition during apical hook early opening.

a-d, Kinetics of apical hook development in WT, *phyb*, *PILS5^{OX}*, and *PILS5^{OX} phyb* dark-grown seedlings (**a**); in WT, *PILS3^{OX}*, *PIF5^{OX}*, and *PILS3^{OX} PIF5^{OX}* expressing dark-grown seedlings (**b**); in WT, *PILS3^{OX}*, *PIF5^{OX}*, and *PILS3^{OX} PIF5^{OX}* expressing seedlings (exposed to light at the end of maintenance phase) (**c**); and in WT, *pils3*, *pif5*, *pils3 pif5* expressing dark-grown seedlings (**d**) (n 12). Statistical significance was evaluated by non-linear regression and a subsequent Extra Sum of squares F test. End of maintenance phase

(X_0) and speed of opening (K) (see Supplementary Fig. 2b) were compared to *PILS5^{OX} phyB* (a), or *PILS3^{OX} PIF5^{OX}* (b) or *pils3 pif5* (d). Significance of X_0 is most informative here and hence indicated in the graphs a,b,d * $p < 0.05$, ** $p < 0.001$ *** $p < 0.0001$; ns: non significant; However, K was also highly significant ($p < 0.0001$) for all kinetics excepted WT compared to *PILS5^{OX} phyB* ($p < 0.05$). In c, speed of opening (K) were compared to *PILS3^{OX} PIF5^{OX}* and is indicated in the graph *** $p < 0.0001$. Error bars represent standard error of the mean. e, Model illustrations: phyB/PIF5 light perception module directly impacts on *PILS2* and *PILS3* transcription, controlling auxin-dependent growth transition (repression to promotion) for apical hook opening (left panel). During the formation phase of the apical hook, growth is repressed which correlates with a progressive increase in auxin signalling in a PIN dependent manner. Ethylene is able to prolong this repression. During the maintenance phase, no growth is occurring. Light induces *PILS* expression terminates the maintenance phase and induces growth by decreasing auxin signalling. *PILS5^{OX}: p35S::PILS5::GFP. PILS3^{OX}: p35S::GFP:PILS3. PIF5^{OX}: p35S::PIF5::HA*