Patterning of Leaf Vein Networks by Convergent Auxin Transport Pathways

Megan G. Sawchuk, Alexander Edgar, Enrico Scarpella*
Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada

Abstract
The formation of leaf vein patterns has fascinated biologists for centuries. Transport of the plant signal auxin has long been implicated in vein patterning, but molecular details have remained unclear. Varied evidence suggests a central role for the plasma-membrane (PM)-localized PIN-FORMED1 (PIN1) intercellular auxin transporter of Arabidopsis thaliana in auxin-transport-dependent vein patterning. However, in contrast to the severe vein-pattern defects induced by auxin transport inhibitors, pin1 mutant leaves have only mild vein-pattern defects. These defects have been interpreted as evidence of redundancy between PIN1 and the other four PM-localized PIN proteins in vein patterning, redundancy that underlies many developmental processes. By contrast, we show here that vein patterning in the Arabidopsis leaf is controlled by two distinct and convergent auxin-transport pathways: intercellular auxin transport mediated by PM-localized PIN1 and intracellular auxin transport mediated by the evolutionarily older, endoplasmic-reticulum-localized PIN6, PIN8, and PIN5. PIN6 and PIN8 are expressed, as PIN1 and PIN5, at sites of vein formation. pin6 synthetically enhances pin1 vein-pattern defects, and pin8 quantitatively enhances pin1pin6 vein-pattern defects. Function of PIN6 is necessary, redundantly with that of PIN8, and sufficient to control auxin response levels, PIN1 expression, and vein network formation; and the vein pattern defects induced by ectopic PIN6 expression are mimicked by ectopic PIN8 expression. Finally, vein patterning functions of PIN6 and PIN8 are antagonized by PIN5 function. Our data define a new level of control of vein patterning, one with repercussions on other patterning processes in the plant, and suggest a mechanism to select cell files specialized for vascular function that predates evolution of PM-localized PIN proteins.

Introduction
Branched structures pervade all levels of organization in living organisms, from molecules to organelles, cells, multicellular organs and entire organisms, and the principles that guide the formation of these branched structures have long been of interest to biologists and mathematicians. However, few branched structures have historically captured more widespread attention than the vein networks of plant leaves. From a developmental standpoint, such observations agree with conceptual formulation [12,13] and mathematical modeling (reviewed in [14–18]) of progressive restriction of nondirectional auxin transport across tissues to polar transport in single files of vascular precursor cells by positive feedback between auxin flow through a cell and the cell’s auxin conductivity. However, in contrast to the severe vein-pattern defects induced by auxin transport inhibitors [6,7], vein pattern defects in pin1 mutant leaves are mild [6,19,20]. PIN1 is member of a family comprising four other PM-localized PIN proteins and three, evolutionarily older, endoplasmic reticulum (ER)-localized PIN proteins [21–24]. Thus, the mild vein-pattern defects of pin1 have been attributed to redundancy among PM-localized PIN proteins (e.g., [6,10,19,25]), redundancy that has been shown to...
Author Summary

The beautiful and varied patterns of veins in plant leaves have intrigued both artists and scientists since antiquity. The seminal work of some of these scientists has shown that the plant hormone auxin and its transport in plant tissues control leaf vein patterning, but molecular details of auxin-transport-dependent vein patterning are still unknown. Here we find that vein patterning is controlled by the concerted action of two spatially separate auxin transport pathways. One pathway transports auxin from cell to cell, the other to and from different compartments within the cell. The cell-to-cell pathway of auxin transport seems to exist only in land plants with veins. The within-cell pathway of auxin transport seems to have appeared earlier in the evolution of plants and exists also in primitive land plants, which form vein-like structures. Our findings suggest an unsuspected level of control of vein patterning, one that regulates patterning of plant features beyond veins and that could even be relevant for the formation of vein-like structures of primitive land plants.

Results

Vein patterning functions of Arabidopsis PIN genes

WT Arabidopsis grown under normal conditions forms separate leaves, whose vein patterns are defined by reproducible features [28,29] (Figure 1A and 1B): a narrow, central midvein that runs the length of the leaf; lateral veins that branch from the midvein and join distal veins to form closed loops; minor veins that branch from midvein and loops and either end freely or join other veins; and minor veins and loops that curve near the leaf margin, lending a scalloped outline to the vein network. By contrast, WT leaves developed in the presence of auxin transport inhibitors often show separation defects (‘fused leaves’), and vein patterns of auxin-transport-inhibited leaves deviate from the norm in at least four respects [6,7]. First, the midvein bifurcates near the leaf tip. Second, the vein network consists of more lateral veins. Third, lateral veins do not join the midvein at the centre of the leaf but run parallel to one another to form a wider midvein. Fourth, lateral veins end in a marginal vein that closely parallels the leaf margin, lending a smooth outline to the vein network. Mutation of PIN1 (AT1G73590) approximates these defects, but vein patterns are only mildly affected in pin1 [6,19] (Figure 1). As PIN1 is one of eight PIN genes [21–24] and other gene families have been implicated in auxin transport (e.g., [30–32]), the mild vein-pattern defects of pin1 might reflect contribution of other auxin transporters to vein patterning; here we tested whether other PIN genes contribute to vein patterning.

We first explored this possibility by analyzing vein patterns of single mutants in PIN2 (AT5G37090), PIN3 (AT1G70940), PIN4 (AT2G01420), PIN5 (AT5G16530), PIN6 (AT1G77110), PIN7 (AT1G23080) and PIN8 (AT5G15100). pin2-pin8 (Table S1) had WT vein patterns (Figure 1), limiting nonredundant vein-patterning functions to PIN1. Thus, to uncover potential vein-patterning functions of PIN2–PIN8, we next analyzed vein patterns of double mutants between pin1 and pin2–pin8. Only mutation of the auxin-transporter-encoding PIN6 [9] had a significant effect on pin1 phenotype spectrum, with ~65% of pin1pin6 (pin1;6) leaves belonging to new, stronger classes (Figure 1); pin6 had a similar effect on pin1 phenotype spectrum in seedlings (Figure S1): ~95% penetrance of cotyledon defects in pin1;6 vs. ~55% in pin1, and appearance in pin1;6 of a cup-shaped cotyledon phenotype resembling that of embryos developed in the presence of auxin transport inhibitors [33,34]. In both leaves and seedlings, the pin1;6 phenotype spectrum was mimicked by expressing an artificial microRNA targeting PIN6 (UBQ10:amiPIN6) in the pin1 background (Figure 1 and Figure S1). We conclude that PIN6 is the PIN gene that most contributes to auxin-transport-dependent vein patterning in the absence of PIN1 function.

PIN6 expression in leaf development

Double-mutant analysis suggests functions for PIN6 in vein patterning (Figure 1; see Discussion). We thus asked whether PIN6 was expressed during vein formation. To address this question, we imaged expression of a functional (Table S1) translational fusion of PIN6 to GFP driven by the PIN6 promoter (PIN6::PIN6:GFP). During leaf development, PIN6::PIN6:GFP expression was initiated in broad subepidermal domains that narrowed to sites of vein formation (Figure 2A–2H), suggesting PIN6 expression during vein formation. Expression of PIN6::PIN6:GFP was recapitulated by expression of a PIN6::YPF::nuc transcriptional fusion (PIN6 promoter driving expression of a nuclear YFP) (Figure S2A–S2E), which overlapped with expression of PIN1::PIN1:CFP [35] in leaf subepidermal tissues (Figure 2I). In pin1, PIN6::PIN6:GFP expression remained confined to subepidermal tissues, but expression was weaker (Figure 2J–2L).

A PIN6 translational fusion localizes to the ER in tobacco suspension cells [36]. To determine PIN6 subcellular localization in Arabidopsis leaves, we quantified degree of colocalization between expression of PIN6::PIN6:GFP and expression of ER, or PM, markers. Expression of PIN6::PIN6:GFP correlated with expression of ER, but not PM, markers (Figure 2M–2T and Figure S1), suggesting ER-localization of PIN6 in vein development.

Genetic interaction between PIN1, PIN5, PIN6, and PIN8 in vein patterning

PIN6 belongs to a subfamily of proteins that includes the ER-localized PIN5 and PIN8 auxin transporters [36–39]. Thus, we asked whether frequencies of vein pattern phenotypes in pin1;6 were affected by additional mutation of PIN5 or PIN8. While pin6 shifted the distribution of pin1;6 phenotypes towards stronger classes, pin5 partially normalized pin1;6;8 phenotype spectrum (Figure 3A). pin5 and pin8 had similar effects in seedlings (Figure S1): (1) complete penetrance of cotyledon defects in pin1;6;8 vs. ~95% in pin1;6;8 (2) ~95% penetrance of the cup-shaped cotyledon phenotype in pin1;6;8 vs. ~60% in pin1;6;8, and (3) partial normalization of cotyledon defects of pin1;6;8 by pin5.

PIN8 expression in leaf development

Triple-mutant analysis suggests functions for PIN8 in vein patterning (Figure 3; see Discussion). We thus asked whether PIN8 was expressed, as PIN1 [10,11], PIN3 [36] and PIN6 (Figure 2 and Figure S2A–S2E), during vein development. To address this question, we imaged expression of a functional (Table S1) translational fusion of PIN8 to GFP driven by the PIN8 promoter (PIN8::PIN8:GFP). Expression of PIN8::PIN8:GFP was restricted
to narrow sites of vein formation in both WT and pin1;6 (Figure 4A–4E), suggesting PIN8 expression in vein development, a conclusion further supported by expression of a PIN8::YFPnuc transcriptional fusion (PIN8 promoter driving expression of a nuclear YFP) (Figure 4H–4K).

In pollen, PIN8 translational fusions localize to the ER [38,39]. To determine PIN8 subcellular localization in leaves, we quantified degree of colocalization between expression of PIN8::PIN8:GFP and staining by the glibenclamide-derived ER-Tracker Red dye, which selectively stains the ER (e.g., [40–42]). Expression of PIN8::PIN8:GFP correlated with staining by ER-Tracker Red (Figure 4F) (mean Manders’ coefficient ‘r’ ± SE: 0.82 ± 0.03; n = 10) and overlapped with staining by the dapoxyl-derived ER-Tracker Blue-White DPX dye [43] (Figure S2G), suggesting ER-localization of PIN8 in vein development.

Necessary functions of PIN5, PIN6, and PIN8 in vein network formation

Single mutation of PIN5, PIN6 or PIN8 had no effect on vein patterns (Figure 1). Thus, to test whether a function in vein patterning could be assigned to ER-localized PIN proteins in the presence of PIN1-mediated intercellular auxin-transport, we analyzed vein patterns of combinations of pin5, pin6 and pin8. Double and triple mutants had WT vein patterns (sample population sizes: WT, 26; pin2, 68; pin3, 68; pin4, 68; pin5, 68; pin6, 68; UBQ10::amiPIN6-5, 65; UBQ10::amiPIN6-10, 65; pin7, 68; pin8, 68; pin1, 71; pin1,2, 71; pin1,3, 77; pin1,4, 69; pin1,5, 72; pin1,6, 65; UBQ10::amiPIN6-5pin1, 65; UBQ10::amiPIN6-10pin1, 67; pin1,7, 77; pin1,8, 68. Bars: (B–F) 1.5 mm; (G,H) 0.75 mm.

doi:10.1371/journal.pgen.1003294.g001

Figure 1. Vein patterning functions of Arabidopsis PIN genes. (A,B) Vein pattern of WT mature first leaf. In (A), dark grey, midvein; grey, loops; light grey, minor veins. (B–H) Dark-field illumination of cleared mature first leaves illustrating phenotype classes: unbranched, narrow midvein and scalloped vein-network outline (B); bifurcated midvein and scalloped vein-network outline (C); fused leaves with scalloped vein-network outline (D); conspicuous marginal vein (E); fused leaves with conspicuous marginal vein (F); wide midvein (G); fused leaves with wide midvein (H). (I) Percentages of leaves in phenotype classes. Difference between pin1 and WT, between pin1;6 and pin1, and between UBQ10::amiPIN6pin1 and pin1 was significant at P<0.001 (***) by Kruskal-Wallis and Mann-Whitney test with Bonferroni correction. Sample population sizes: WT, 65; pin2, 68; pin3, 68; pin4, 68; pin5, 68; pin6, 68; UBQ10::amiPIN6-5, 65; UBQ10::amiPIN6-10, 65; pin7, 68; pin8, 68; pin1, 71; pin1,2, 71; pin1,3, 77; pin1,4, 69; pin1,5, 72; pin1,6, 65; UBQ10::amiPIN6-5pin1, 65; UBQ10::amiPIN6-10pin1, 67; pin1,7, 77; pin1,8, 68. Bars: (B–F) 1.5 mm; (G,H) 0.75 mm.
response reporter [44]. In subepidermal tissues of WT leaves, DR5rev::YFPnuc was strongly expressed at sites of vein formation (Figure 5E); in subepidermal tissues of pin6;8 leaves, DR5rev::YFPnuc expression was weaker (Figure 5F), a defect that was normalized by pin5 (Figure 5G).

We then asked whether pin6;8 defects in auxin response levels and vein network formation were associated with changes in auxin-responsive PIN1 expression [10,44–46]. To address this question, we imaged expression of PIN1::PIN1:YFP [47] in the leaf area enclosed by the first loop. In both WT and pin6;8, PIN1::PIN1:YFP expression was initiated in broad domains that narrowed to sites of vein formation (Figure 5H–5M). However, in pin6;8 PIN1::PIN1:YFP expression was weaker (Figure 5H–5M), vein-associated domains of PIN1::PIN1:YFP expression became visible at earlier time-points (Figure 5H and 5I), and at each time point we observed more vein-associated domains of PIN1::PIN1:YFP expression (Figure 5H–5M). Defects in PIN1::PIN1:YFP expression of pin6;8 were normalized by pin5 (Figure 5N).

In summary, pin6;8 had defects in expression of DR5 and PIN1 and in formation of vein networks, and such defects were normalized by pin5.

Sufficient functions of PIN5, PIN6, and PIN8 in vein network formation

We next asked whether PIN6 function was sufficient to control expression of DR5 and PIN1 and formation of vein networks. To address this question, we expressed the PIN6 cDNA by the promoter of RIBOSOMAL PROTEIN S5A (AT3G11940) (RPS5A::PIN6) and by the promoter of MONOPTEROS (AT1G19850) (MP::PIN6), each highly active in developing leaves [48] (Figure S2F).

![Figure 2. PIN6 expression in leaf development.](image)

(A–O,Q–S) Top right: leaf age in days after germination (DAG), marker and genotype. Bottom left: reproducibility index. First leaves. (A–D) Midvein, loops and minor veins differentiate progressively later in the same region of the developing leaf, and loops and minor veins differentiate in a tip-to-base sequence during leaf development [6,7,57]; purple, green and magenta: successive stages of vein differentiation. Box in (D) illustrates position of close-ups in (I–L). (E–O) Confocal laser scanning microscopy with (E–H,L) or without (I–K,M–O,Q–S) transmitted light. (E–H) PIN6::PIN6:GFP expression. (I) Expression of PIN6::YFPnuc and PIN1::PIN1:CFP at 4 DAG; blue: chlorophyll. (J–L) PIN6::PIN6:GFP expression in WT (J) and pin1 (K) at 4 DAG. LUT (in K) visualizes expression levels. Dotted line: leaf outline. (M–O) Expression of PIN6::PIN6:GFP (M,Q), 35S::YFPer (N), 35S::LTI6B:YFP (R) and respective overlays displayed with a dual-channel LUT [66] (O,S); prevalence of cyan over colocalized magenta signal is shown in green, opposite in red, and colocalized cyan and magenta signals of equal intensity in yellow. (P,T) Quantification of colocalized GFP and YFP signals (as mean ± SE of Manders’ coefficient ‘r’) in populations (n = 11) of positive controls: J1721::GFPer·35S::YFPer (P), 35S::YFPpm;FM4-64 (T); negative controls: ATHB8::GFPnuc·35S::YFPer (P), J1721::GFPer·35S::YFPpm (T); and samples: PIN6::PIN6:GFP·35S::YFPer (P), PIN6::PIN6:GFP·35S::YFPpm (T). (P) Difference between negative control and positive control, and between negative control and sample was significant at P < 0.01 (** by one-way ANOVA and Tukey’s test. (T) Difference between positive control and negative control, and between positive control and sample was significant at P < 0.01 (** by one-way ANOVA and Tukey’s test. e, epidermis; hv, minor vein; l1, first loop; l2, second loop; mv, midvein. Bars: (E,I,L) 10 μm; (F,G) 20 μm; (H,J,K) 50 μm; (M–O) 5 μm; (Q–S) 2.5 μm.

doi:10.1371/journal.pgen.1003294.g002

![Figure 3. Genetic interaction between PIN1, PIN5, PIN6, and PIN8 in vein patterning.](image)

Percentages of leaves in phenotype classes (defined in Figure 1). Difference between pin1;6;8 and pin1;6, and between pin1;5;6;8 and pin1;6;8 was significant at P < 0.05 (*) or P < 0.001 (**) by Kruskal-Wallis and Mann-Whitney test with Bonferroni correction. Sample population sizes: pin1;6, 114; pin1;5;6, 92; pin1;6;8, 95; pin1;5;6;8, 114.

doi:10.1371/journal.pgen.1003294.g003

PLOS Genetics | www.plosgenetics.org 5 February 2013 | Volume 9 | Issue 2 | e1003294
Expression of the auxin-responsive markers DR5rev::YFPnuc, ATHB8::CFPnuc [49] and PIN1::PIN1::YFP was stronger in leaves of RPS5A::PIN6 and MP::PIN6 (Figure 6A–6G). Furthermore, in both these backgrounds lateral domains of PIN1::PIN1::YFP expression often failed to join distal veins (Figure 6E–6G), defects that correlated with the simpler, open vein networks of the mature leaves (Figure 6I–6M).

We then asked whether ectopic expression of PIN8 or PIN5 had any effect on vein network formation. To address this question, we analyzed vein patterns of plants expressing the cDNA of PIN8 or PIN5 by the MP promoter (MP::PIN8 or MP::PIN5, respectively). We found that MP::PIN8 leaves had simpler, open vein networks and that MP::PIN5 leaves had a more complex vein network (Figure 6I and 6N–6Q).

In summary, ectopic PIN6 expression was sufficient to control expression of DR5 and PIN1. Furthermore, ectopic expression of PIN6 or PIN8, on the one hand, and of PIN5, on the other, had opposite effects on vein network formation.

Simulation of pin1;6 defects by reduction of auxin levels in pin1

Weaker expression of auxin responsive markers in pin6;8 leaves and stronger expression of auxin responsive markers in leaves of RPS5A::PIN6 and MP::PIN6 suggest that PIN6-mediated auxin...
Figure 5. Necessity of PIN5, PIN6, and PIN8 for vein network formation. (A) Vein network complexity as mean ± SE number of vein branching points per first-leaf area unit in mm² [28]. Difference between pin6;8 and all other genotypes was significant at P<0.01 (**) by one-way ANOVA and Tukey’s test. Sample population sizes: WT, 30; pin5, 30; pin6, 30; pin8, 27; pin5;6, 28; pin5;8, 28; pin6;8, 28; pin5;6;8, 28. (B–N) Top right: genotype, markers and leaf age in days after germination (DAG). Bottom left: reproducibility index. (B–D) Dark-field illumination of cleared mature first leaves. (E–G) Confocal laser scanning microscopy; first leaves. LUT (in Figure 2K) visualizes expression levels. (E–G) DR5rev::YFPnuc expression, 4 DAG. Dotted line: leaf outline. (H–N) PIN1::PIN1:YFP expression. (O) 4-DAG first leaf illustrating positions of close-ups in (E–G) and (H–N). Bars: (B–D) 1.5 mm; (E–G,L–N) 50 µm; (H–K) 25 µm.

doi:10.1371/journal.pgen.1003294.g005
Figure 6. Sufficiency of PIN5, PIN6, and PIN8 for vein network formation. (A–G,J–P) Top right: genotype, markers and leaf age in days after germination (DAG). Bottom left: reproducibility index. (A–G) Confocal laser scanning microscopy; first leaves. LUT (in Figure 2K) visualizes expression levels. (J–P) Dark-field (J–LN,P) or differential-interference-contrast (M,O) illumination of cleared mature first leaves. (A,B) DR5rev::YFP:nuc expression. (C,D) ATHB8::CFP:nuc expression. Magenta line connects nuclei in the first loop (l1). Dotted line: leaf outline. (E–G) PIN1::PIN1::YFP expression. (H) 4-DAG first leaf illustrating positions of close-ups in (A–D), (E–G) and (M,O). (I) Percentage of first leaves with 0, 1, 2 or 3 free auxin by its conjugation to lysine [50,51]. Expression of PIN6:iaaL (PIN6 promoter driving expression of IAA) in pin1 mimicked the phenotype spectrum of pin1:6 leaves (Figure 7) and cotyledons (Figure S1), a finding that is consistent with the hypothesis that PIN6-mediated intracellular auxin transport increases auxin levels.

Discussion

The mechanisms that control the patterning of vein networks of plant leaves have long fascinated biologists and mathematicians. Varied evidence has increasingly been accumulating that supports an inductive and orienting role for the transport of the plant signal auxin in leaf vein patterning [3,5–7,10–13], but molecular details have remained unclear. Here we show that the vein network of the Arabidopsis leaf is patterned by two distinct and convergent auxin-transport pathways: an intercellular pathway mediated at the plasma membrane (PM) by the PIN1 auxin transporter and an intracellular pathway mediated at the endoplasmic reticulum (ER) by the PIN6, PIN8 and PIN5 auxin transporters. While a role for other families of auxin transporters (e.g., [30–32]) in vein patterning is by no means precluded, our results suggest a new, unsuspected level of control of vein patterning by PIN-mediated auxin transport, one with repercussions on patterning of other plant features.

The localization of PIN1 to the PM [8] and of PIN6 to the ER together with the appearance in pin1:6 of vein pattern defects that exceed the sum of the single-mutant defects suggest that PIN1 and PIN6 act in distinct auxin-transport pathways whose functions converge on vein patterning. By contrast, the overlapping subcellular localizations of PIN6 and PIN8 [36–39] together with the purely quantitative enhancement of pin1:6 vein pattern defects by pin8 suggest redundant function of PIN6 and PIN8 in PIN1-independent vein patterning. Further, the overlapping subcellular localization of PIN8 [36–39] and PIN5 [36,37,39] together with the partial normalization of pin1:6;8 vein pattern defects by pin5 suggest antagonistic functions of PIN8 and PIN5 in PIN1/PIN6-dependent vein patterning. The interaction between the intercellular auxin-transport pathway controlled by PIN1 and the intracellular auxin-transport pathway controlled by PIN6, PIN8 and PIN5 is relevant for at least two other patterning events, separation of cotyledons and of leaves, and might have implications for other developmental processes. The redundancy between PIN6 and PIN8 and the antagonism between PIN8 and PIN5 are, however, independent of PIN1-mediated intercellular auxin transport: in the presence of PIN1 function, PIN6 and PIN8 redundantly control vein network formation, and such redundant functions are antagonized by PIN5. Redundant functions of PIN6 and PIN8 and antagonistic functions of PIN5, as inferred from loss-of-function data, are also consistent with gain-of-function evidence: ectopic PIN8 expression induces vein pattern defects similar to those induced by ectopic PIN6 expression and opposite to those of pin6;8, suggesting that PIN8 can supply vein patterning functions similar to those of PIN6; and ectopic PIN5 expression induces vein pattern defects opposite to those induced by ectopic expression of PIN6 or PIN8 and similar to those of pin6;8, suggesting that PIN5 can supply vein patterning functions opposite to those of PIN6 and PIN8. The internally consistent relationship between the effects of loss of function of PIN6, PIN8 and PIN5 and the effects of gain of function of these genes is not limited to vein patterning but extends to auxin response: auxin response levels are decreased by simultaneous mutation of PIN6 and PIN8 and by ectopic expression of PIN5 [36] and increased by ectopic expression of PIN6 or PIN8 [39] and by mutation of PIN5 [36]. Thus, the genetic interaction between PIN6, PIN8 and PIN5 might reflect general properties of the mechanism with which these proteins function, a conclusion that is also supported by the antagonistic functions of PIN8 and PIN5 in a process unrelated to vein patterning such as pollen development [39]. The most parsimonious explanation for the antagonistic interaction between PIN6/PIN8 and PIN5, which transports auxin from the cytoplasm to the ER lumen [36], is that PIN6 and PIN8 transport auxin from the ER lumen to the cytoplasm or to the nucleus, whose envelope is continuous with the ER membrane [52,53] (Figure 8C). This scenario is also supported by the observation that reducing auxin levels in pin1 leads to pin1:6 characteristic defects, and is consistent with higher levels of auxin measured in PIN8 overexpressors [38,39] and pin5 mutants [36]. Alternatively, or in addition, PIN6, PIN8 and PIN5 could all transport in the same direction but have different affinities for auxins or auxin conjugates with different, even opposing, developmental functions reviewed in [54] (Figure 8D).

Our data suggest that auxin transported by PIN6 and PIN8 increases levels of auxin-responsive PIN1 expression during vein formation (Figure 8A). In pin5;8, low auxin levels at sites of vein formation could accelerate formation of narrow vein-associated domains of PIN1 expression, thus resulting in high-complexity vein networks [13,55–58]. Conversely, in leaves ectopically expressing PIN6, high auxin levels at sites of vein formation could hinder or prevent formation and connection of vein-associated PIN1-expression domains, thus resulting in simple, open vein networks [13,36,57,59–61]. Alternatively, PIN6/PIN8-mediated auxin transport could control vein network formation exclusively through PIN1-independent pathways; defects in auxin response or PIN1 expression would then be consequence, rather than cause, of defective vein-network formation (Figure 8B). In any case, the synthetic enhancement of pin1 defects by pin5 and pin6;8, as opposed to the epistasis of pin6;8 to pin1, suggests vein patterning functions of PIN6/PIN8-mediated intracellular auxin transport beyond regulation of PIN1 expression or of intercellular auxin transport mediated by PM-localized PIN proteins (Figure 8A and 8B). We can probably exclude that such functions of PIN6 and PIN8 require physical interaction with PIN1 because PIN1 and PIN6/PIN8 localize to different cellular compartments and because PIN1/pin1/pin6/pin8 lacks defects in cotyledons and vein patterns (n>50), defects that would be expected for interacting
proteins (reviewed in [62]). Because the localization of PIN6 and PIN8 is maintained in pin1 backgrounds, it seems unlikely that these two proteins have auxin transport functions that are redundant and homologous with those of PIN1. Nevertheless, PIN6/PIN8/PIN5-mediated intracellular auxin-transport could contribute to intercellular auxin transport by a mechanism nonhomologous to that used by PM-localized PIN proteins [63] (Figure 8A and 8B), a function of ER-localized PIN proteins that would be particularly relevant in primitive land plants like mosses, which seem to lack PM-localized PIN proteins [36] but do select cell files for vascular function [64].

**Materials and Methods**

**Plants**

Origin and nature of lines, genotyping strategies and oligonucleotide sequences are in Tables S1, S2, S3, respectively. Seeds were sterilized and germinated, and plants were grown and transformed as in [65].

**Imaging**

For plasma-membrane stainings, seedlings were incubated in 10 μg ml⁻¹ FM 4-64 (Invitrogen) for ~4 min under vacuum before mounting. For endoplasmic-reticulum stainings, dissected leaves were incubated in 10 μM ER-Tracker Red (Invitrogen/Life Technologies) or ER-Tracker Blue-White DPX (Invitrogen/Life Technologies) for ~30 min under vacuum before mounting. Leaf primordia were mounted in water under 0.17-mm-thick coverslips (Fischer Scientific) and imaged with the 20×/0.8 Plan-Apochromat or 40×/1.2 W C-Apochromat objective of an Axio Imager.M1/LSM 510 META confocal microscope (Carl Zeiss). 512×512-pixel frames were scanned unidirectionally at 8-bit depth with 1.6-μsec pixel dwell time.

---

**Figure 7. Control of PIN1-dependent vein patterning by intracellular auxin levels.** (A) Percentages of leaves in phenotype classes (defined in Figure 1). Difference between pin1 and WT, between pin1;6 and pin1, and between PIN6::iaaL::pin1 and pin1 was significant at P<0.001 (**) by Kruskal-Wallis and Mann-Whitney test with Bonferroni correction. Sample population sizes: WT, 50; PIN6::iaaL-15, 50; PIN6::iaaL-12, 50; pin1, 61; pin1;6, 61; PIN6::iaaL-15::pin1, 60; PIN6::iaaL-12::pin1, 62. (B,C) Dark-field illumination of cleared mature first leaves. Top right: genotype. Bottom left: phenotype class. Bars: (B,C) 2 mm. doi:10.1371/journal.pgen.1003294.g007
and 8-fold averaging. Scanning zoom was adjusted to set pixel size to half the spacing of the features to be resolved, with minimum pixel size equal to half the objective lateral resolving power. Emission was collected from 5-mm-thick (single-fluorophore imaging) or 1-mm-thick (multi-fluorophore imaging) optical slices. Amplifier gain was set at 1; detector gain at 50–60%. Laser transmission and offset value were adjusted to match signal to detector's input range. Marker-line-specific imaging parameters are in Tables S4 and S5. For multi-fluorophore imaging, sequential excitation and collection of emission were performed in line-by-line channel-switching mode, which did not result in signal crossover under our imaging conditions. Signal levels in images acquired at identical settings were visualized as in [65]. Signal colocalization was visualized as in [66] and quantified as in [67]. Mature leaves were imaged as in [49]. Images were analyzed and processed with ImageJ (National Institutes of Health), and figures were generated with Canvas (ACD Systems International Inc.).

Supporting Information

Figure S1 Cotyledon patterns of pin mutants. (A–G) Dark-field illumination of 4-day-old seedlings illustrating phenotype classes: two separate cotyledons (A); fused cotyledons and separate single cotyledon (B); three separate cotyledons (C); fused cotyledons (D); single cotyledon (E); partially fused cup-shaped cotyledon, side view; inset: top view (F); completely fused cup-shaped cotyledon, side view; inset: top view (G). (H) Percentages of seedlings in phenotype classes. Difference between pin₁ and WT, between pin₁;₆ and pin₁;₁₈, between UBQ10::amiPIN₆-₁₀ and pin₁, between pin₁;₆;₈ and pin₁;₁₆, and between PIN6::iaaL;pin₁ and pin₁ was significant at P < 0.001 (***). Sample population sizes: WT, 50; pin₂, 50; pin₃, 50; pin₄, 50; pin₅, 50; pin₆, 50; UBQ10::amiPIN₆-₃, 50; UBQ10::amiPIN₆-₁₀, 50; pin₇, 50; pin₈, 50; PIN6::iaaL-₁₅, 50; PIN6::iaaL-₁₂, 50; pin₁, 61; pin₁, 2₅; pin₁;₃, 4₅; pin₁;₄, 4₉; pin₁;₅, ₄₇; pin₁;₆, 4₃; pin₁;₇, 4₈; pin₁;₈, 4₉; pin₁;₉, 4₅; pin₁;₁₀, 4₁; pin₁;₁₁, 3₉; pin₁;₁₂, 3₁; pin₁;₁₃, 3₇; pin₁;₁₄, 3₄; pin₁;₁₅, 3₂; pin₁;₁₆, 2₉; pin₁;₁₇, 2₇; pin₁;₁₈, 2₂; pin₁;₁₉, 2₁; pin₁;₂₀, 1₉; pin₁;₂₁, 1₇; pin₁;₂₂, 1₅; pin₁;₂₃, 1₃; pin₁;₂₄, 1₂; pin₁;₂₅, 1₁; pin₁;₂₆, 1₀; pin₁;₂₇, 9; pin₁;₂₈, 8; pin₁;₂₉, 7; pin₁;₃₀, 6; pin₁;₃₁, 5; pin₁;₃₂, 4; pin₁;₃₃, 3; pin₁;₃₄, 2; pin₁;₃₅, 1; pin₁;₃₆, 0; pin₁;₃₇, 0; pin₁;₃₈, 0; pin₁;₃₉, 0; pin₁;₄₀, 0; pin₁;₄₁, 0; pin₁;₄₂, 0; pin₁;₄₃, 0; pin₁;₄₄, 0; pin₁;₄₅, 0; pin₁;₄₆, 0; pin₁;₄₇, 0; pin₁;₄₈, 0; pin₁;₄₉, 0; pin₁;₅₀, 0.
Figure S2  Expression of PIN6, PIN8 and MP in leaf development. (A–G) Confocal laser scanning microscopy with (A–C,E,F) or without (D,G) transmitted light. Top right: reporters and leaf age in days after germination (DAG). Bottom left: reproducibility index. First leaves. (D,E) Blue: chlorophyll. (E) Close-up of area as boxed in D. (G) Expression at 3.25 DAG of PIN8::PIN8:GFP (left), staining by ER-Tracker Blue-White DPX (centre) and their overlay displayed with a dual-channel LUT (defined in Figure 2) (right). e, epidermis. Bars: (A–L) 10 μm; (C,F) 50 μm; (D) 100 μm; (E) 20 μm; (G) 2 μm. (TIF)

Figure S3  Colocalization analysis of PIN6 with endoplasmic-reticulum or plasma-membrane markers. (A–O) Confocal laser scanning microscopy. First leaves. Expression of J1721:GFPPer (A,M), 35S:YFPPer (B,E), ATHB8:GFPnuc (D), PIN6::PIN6:GFP (G), 35S:RTNLB4:YFP (H), 35S:YFPpm (J,N), FM4-64 (K) and respective overlays displayed with a dual-channel LUT (defined in Figure 2) (C,F,I,L,O). Bars: (A–L) 5 μm; (M–O) 2.5 μm. (TIF)

References

1. Foster AS (1952) Foliar Vascularization in Angiosperms from an Oogenetic Standpoint. American Journal of Botany 39: 752–766.
2. Pray TR (1955) Foliar vascularization in Angiosperms. II. Histogenesis of the venation of Liriodendron. American Journal of Botany 42: 18–27.
3. Sachs T (1989) The development of vascular networks during leaf development. Current Topics in Plant Biochemistry and Physiology 8: 168–183.
4. Berleth T, Mattsson J, Hardiké CS (2000) Vascular continuity and auxin signals. Trends in Plant Science 5: 367–373.
5. Gersani M (1987) The Induction of Differentiation of Organized Vessels in a Storage Organ. Annals of Botany 59: 31–34.
6. Mattsson J, Sunf ZK, Berleth T (1999) Responses of plant vascular systems to auxin transport inhibition. Development 126: 2979–2991.
7. Sieburth LE (1999) Auxin is required for leaf vein pattern in Arabidopsis. Plant Physiology 121: 1179–1190.
8. Galweiler L, Guan C, Muller A, Wisman E, Mendgen K, et al. (1998) Regulation of polar auxin transport by AtPIN1 in Arabidopsis vascular tissue. Science 282: 2226–2230.
9. Petrova J, Mraevc J, Rouchaud R, Blakeslee JJ, Abas M, et al. (2006) PIN proteins perform a rate-limiting function in cellular auxin efflux. Science 314: 918–919.
10. Searpella E, Marcos D, Friml J, Berleth T (2006) Control of leaf vascular patterning by polar auxin transport. Genes & Development 20: 1015–1027.
11. Wenzel CL, Schuetz M, Yu Q, Mattsson J (2007) Dynamics of MONOPTEROS and PIN-FORMED1 expression during leaf vein pattern formation in Arabidopsis thaliana. The Plant Journal 49: 387–398.
12. Sachs T (1991) Cell Polarity and Tissue Patterning in Plants. Development Supplement 1: 83–93.
13. Sachs T (1981) The control of the patterned differentiation of vascular tissues. Advances in Botanical Research 9: 151–262.
14. Garnett P, Steinacher A, Stepney S, Clayton R, Leyer O (2010) Computer simulation: the imaginary friend of auxin transport biology. Biosciences 32: 828–835.
15. Krupinski P, Josson H (2010) Modeling auxin-regulated development. Cold Spring Harbor Perspectives in Biology 2: a001560. doi: 00150100011005010001560.
16. Santos F, Teale W, Fleck C, Volpers M, Ruperti B, et al. (2010) Modelling polar auxin transport in developmental patterning. Plant Biology 12 Supplement 1: 3–14.
17. Smith RS, Bayer EM (2009) Auxin transport-feedback models of patterning in plants. Plant Cell & Environment 32: 1250–1271.
18. Unnik K, Gosciewski W, Friml J, Käfer-Vehn, J (2011) Feedback models for polarized auxin transport: an emerging trend. Molecular Biosystems 7: 2352–2359.
19. Billborough GD, Runions A, Barkoulas M, Jenkins HW, Hasson A, et al. (2011) Models for the regulation of Arabidopsis thaliana leaf margin development. Proceedings of the National Academy of Sciences, USA 108: 3424–3429.
20. Guentz B, Bayer E, Kierzkowski D, Smith RS, Mandel T, et al. (2012) PIN1-Independent Leaf Initiation in Arabidopsis. Plant Physiology 159: 1301–1510.

Table S1  Origin and nature of lines. (DOC)

Table S2  Genotyping strategies. (DOC)

Table S3  Oligonucleotide sequences. (DOC)

Table S4  Imaging parameters: single-marker lines. (DOC)

Table S5  Imaging parameters: multi-marker lines. (DOC)

Acknowledgments

We thank the Arabidopsis Biological Resource Center, Ikram Blilou, Sean Cutler, Taku Demura, Mark Estelle, Harry Klee, Elliot Meyerowitz, Ben Scheres, Benoît Schoefs, and Tyler Donner for kindly providing material and Thomas Berleth, Tyler Donner, Giorgio Morelli, and Osama Odat for invaluable comments on the manuscript.

Author Contributions

Conceived and designed the experiments: MGS ES. Performed the experiments: MGS AE ES. Analyzed the data: MGS ES. Wrote the paper: MGS ES.
Leaf Vein Patterning by Auxin Transport

38. Bosco CD, Dovzenko A, Liu X, Woerner N, Rensch T, et al. (2012) The endoplasmic reticulum localized PIN8 is a pollen specific auxin carrier involved in intracellular auxin homeostasis. The Plant Journal 71: 860–870.

39. Ding Z, Wang B, Moreno I, Duglaskova N, Simon S, et al. (2012) ER-localized auxin transporter PINII regulates auxin homeostasis and male gametophyte development in Arabidopsis. Nature Communications 3: 941. doi: 10.1038/ncomms1941.

40. Yamamuku S, Sakata-Sogawa K, Hasegawa A, Suzuki T, Kubo K, et al. (2007) Zinc is a novel intracellular second messenger. Journal of Cell Biology 177: 657–665.

41. Don AS, Martinez-Lamenca C, Webb VR, Proia RL, Roberts E, et al. (2007) Essential requirement for sphingosine kinase 2 in a sphingolipid apoptosis pathway activated by FasL2 analogues. Journal of Biological Chemistry 282: 15033–15042.

42. Tan Y, Dourlin N, Wu C, De Veyra T, Elce JS, et al. (2006) Ubiquitous calpains promote caspase-12 and JNK activation during endoplasmic reticulum stress-induced apoptosis. Journal of Biological Chemistry 281: 16016–16024.

43. Cole I, Davies D, Hyde CJ, Ashford AE (2009) ER-Tracker dye and BODIPY-brefeldin A differentiate the endoplasmic reticulum and golgi bodies from the tubular-vacuole system in living hyphae of Podosphaera xanthii. Journal of Microscopy 197: 239–249.

44. Heidler MG, Ohno C, Das P, Sieber P, Reddy GV, et al. (2005) Patterns of Auxin Transport and Gene Expression during Primordium Development Revealed by Live Imaging of the Arabidopsis Inflorescence Meristem. Current Biology 15: 1899–1911.

45. Vierten A, Vanneste S, Wisniewska J, Benkova E, Benjamin R, et al. (2005) Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. Development 132: 4521–4531.

46. Nembrainer J, Hong F, Chory J (2006) Different plant hormones regulate similar processes through largely nonoverlapping transcriptional responses. Cell 126: 467–475.

47. Xu J, Hoffman H, Heidstra R, Sauer M, Frimel J, et al. (2006) A molecular framework for plant regeneration. Science 313: 385–390.

48. Weijers D, Fraenke-van Dijk M, Venken RJ, Quint A, Hoogenraad, P, et al. (2001) An Arabidopsis Minute-like phenotype caused by a semi-dominant mutation in a RIBOSOMAL PROTEIN S5 gene. Development 129: 4289–4299.

49. Donner TJ, Sherr I, Scarpella E (2009) Regulation of prepocambial cell state acquisition by auxin signaling in Arabidopsis leaves. Development 136: 3235–3246.

50. Romano CP, Hein MB, Klee HJ (1991) Inactivation of auxin in tobacco transformed with the indoleacetic acid-lysine synthetase gene of Pseudomonas savastanoi. Genes & Development 5: 438–446.

51. Jensen PJ, Hanger RP, Estelle M (1998) Auxin transport is required for hypocotyl elongation in light-grown but not dark-grown Arabidopsis. Plant Physiology 116: 455–462.

52. Graumann K, Evans DE (2011) Nuclear envelope dynamics during plant cell division suggest common mechanisms between kingdoms. Biochemistry Journal 453: 661–667.

53. Oda Y, Fukushima H (2011) Dynamics of Arabidopsis SUN proteins during mitosis and their involvement in nuclear shaping. The Plant Journal 66: 629–641.

54. Ludwig-Muller J (2012) Auxin conjugates: their role for plant development and in the evolution of land plants. Journal of Experimental Botany 62: 1757–1773.

55. Rolland-Lagan AG, Prusinkiewicz P (2005) Reviewing models of auxin canalization in the context of leaf vein pattern formation in Arabidopsis. The Plant Journal 44: 854–865.

56. Wabnik K, Kleine-Vehn J, Balla J, Sauer M, Naramoto S, et al. (2010) Emergence of tissue polarization from synergy of intracellular and extracellular auxin signaling. Molecular Systems Biology 6: 447. doi: 10.1038/msb.2010.103.

57. Vieten A, Vanneste S, Wisniewska J, Benkova E, Benjamin R, et al. (2005) Functional redundancy of PIN proteins is accompanied by auxin-dependent cross-regulation of PIN expression. Development 132: 4521–4531.

58. McKown AD, Dengler NG (2009) Shifting in leaf vein density through accelerated vein formation in C4 Flaveria (Asteraceae). Annals of Botany 104: 1085–1096.

59. Prusinkiewicz P, Crawford S, Smith RS, Ljung K, Bennett T, et al. (2009) Control of bud activation by an auxin transport switch. Proceedings of the National Academy of Sciences, USA 106: 17431–17436.

60. Balla J, Kaloszsek P, Reinold V, Frimel J, Posezhak S (2011) Competitive canalization of PIN-dependent auxin flow from auxiliary buds controls pea bud outgrowth. The Plant Journal 65: 571–577.

61. Sachs T (1970) A control of bud growth by vascular tissue differentiation. Israel Journal of Botany 19: 484–498.

62. Hovey RS, Gilliland WD (2006) Sometimes the result is not the answer: the truths and the lies that come from using the complementation test. Genetics 174: 3–13.

63. Wabnik K, Kleine-Vehn J, Govaerts W, Frimel J (2011) Prototype cell-to-cell auxin transport mechanism by intracellular auxin compartmentalization. Trends in Plant Science 16: 468–475.

64. Lajtay M, Dautt JG, Renzaglia KS (2000) Conducting tissues and phyletic relationships of bryophytes. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences 355: 795–813.

65. Sawchuk MG, Donner TJ, Head P, Scarpella E (2008) Unique and overlapping expression patterns among members of photosynthesis-associated nuclear gene families in Arabidopsis. Plant Physiology 148: 1980–1992.

66. Demondi D, Davoust J (1997) Multicolor analysis and local image correlation in confocal microscopy. Journal of Microscopy 185: 21–36.

67. Manders EMM, Verbeek IJ, Aten JA (1993) Measurement of Colocalization of Objects in Dual-Color Confocal Images. Journal of Microscopy 169: 375–382.