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Directional Auxin Transport Mechanisms in Early Diverging Land Plants

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Summary

The emergence and radiation of multicellular land plants was driven by crucial innovations to their body plans [1]. The directional transport of the phytohormone auxin represents a key, plant-specific mechanism for polarization and patterning in complex seed plants [2–5]. Here, we show that already in the early diverging land plant lineage, as exemplified by the moss *Physcomitrella patens*, auxin transport by PIN transporters is operational and diversified into ER-localized and plasma membrane-localized PIN proteins. Gain-of-function and loss-of-function analyses revealed that PIN-dependent intercellular auxin transport in *Physcomitrella* mediates crucial developmental transitions in tip-growing filaments and waves of polarization and differentiation in leaf-like structures. Plasma membrane PIN proteins localize in a polar manner to the tips of moss filaments, revealing an unexpected relation between polarization mechanisms in moss tip-growing cells and multicellular tissues of seed plants. Our results trace the origins of polarization and auxin-mediated patterning mechanisms and highlight the crucial role of polarized auxin transport during the evolution of multicellular land plants.

Results and Discussion

During plant diversification, a spectacular evolutionary transition from anatomically simple green algae to developmentally complex multicellular land plants took place, presumably in order to adapt to new and challenging environments [1]. The phytohormone auxin is the most versatile plant-specific signal that governs many crucial aspects of the seed plant body organization [2]. A unique property of auxin among plant signaling molecules is its directional (polar) transport through tissues, which is essential for most auxin-regulated developmental processes, such as the establishment of the polarity axis during embryogenesis, de novo formation of organs, and vascular tissue formation [3–5]. Auxin transport depends largely on specific auxin transporters, namely the PIN-FORMED (PIN) proteins [6]. Their typically asymmetrical (polar) localization at the plasma membrane (PM) determines the direction of auxin flow between cells [7], which in turn provides directional and positional information for the development of multicellular tissues by linking information at the level of individual cells to a coordinated developmental output [8]. On account of its universal roles in polarization and patterning processes in seed plants, it is believed that this PIN-mediated auxin transport played a key role in important developmental innovations during the diversification of land plants [9, 10]. However, in planta data on the evolution of auxin transport and polarization machineries in early diverging land plants are missing.

In order to reconstruct the role of PIN proteins during land plant evolution, we used the moss model species *Physcomitrella patens*, which is a representative of one of the earliest diverging lineages of land plants [11]. Similar to that of the angiosperm *Arabidopsis*, the *P. patens* genome encodes two different types of PIN proteins, characterized by either a short or a long hydrophylic loop between the transmembrane regions (Figure 1A), the latter designated as the canonical PIN protein [12, 13]. We assessed the auxin transport capabilities of these different PIN proteins by several means. We performed auxin transport assays using radioactively labeled substrates in mesophyll protoplasts from transfected *Nicotiana benthamiana* leaves [14] and found that *P. patens* PINs with a long and short loop enhanced the export of indole-3-acetic acid (IAA), but not the chemically related benzoic acid (Figure S1F available online). Using root hair growth as an established measure of auxin export capacities, we showed that overexpression (OE) of long moss PINs in *Arabidopsis*, similar to OE of a typical PM-localized long PIN from *Arabidopsis* (AtPIN1), inhibits root hair growth, suggesting action in stimulating auxin efflux. In contrast, OE of the short moss PIN did not affect root hair growth, similar to OE of the ER-localized, short AtPIN5 from *Arabidopsis* [15] (Figures 1B and S1G). Finally, *P. patens* filaments export auxin [16, 17] that can be detected in the cultivation medium (Figure 1C). Using this in vivo assay, we observed that OE of moss and *Arabidopsis* PINs strongly enhanced auxin export into the medium. Also, a double mutant line in long moss PINs (*pinapinb*) slightly reduced auxin export into the medium (Figures 1C, S1A–S1E, and S1H). These results suggest that already in one of the early diverging land plant lineages, similar to seed plants, the PIN proteins diversified into long PINs with auxin export function and short PINs with presumable roles in auxin homeostasis and metabolism.

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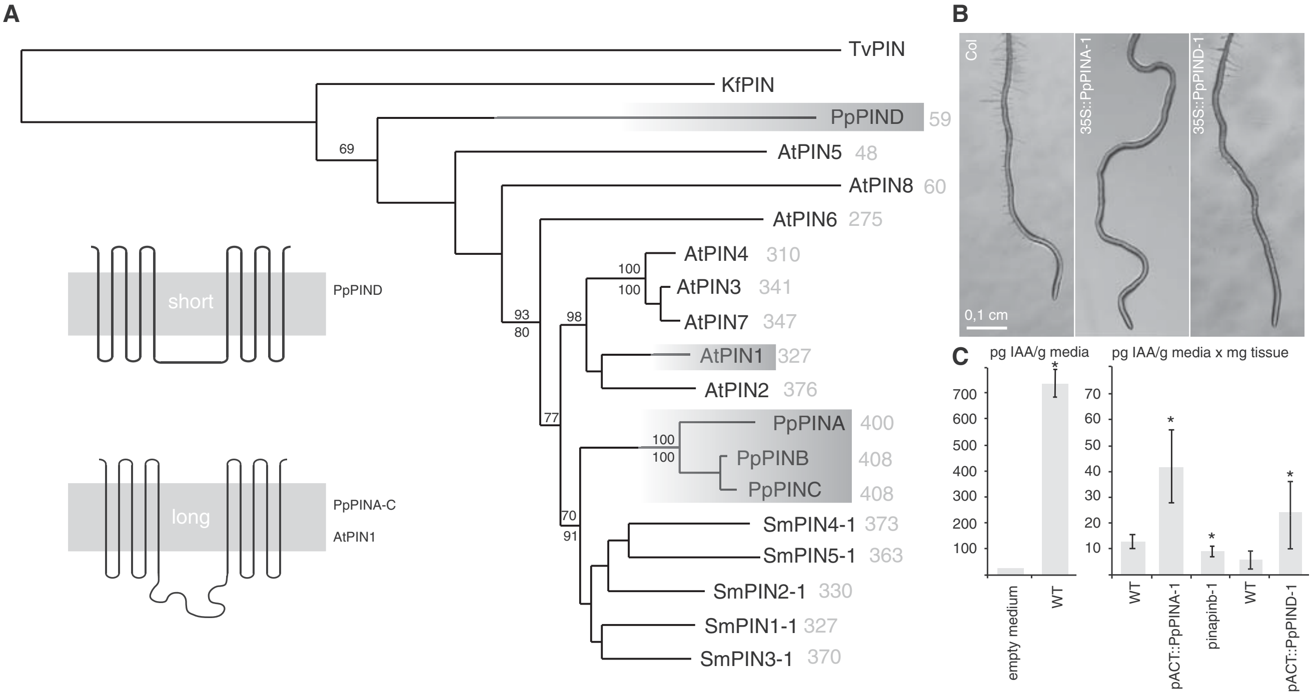

Figure 1. Evolution and Auxin Transport Activities of PIN Proteins from the Moss *P. patens*

(A) Evolution of PIN proteins from the seed plant *Arabidopsis* (AtPIN), the lycophyte *Selaginella moellendorffii* (SmPIN), the moss *P. patens* (PpPIN), and the alga *Klebsormidium flaccidum* (KfPIN). A putative PIN-like sequence from the protist *Trichomonas vaginalis* (TvPIN) is used to root the tree. A maximum-likelihood tree is shown with neighbor-joining bootstrap support values (>70%) and bootstrap support values from maximum-likelihood analyses (>70%) indicated above and below branches, respectively. As predicted by the TMHMM server (v.2.0), the number of amino acids in the hydrophilic loop in between the transmembrane regions for each PIN protein is indicated next to the terminal taxa. PIN proteins highlighted in gray are investigated: a long-looped PIN from *Arabidopsis* (AtPIN1) and moss long (PpPINA-C) and short (PpPIND) PINs.

(B) Overexpression (OE) of the long moss PpPINs inhibits root hair elongation in *Arabidopsis*, suggesting auxin export activity, whereas OE of the short PpPIND does not affect root hair development.

(C) *P. patens* protonemal tissue grown in liquid medium exports IAA into the medium. OE of long and short PpPIN in *P. patens* enhances IAA export into the medium. IAA export is reduced in the *pinapinb* double mutant line compared to WT. Free IAA in liquid media per mg moss tissue is shown.

Data are represented as mean ± SD; *p < 0.05 (by Student's t test). See also Figure S1.

The subcellular localization of PIN proteins in seed plants was crucial for understanding their role in auxin-mediated developmental processes with long PINs polarly localizing at the PM and short PINs showing subcellular localization at the ER [7, 18]. We investigated PpPIN expression and subcellular localization in vivo by generating EGFP transcriptional and translational fusions for both long and short *P. patens* PINs (Figure S2A). Both fusion constructs revealed a gradual expression of the long PpPINA in moss filaments, peaking toward the tip (Figures 2A, S2B, and S2C). Close examination of the protonemal apical and subapical cells revealed a clear polarization of the PpPINA-GFP signal at the PM (as evidenced by costaining with FM4-64 lipophilic dye) of the distal cell sides toward the filament tip (Figures 2B, 2C, and S2D). We also expressed the translational fusions of long and short moss PINs under the rice actin promoter in *P. patens* and confirmed their functionality by comparing phenotypic effects to the nontagged OE lines (Figures S3A and S3B; Table S2). For the constitutively expressed long PpPINA and PpPINB, we again observed a clear asymmetric signal toward the growing tip of the filament (Figures 2D and S2E). In contrast, the localization of the short PpPIND did not show any colocalization with the FM4-64-staining of the PM. Instead, the signal was predominantly intracellular and could be observed close to the PM and around the nucleus, consistent with localization at the ER and similar to short PINs in seed plants [18–20] (Figure 2E).

The polar PM localization of long moss PINs and the ER localization of short moss PINs are in line with the transport studies and support an extensive diversification of the PIN auxin transport family in early land plants, both in terms of auxin transport and subcellular localization. Also, if a canonical or long PIN protein was present in the last common ancestor of land plants [13], our data would suggest that PM-localized PIN proteins predate the origin of land plants.

A multicellular filamentous system (protonemata) represents the first stage of the gametophytic part of the moss life cycle and consists of two different types of filaments that grow by apical cell divisions, the photosynthetically active chloronema and the colony-spreading caulonema. Protonemal filaments show a cell identity gradient, with the proximal cells being more chloronemal-like and a gradual transition toward caulonemal cell identity along the filament. The transition, which occurs only in tip cells, is positively regulated by auxin [16, 21, 22], and it has been proposed that the highest auxin level is present in the tip cell and further declines toward the base of the filament [23, 24]. The second phase of the moss gametophytic life cycle starts with the formation of buds on caulonemal cells to generate gametophores producing leaf-like structures (hereafter referred to as leaves) and, eventually, organs for sexual reproduction.

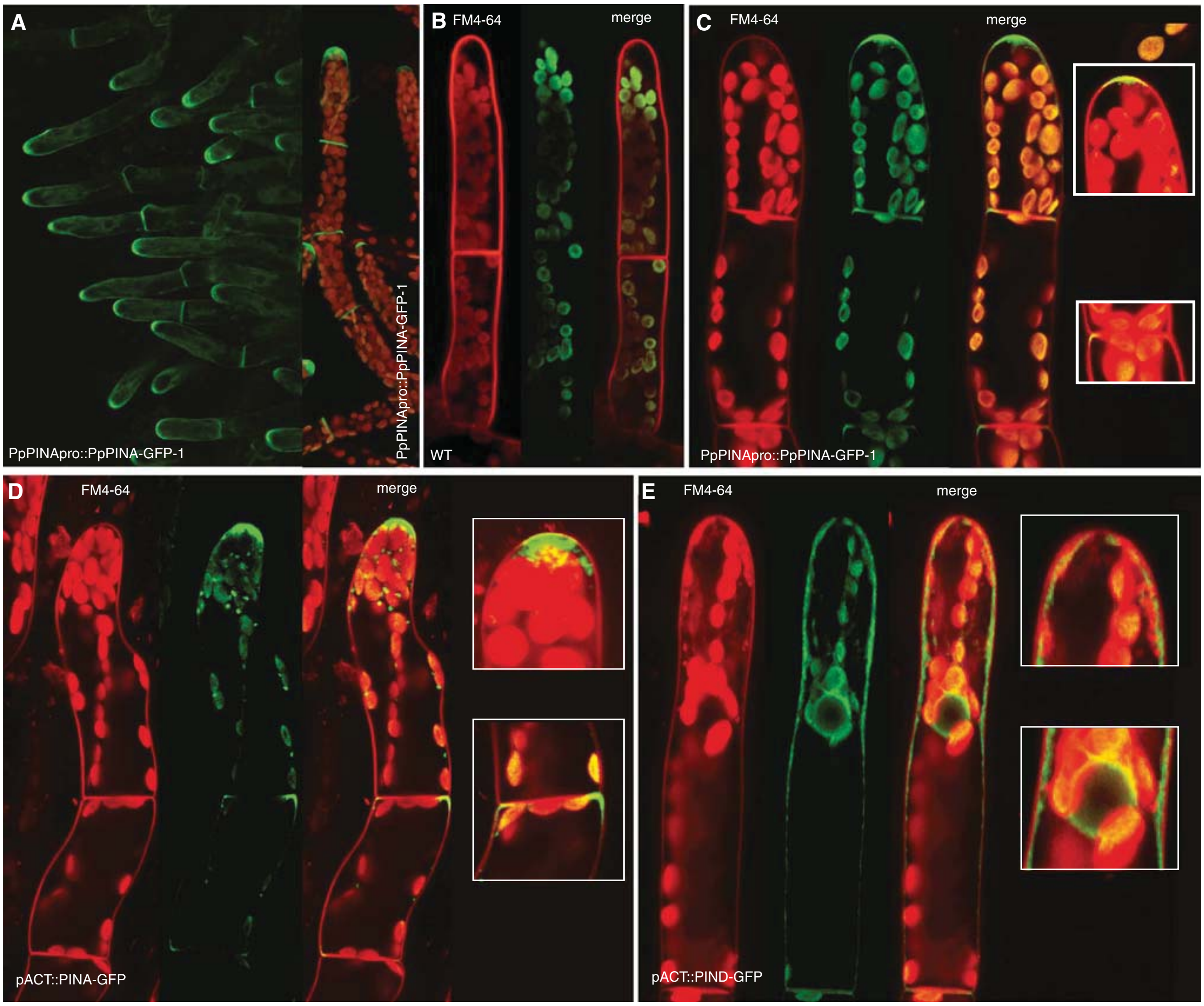

Figure 2. Long PpPINs Are Polarly Localized at the PM, whereas the Short PpPIN is ER Localized

(A) Long PpPINA expression gradually increases toward the tip of moss protonemal filaments and is strongest in the apical tip cell.

(B) WT filaments stained with PM-staining dye FM4-64.

(C) PpPINA colocalizes with FM4-64 and shows a clear polarization at the cell sides toward the filament tip.

(D) OE of the long moss PpPINA translational fusion to GFP also shows colocalization with FM4-64 at the cell side toward the filament tip.

(E) The translational fusion of the short moss PpPIND shows predominantly intracellular signal with ER-like perinuclear pattern.

Short staining with the red fluorescent dye FM4-64 is used as a PM marker, and autofluorescence of chloroplasts is obvious in both the green and the red channels. See also Figure S2.

To analyze the developmental role of PM-localized long PIN proteins, we generated *P. patens* PIN mutants and OE lines (Figure S1). Long PIN double mutants (*pinapinb*) showed consistently smaller colonies and a premature appearance of gametophores, in contrast to OE lines that produced small dense circular colonies with a strong delay in gametophore formation (Figures 3A–3D, S3A, S3B, S4A, and S4B; Table S2). Also, the rate of dark-grown caulonema production is higher in the mutant, compared to wild-type (WT) (Figure S4C). As gametophores mainly develop from caulonemal cells, this suggested an earlier transition to caulonema cell identity in the *pinapinb* mutant and chloronema identity maintenance in the OE lines. We addressed this hypothesis by analyzing protonemal regeneration from protoplasts. By identifying the first caulonemal cell in the primary regenerating protonemal filament through identification of the first oblique cell wall, we confirmed that in contrast to OE lines, the *pinapinb* mutant establishes caulonemal identity earlier than WT (Figures 3E and S4D). Likewise, the second cell in the regenerating filaments of a *pinapinb* mutant already shows a significant increase in length and a decrease in width (hallmark of caulonemal cell identity), whereas OE lines show a significant reduction in cell length and an increase in cell width, mimicking chloronemal cell identity (Figures 3F and S4E).

The loss-of-function and gain-of-function phenotypes, the auxin transport activities, and the gradual expression of polarly localized long moss PINs support a model in which polarized transport of auxin toward the filament tip regulates the chloronema-to-caulonema transition. In this scenario,

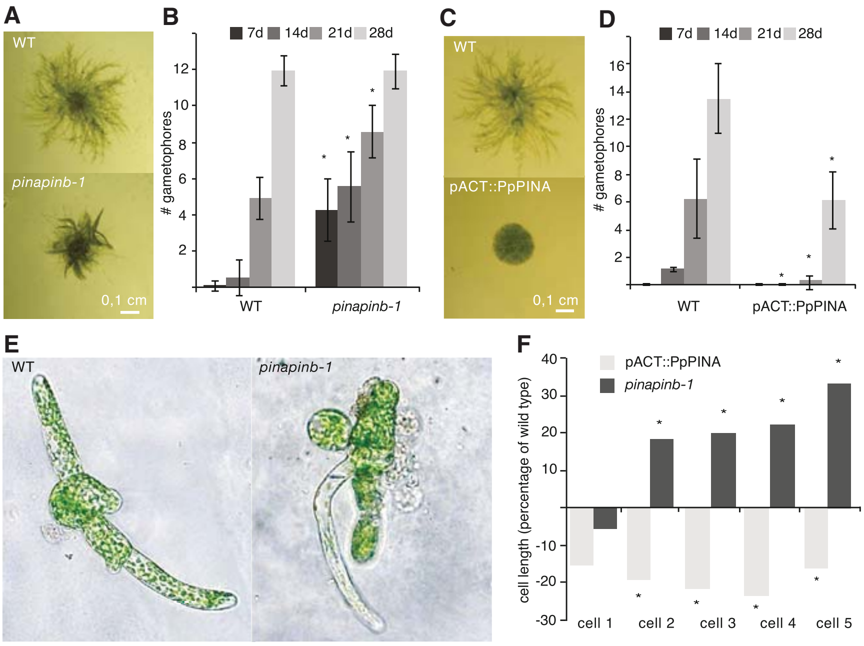

Figure 3. PpPIN Activity Mediates a Prominent Cell-Fate Switch during Moss Protonemal Development

(A–D) A *pinapinb* double mutant line produces smaller colonies and a sooner appearance of gametophores as compared to WT, whereas a PpPINA OE line produces small dense circular colonies, and the appearance of gametophores is strongly delayed compared to WT. Two-week-old colonies are shown.

(E) Regenerating moss filaments from protoplasts show an earlier transition to caulonemal cell identity in a *pinapinb* double mutant line.

(F) Loss of function of long PIN proteins stimulates cell elongation (hallmark of caulonemal identity) in regenerating protonemal filaments from protoplasts, whereas OE inhibits cell elongation in these cells.

Data are represented as mean ± SD; *p < 0.05 (by Student's t test). See also Figures S3 and S4 and Table S2.

PIN-dependent auxin transport from the base of protonemal filaments to and out of the tip regulates the cellular auxin levels in cells along the filament. Loss of PIN function promotes early caulonemal transition, presumably as an effect of ectopic auxin accumulation caused by blocked export. Conversely, PIN gain of function delays the transition to caulonema, presumably by enhancing auxin export from the tip cell and reducing cellular auxin concentration. An attractive hypothesis would be that the auxin levels needed to induce this transition will only occur when a critical number of chloronema cells exporting auxin toward the tip cell have formed. The tip cell will thus be primed that the filament is long enough to provide photosynthates to caulonema cells, hence allowing for differentiation to occur. Although the details of this mechanism remain unclear, the loss-of-function and gain-of-function analyses show that PIN activity in protonemal filaments mediates the transition between chloronemal and caulonemal cell identity, a prominent cell-fate change during moss gametophytic development [22].

The development of leaves on the gametophore also strongly depends on long PIN protein activity. Whereas the leaves of both the PIN OE and mutant lines are narrower, the mutant leaves are longer and the OE leaves are shorter, compared to WT (Figures 4A, 4B, and S4F). A reduced number of longitudinal cell files in both mutant and OE lines can explain the reduced leaf width, suggesting that the number of transverse cell divisions during early leaf development has been restricted. In addition, the leaf cells in the mutant are significantly longer and wider, whereas in the OE cells, they are shorter and narrower, explaining the difference in leaf length (Figures 4A, 4B, and S4F). The phenotype of the mutant leaves can be phenocopied by growing moss colonies on the auxin efflux inhibitor NPA, linking PIN involvement to its auxin transport activity (Figure S4G). During gametophore development, long PpPIN activity was never detected in the youngest leaf but was clearly detected in the third leaf from the apex (P3) and sometimes also in the second leaf from the apex, where it was detected in the apical-most cells. The PpPIN expression zone then expanded in consecutive leaves and moved toward the base of the leaf (Figures 4C–4E and S4H–S4K). At the subcellular level, both PpPINA and PpPINB show bipolar localization at both the apical and basal PM of the cells in the expression domain closest to the leaf tip, whereas in more-basal parts of the expression domain, they are located symmetrically at all sides of the cells (Figure 4F). Notably, this wave of expression and polarization of long PpPINs correlates with a similarly moving wave of cell elongation during leaf development. Together with gain-of-function and loss-of-function phenotypes, this correlation suggests a role for PIN-mediated auxin transport in regulating the transition from cell division to cell elongation during gametophore leaf development.

Conclusions

Our genetic analysis revealed that PIN-dependent auxin transport mediates important developmental decisions functional in early diverging land plants. During moss gametophytic development in general, reduced long PIN activity increases cellular auxin content, leading to premature cell-fate switches toward differentiation and/or elongation, whereas elevated PIN expression reduces the cellular auxin content and delays these developmental transitions. Changes in PIN activity also interfere with cell division, suggesting a role to fine-tune the balance between cell division and cell differentiation in auxin-regulated developmental processes in *P. patens*. These observations show that a blueprint of developmentally regulated cell-fate changes by PIN-mediated distribution of auxin was already operational in the common ancestor of early diverging land plant lineages. Together with the observations that other auxin signaling components, such as biosynthesis regulators, receptors, and downstream effectors, function in a similar way in *P. patens* as in seed plants, this demonstrates the conserved role of auxin action in land plants [21, 25–27].

Our evolutionary comparison of auxin transport mechanisms provides insights into the origin of plant-specific mechanisms that underlie cell polarity and tissue polarization. The tip-growing moss filaments represent a simple level of structural organization of the plant body plan, with growth along a single

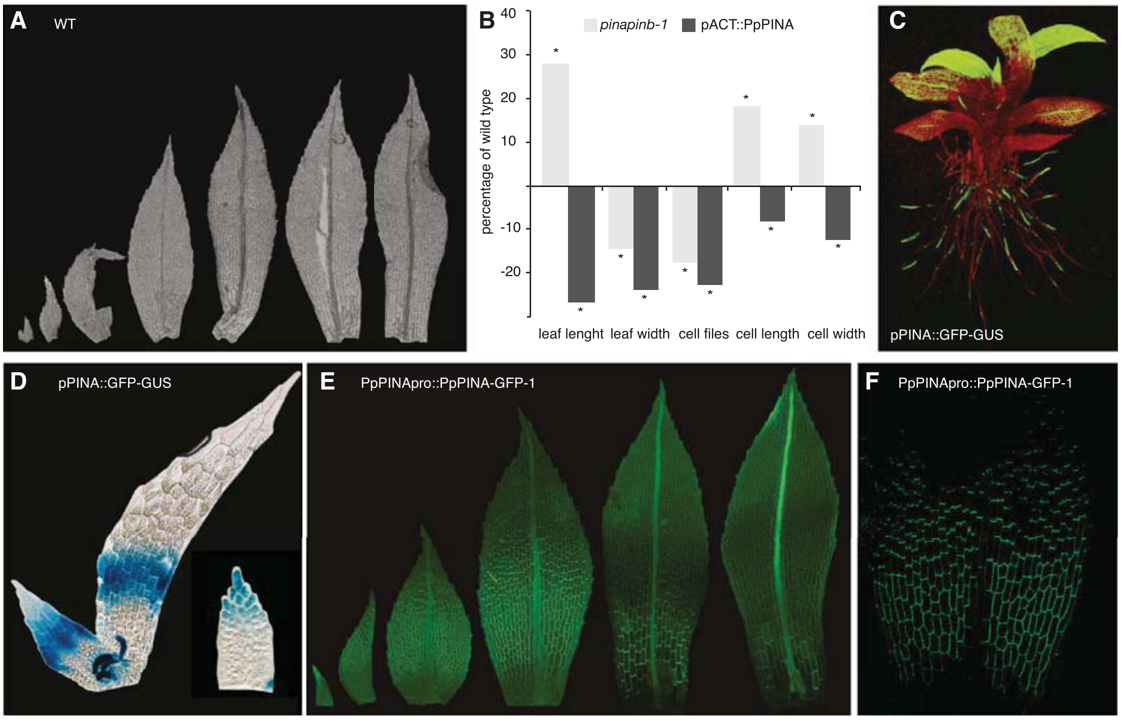

Figure 4. Wave of PIN Expression and Polarization Is Required for the Transition from Cell Division to Elongation during Moss Leaf Development

(A) Leaf developmental series of WT (leaves P1–P8).

(B) A comparison of the developing leaves and leaf cells in *pinapinb* mutant, PpPINA OE and WT, suggests that both cell division and cell elongation are regulated by the activity of the long PpPINs. Whereas the KO leaves are longer and narrower, OE reduces both leaf length and width. Also, leaf cells are elongated in KO and reduced in length in OE lines. Data are represented as mean ± SD; *$p < 0.05$ (by Student's t test).

(C) PpPINA promoter activity is strong in developing leaves of the moss gametophytic shoot.

(D and E) Expression is initially at the tip of young leaves and gradually moves toward the base of the leaves as they elongate.

(F) The PpPINA protein localizes at the apical and basal cell sides in the expression domain closest to the leaf tip and becomes symmetric at all sides of the cells in the more-basal parts of the leaf expression domain.

See also Figure S4.

axis. The polar PIN localization in this tissue together with the growth phenotypes observed through manipulation of PIN activity suggest that polarized PIN-mediated auxin transport, which is extremely versatile in the regulation of seed plant development [28], was first established during polarized growth of tip-growing plant cells. An evolutionary relationship between tip-growing cells and polarized multicellular tissues from seed plants is unexpected because this mechanism is not present in tip-growing cells of angiosperms, such as pollen tubes and root hairs. The next step in the evolution of multicellular plant tissues is exemplified by growth along two axes in the leaf blade of moss gametophores. In these leaves, the polar PIN localization at young stages was detected (see Bennett et al. [29] in this issue of *Current Biology*), and the capacity to change the PM PIN localization from bipolar to symmetrical is obvious and correlates with the developmental switch to cell elongation. This suggests that the elaborated regulations of PIN polarity in complex tissues of angiosperms can be traced back to more-simple polarity changes in the ancestor and that PIN-mediated auxin transport and its downstream effects in gametophytes were co-opted to drive crucial innovations to the body plan of the sporophyte. PIN-mediated auxin transport was thus a key part of the molecular toolkit that allowed land plants to evolve the structural and developmental complexity crucial to their adaptation to the terrestrial environment, and it helped give rise to the enormous variety of land plants that populate Earth now [1].

Experimental Procedures

Moss Transformation

We used the *P. patens* ssp. *patens* strain Gransden 2004 as a background to generate transgenic lines. PEG-mediated transformation of OE and knockout (KO) constructs and transcriptional and translational fusion constructs following protoplast isolation were completed according to methods that have been previously described [30]. Transformants were selected using 50 μg/ml *G418* (G9516, Sigma) or 50 μg/ml zeocine (Invitrogen), depending on the construct. gDNA was isolated using QuickExtract Plant DNA Extraction Solution (Epicenter), and PCR genotyping of stable transformants was performed with primers shown in Table S1 (Figure S1). For each construct, at least two independently generated transgenic lines were fully analyzed. These lines showed similar expression profiles (transcriptional and translational fusions) and similar phenotypes (KO and OE lines). The only identified difference was quantitative, and this was mainly detected between different OE lines due to different level of transgene expression. Because of space limitation, data for only one transgenic line for each construct are presented.

Liquid Cultures and IAA Measurements of *P. patens*

Freshly subcultured protonemal tissue of the different genotypes was used to start the liquid cultures. In a 15 ml falcon with 9 ml of liquid BCD medium (with 5 mM ammonium tartrate), about $^{1}/_{5}$ of a full plate with fresh protonemal tissue was added. The tubes were placed horizontally on a shaker and grown under standard conditions. Lids were opened 1–2 times a day to allow for gas exchange. After 4 days of growth, the total amount of tissue was weighed, and 1 ml of the liquid medium from each tube was collected for IAA quantification. We added 500 pg $^{13}C_6$-IAA internal standard to each sample and carried out extraction and purification as described [31]. IAA was quantified using gas chromatography-tandem mass spectrometry [32]. The amount of IAA was based on 5–7 independent biological samples per genotype and presented as pg IAA per ml media and per gram of tissue added to the liquid culture.

Supplemental Information

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.09.056.

Author Contributions

T.V., E.S., M.T., E.F., and J.F. designed the research. T.V., K. Landberg, M.T., E.M., E.P., E.F., E.D.C., M.K., K. Ljung, and M.G. performed the experiments. T.V., K. Landberg, M.T., E.D.C., K. Ljung, M.G., E.S., and J.F. analyzed the data. T.V., M.T., E.S., and J.F. wrote the paper.

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