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RESEARCH REPORT

Vein patterning by tissue-specific auxin transport

Priyanka Govindaraju, Carla Verna*, Tongbo Zhu‡ and Enrico Scarpella§

ABSTRACT

Unlike in animals, in plants, vein patterning does not rely on direct cell-cell interaction and cell migration; instead, it depends on the transport of the plant hormone auxin, which in turn depends on the activity of the PIN-FORMED1 (PIN1) auxin transporter. The current hypotheses of vein patterning by auxin transport propose that, in the epidermis of the developing leaf, PIN1-mediated auxin transport converges to peaks of auxin level. From those convergence points of epidermal PIN1 polarity, auxin would be transported in the inner tissues where it would give rise to major veins. Here, we have tested predictions of this hypothesis and have found them unsupported: epidermal PIN1 expression is neither required nor sufficient for auxin transport-dependent vein patterning, whereas inner-tissue PIN1 expression turns out to be both required and sufficient for auxin transport-dependent vein patterning. Our results refute all vein patterning hypotheses based on auxin transport from the epidermis and suggest alternatives for future tests.

KEY WORDS: *Arabidopsis thaliana*, Leaf development, Vascular patterning, Auxin, PIN genes, Necessity and sufficiency

INTRODUCTION

Most multicellular organisms solve the problem of long-distance transport of signals and nutrients by means of tissue networks such as the vascular system of vertebrate embryos and the vein networks of plant leaves; therefore, how vascular networks form is a key question in biology. In vertebrates, the formation of the embryonic vascular system relies on direct cell-cell interaction and at least in part on cell migration (e.g. Noden, 1988; Xue et al., 1999). Both direct cell-cell interaction and cell migration are precluded in plants by a wall that keeps cells apart and in place; therefore, vascular networks form differently in plant leaves.

How leaf vein networks form is unclear, but available evidence suggests that polar transport of the plant hormone auxin is non-redundantly required for vein patterning (Mattsson et al., 1999; Sieburth, 1999). Such non-redundant functions of polar auxin transport in vein patterning in turn depend on non-redundant functions of the PIN-FORMED1 (PIN1) auxin transporter (Galweiler et al., 1998; Petrasek et al., 2006; Sawchuk et al., 2013; Zourelidou et al., 2014; Verna et al., 2019). In developing leaves, PIN1 polar localization at the plasma membrane of epidermal cells is directed toward single cells along the marginal epidermis (Benková et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005; Hay et al., 2006; Scarpella et al., 2006; Wenzel et al., 2007; Bayer et al., 2009). These convergence points of epidermal PIN1 polarity are associated with broad domains of PIN1 expression in the inner tissue of the developing leaf; over time, these broad domains will become restricted to the narrow sites where midvein and lateral veins will form.

Consistent with these observations, the prevailing hypotheses of vein patterning propose that convergence points of epidermal PIN1 polarity contribute to the formation of local peaks of auxin level in the epidermis, and that auxin is transported by PIN1 from the epidermal convergence points into the inner tissue of the leaf, where it will lead to vein formation (reviewed by Prusinkiewicz and Runions, 2012; Bennett et al., 2014; Runions et al., 2014; Linh et al., 2018). Similar hypotheses propose that convergence points of epidermal PIN1 polarity lead to the positioning, growth and differentiation of flower primordia; the positioning of leaf primordia; the formation of dissected leaves; and the formation of leaf serrations (Benková et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005; Hay et al., 2006; Barkoulas et al., 2008). All these hypotheses share the prediction that epidermal PIN1 expression is required for the process controlled by *PIN1*. Such a prediction is supported by experimental evidence for all those processes except vein patterning, for which the prediction has not been tested (Bilsborough et al., 2011; Kierzkowski et al., 2013, 2019; Li et al., 2019 preprint).

Here we have tested this prediction and have found it unsupported: unlike other *PIN1*-dependent processes, epidermal PIN1 expression is neither required nor sufficient for auxin transport-dependent vein patterning; instead, PIN1 expression in the inner tissues turns out to be both required and sufficient for auxin transport-dependent vein patterning. Our results point to a mechanistic difference between vein patterning and other auxin transport-dependent processes, refute all the current hypotheses of vein formation that depend on auxin transport from the epidermis, and suggest alternatives for future testing.

RESULTS AND DISCUSSION

PIN1 expression during *Arabidopsis* vein patterning

In *Arabidopsis* leaf development, the formation of the midvein precedes the formation of the first loops of veins (‘first loops’), which in turn precedes the formation of the second loops (Mattsson et al., 1999; Sieburth, 1999; Kang and Dengler, 2004; Scarpella et al., 2004; Sawchuk et al., 2007) (Fig. 1A-C). The formation of second loops precedes the formation of third loops and that of minor veins in the area delimited by the midvein and the first loops (Fig. 1C,D). Loops and minor veins form first near the top of the leaf and then progressively closer to its bottom, and minor veins form after loops in the same area of the leaf (Fig. 1B-D).

Consistent with previous reports (Benková et al., 2003; Reinhardt et al., 2003; Heisler et al., 2005; Scarpella et al., 2006; Sawchuk et al., 2007, 2013; Wenzel et al., 2007; Bayer et al., 2009; Marcos and Berleth, 2014; Verna et al., 2019), a fusion of the

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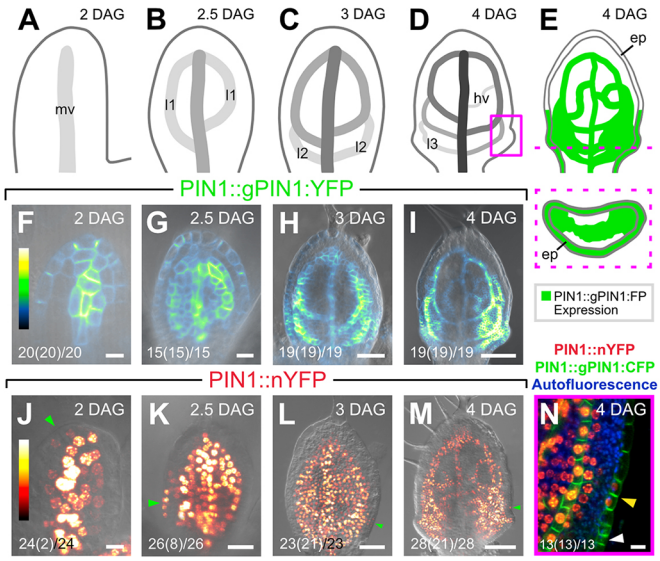

Fig. 1. PIN1 expression during *Arabidopsis* vein patterning. (A-N) Top right: leaf age in days after germination (DAG). Abaxial side to the left in A,F,J. (A-D) Midvein, loops and minor veins form sequentially during leaf development (Mattsson et al., 1999; Sieburth, 1999; Kang and Dengler, 2004; Scarpella et al., 2004; Sawchuk et al., 2007); increasingly darker grays depict progressively later stages of vein development. Box in D illustrates the position of close-ups in N, and in Figs 2D,J and 4D,J. (E) Map of PIN1::gPIN1:FP expression in developing leaves. For simplicity, changes in expression level occurring during vein development have not been represented. Dashed line illustrates the position of the transverse section shown in the dashed box. See text for details. (F-N) Confocal laser scanning microscopy with (F-M) or without (N) transmitted light. Bottom left: reproducibility index, i.e. number of leaves with the displayed inner-tissue expression (number of leaves with the displayed epidermal expression)/number of leaves analyzed. Lookup tables in F-I (ramp in F) and in J-M (ramp in J). These visualize expression levels. Green arrowheads in J-M and yellow arrowhead in N indicate epidermal expression; white arrowhead in N indicates a convergence point of PIN1 polarity. ep, epidermis; hv, minor vein; l1, first loop; l2, second loop; l3, third loop; mv, midvein. Scale bars: 10 µm in F,J,N; 20 µm in G,K; 50 µm in H,L; 100 µm in I,M.

PIN-FORMED1 (*PIN1*) open reading frame to YFP driven by the *PIN1* promoter (PIN1::gPIN1:YFP) (Xu et al., 2006) was expressed in all the cells of the leaf at early stages of tissue development; over time, however, epidermal expression became restricted to the basalmost cells, and inner-tissue expression became restricted to developing veins (Fig. 1E–I).

We asked whether PIN1::gPIN1:YFP expression were recapitulated by the activity of the *PIN1* promoter. To address this question, we imaged expression of a nuclear YFP driven by the *PIN1* promoter (PIN1::nYFP) in first leaves 2, 2.5, 3 and 4 days after germination (DAG).

Just like PIN1::gPIN1:YFP (Fig. 1E–I), PIN1::nYFP was expressed in all the inner cells of the leaf at early stages of tissue development, and over time this inner-tissue expression became restricted to developing veins (Fig. 1J-M). However, unlike PIN1::gPIN1:YFP and PIN1::gPIN1:CFP (Gordon et al., 2007) (Fig. 1E-I, N), PIN1::nYFP was expressed in very few epidermal cells at the tip of 2-DAG primordia and at the margin of 2.5-DAG primordia, and this epidermal expression was very rare (Fig. 1J,K). PIN1::nYFP expression in epidermal cells at the leaf margin was more frequent at 3 and 4 DAG but was still limited to very few cells (Fig. 1L-N). Moreover, these PIN1::nYFP-expressing epidermal cells were not those that contributed to convergence points of epidermal PIN1 polarity (Fig. 1N).

Because a fusion of the *PIN1* coding sequence to GFP driven by the *PIN1* promoter (PIN1::cPIN1:GFP) was hardly expressed in leaf epidermal cells (Fig. 2C,D,I,J), we conclude that the already limited activity of the *PIN1* promoter in the leaf epidermis is suppressed by the *PIN1* coding sequence and that the leaf epidermal expression characteristic of PIN1 is encoded in the introns of the gene.

Tissue-specific PIN1 expression in *PIN1* non-redundant functions in vein patterning

During leaf development, PIN1 is expressed in all the tissues – the epidermis, the vascular tissue and the nonvascular inner tissue (Fig. 1). We asked what the function in *PIN1*-dependent vein patterning was of PIN1 expression in these tissues. To address this question, we expressed the following in the wild-type and *pin1* mutant backgrounds: (1) PIN1::gPIN1:GFP, which, like PIN1::gPIN1:YFP and PIN1::gPIN1:CFP (Fig. 1E-I,N), is expressed in all the tissues of the developing leaf (Fig. 2A,G); (2) cPIN1:GFP driven by the epidermis-specific *ARABIDOPSIS THALIANA MERISTEM LAYER1* promoter (Sessions et al., 1999) (ATML1::cPIN1:GFP) (Fig. 2B,H); (3) PIN1::cPIN1:GFP, which is expressed in the leaf inner tissues (Fig. 2C,D,I,J); (4) cPIN1:GFP driven by the vascular-tissue-specific *SHORT-ROOT* promoter (Gardiner et al., 2011) (SHR::cPIN1:GFP) (Fig. 2E,K); and (5) cPIN1:GFP driven by the *SCARECROW-LIKE32* promoter, which is active in the nonvascular inner tissue of the leaf (Gardiner et al., 2011) (SCL32::cPIN1:GFP) (Fig. 2F,L). We then compared vein patterns of mature first leaves of the resulting backgrounds.

Consistent with previous reports (Sawchuk et al., 2013; Verna et al., 2019), the vein patterns of nearly 50% of *pin1* leaves were abnormal (Fig. 2M-P). The vein patterns of PIN1::gPIN1:GFP, ATML1::cPIN1:GFP, PIN1::cPIN1:GFP, SHR::cPIN1:GFP and SCL32::cPIN1:GFP were no different from the wild-type vein pattern (Fig. 2M-P). PIN1::gPIN1:GFP and PIN1::cPIN1:GFP normalized the phenotype spectrum of *pin1* vein patterns (Fig. 2M-P; Fig. S1A,C), and SHR::cPIN1:GFP shifted the phenotype spectrum of *pin1* vein patterns toward the wild-type vein pattern (Fig. 2M-P; Fig. S1D). By contrast, the vein pattern defects of ATML1::cPIN1:GFP;*pin1* and SCL32::cPIN1:GFP;*pin1* were no different from those of *pin1* (Fig. 2M-P; Fig. S1B,E). We observed a similar effect of tissue-specific PIN1 expression in *PIN1*-dependent cotyledon patterning (Fig. S2).

Consistent with interpretation of similar findings in other organisms (e.g. Cherbas et al., 2003; Soloviev et al., 2011; Topalidou and Miller, 2017; Wisidagama et al., 2019), we conclude that PIN1 expression in the epidermis is neither required nor sufficient for *PIN1*-dependent vein patterning. By contrast, PIN1 expression in the inner tissues of the leaf is both required and sufficient for *PIN1*-dependent vein patterning. Such function of PIN1 expression seems to mainly depend on PIN1 expression in the vascular tissue: only PIN1 expression in the vascular tissue, and not PIN1 expression in the nonvascular inner tissues of the leaf, is required for *PIN1*-dependent vein patterning. Even though it is only the combined expression of PIN1 in the vascular and nonvascular inner tissues of the leaf that is sufficient for *PIN1*-dependent vein patterning, the contributions to such sufficiency of PIN1 expression in the vascular tissue and of PIN1 expression in the nonvascular inner tissues of the leaf are unequal: PIN1 expression in the vascular tissue is sufficient for most of the *PIN1* functions in vein patterning, but PIN1 expression in the nonvascular tissues of the leaf is sufficient for none.

Unlike for *PIN1*-dependent vein patterning, PIN1 expression by the *ATML1* promoter is required and sufficient for the positioning,

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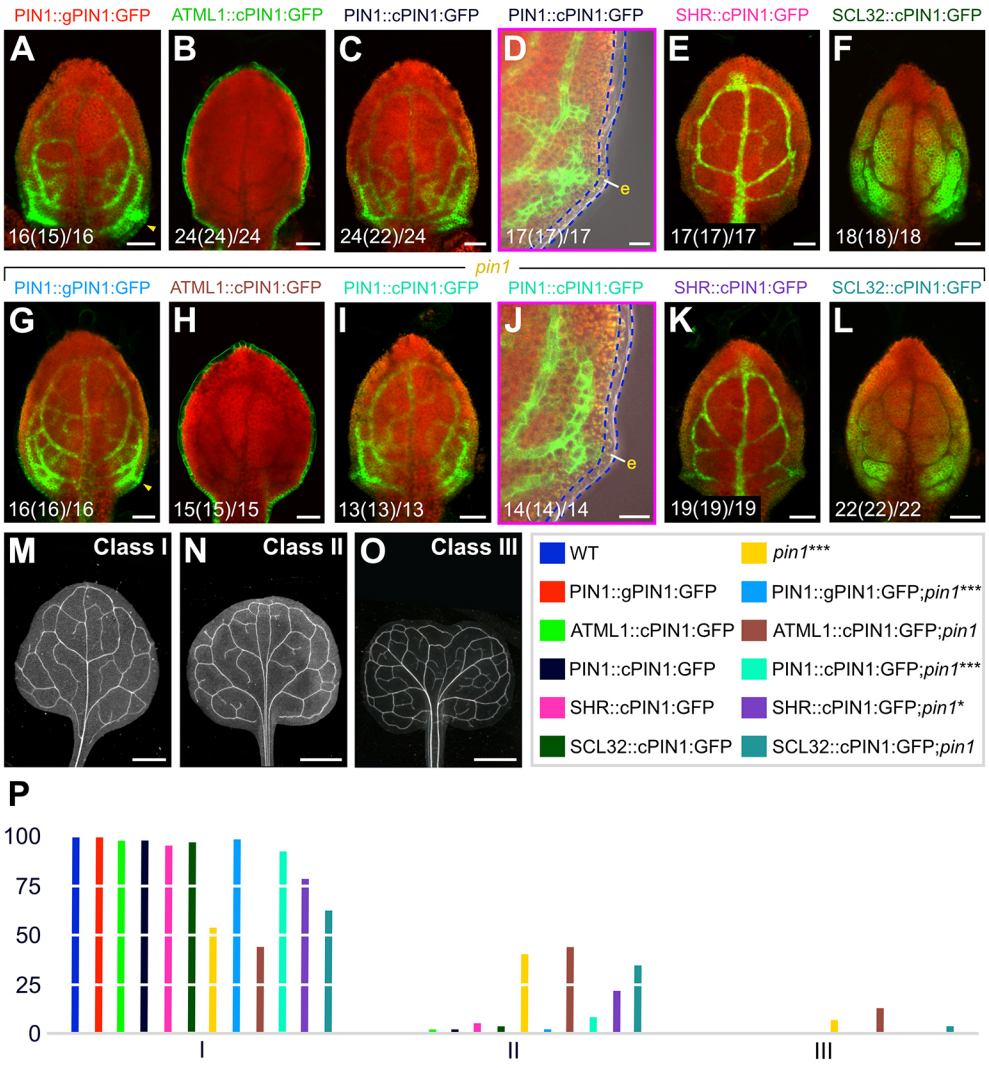

Fig. 2. Tissue-specific PIN1 expression in *PIN1*-dependent vein patterning. (A-L) Confocal laser scanning microscopy with (D,J) or without (A-C,E-I,K,L) transmitted light; first leaves 4 DAG. Green, GFP expression; red, autofluorescence. Yellow arrowheads in A,G indicate epidermal expression. Bottom left: reproducibility index, i.e. number of leaves with the displayed inner-tissue expression (number of leaves with the displayed epidermal expression)/number of leaves analyzed. (M-O) Dark-field illumination of mature first leaves illustrating phenotype classes (top right): class I, I-shaped midvein (M); class II, Y-shaped midvein (N); class III, fused leaves (O). (P) Percentages of leaves in phenotype classes. Differences between *pin1* and wild type, between PIN1::gPIN1:GFP;*pin1* and *pin1*, and between PIN1::cPIN1:GFP;*pin1* and *pin1* were significant (***P<0.001; Kruskal-Wallis and Mann-Whitney test with Bonferroni correction). Differences between SHR::cPIN1:GFP;*pin1* and wild type, and between SHR::cPIN1:GFP; *pin1* and *pin1* were significant (*P<0.05 by Kruskal-Wallis and Mann-Whitney test with Bonferroni correction). Sample population sizes: wild type, 40; *pin1*, 60; PIN1::gPIN1:GFP, 55; ATML1::cPIN1: GFP, 49; PIN1::cPIN1:GFP, 48; SHR:: cPIN1:GFP, 59; SCL32::cPIN1:GFP, 60; PIN1::gPIN1:GFP;*pin1*, 60; ATML1:: cPIN1:GFP;*pin1*, 55; PIN1::cPIN1:GFP; *pin1*, 51; SHR::cPIN1:GFP;*pin1*, 60; and SCL32::cPIN1:GFP;*pin1*, 58. e, epidermis. Scale bars: 60 µm in A-C,E-I,K, L; 20 µm in D,J; 1 mm in M; 2 mm in N,O.

growth and differentiation of flower primordia (Bilsborough et al., 2011; Kierzkowski et al., 2013, 2019; Li et al., 2019 preprint). We therefore asked whether our ATML1::cPIN1:GFP construct normalized, as previously reported ATML1::gPIN1:GFP constructs did (Bilsborough et al., 2011; Kierzkowski et al., 2013, 2019), the pin-shaped inflorescence phenotype of *pin1*. We found that ATML1::cPIN1:GFP and PIN1::gPIN1:GFP did but that PIN1::cPIN1:GFP, SHR::cPIN1:GFP and SCL32::cPIN1:GFP failed to do so (Fig. S3). These findings exclude the possibility that the inability of ATML1::cPIN1:GFP to rescue the vein pattern defects of *pin1* is an experimental artifact and instead point to a mechanistic difference between vein patterning and the positioning, growth and differentiation of flower primordia.

Expression of PIN3, PIN4 and PIN7 during vein patterning

Collectively, *PIN3*, *PIN4* and *PIN7* act redundantly with *PIN1* in *PIN1*-dependent vein patterning, and like *PIN1* they are expressed in both epidermis and inner tissues of young leaves (Verna et al., 2019). In those leaves, however, the most reproducible features of the *Arabidopsis* vein pattern can already be recognized (Donner et al., 2009; Gardiner et al., 2010, 2011; Donner and Scarpella, 2013; Sawchuk et al., 2013; Verna et al., 2015; Amalraj et al., 2020; Verna et al., 2019). Therefore, to test the possibility that compensatory functions provided by *PIN3*, *PIN4* and *PIN7* might account for the observation that PIN1 expression in the epidermis is dispensable and that PIN1 expression in the inner tissues of the leaf is sufficient for *PIN1*-dependent vein patterning, we first asked what the expression of PIN3, PIN4 and PIN7 was during vein patterning. To address this question, we imaged expression of PIN3::gPIN3: YFP, PIN4::gPIN4:YFP and PIN7::gPIN7:YFP in first leaves 2, 2.5, 3 and 4 DAG. As shown in Fig. 3 and quantified in Table S1, PIN3, PIN4 and PIN7 are collectively expressed in the epidermis, in developing veins, and – more weakly – in the nonvascular inner tissue of the leaf during vein patterning.

Tissue-specific PIN1 expression in *PIN1* redundant functions in vein patterning

Collectively, *PIN3*, *PIN4* and *PIN7* act redundantly with *PIN1* in *PIN1*-dependent vein patterning (Verna et al., 2019), and they are expressed in the leaf epidermis and inner tissues during vein patterning (Fig. 3). Therefore, to test the possibility that compensatory functions provided by *PIN3*, *PIN4* and *PIN7* may account for the observation that PIN1 expression in the epidermis is dispensable and that PIN1 expression in the inner tissues of the leaf is sufficient for *PIN1*-dependent vein patterning, we next expressed the following in the *pin3*;*pin4*;*pin7* (*pin3*;*4*;*7* hereafter) and *pin1*,*3*;*4*;*7* mutant backgrounds: (1) PIN1::gPIN1:GFP, which is expressed in all the tissues of the developing leaf (Fig. 4A,G); (2) ATML1::cPIN1:

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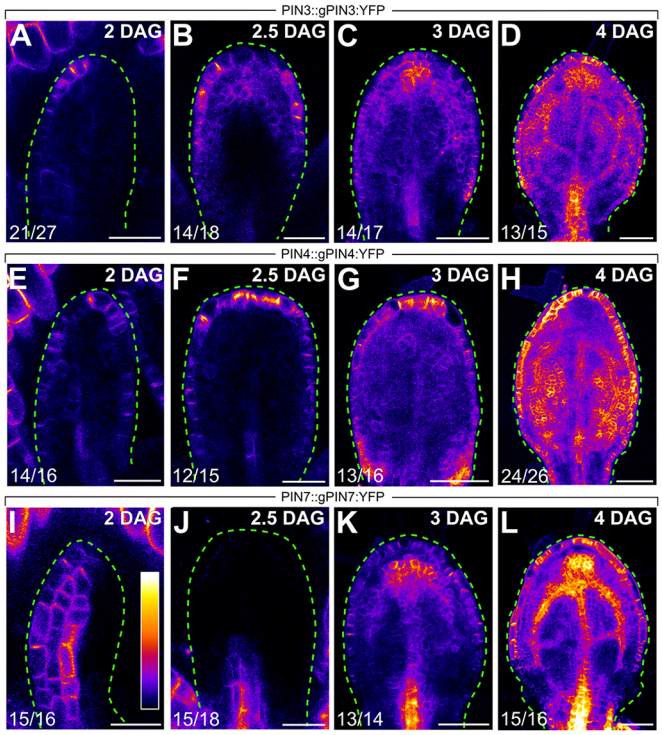

Fig. 3. Expression of PIN3, PIN4 and PIN7 during vein patterning. (A-L) Confocal laser scanning microscopy. Top right, leaf age in DAG; bottom left, reproducibility index, i.e. number of leaves with the displayed expression/number of leaves analyzed. Lookup table (ramp in I) visualizes expression levels. Abaxial side to the left in A,E,I. Scale bars: 30 µm in A,B,E,F,I,J; 60 µm in C,D,G,H,K,L.

GFP, which is only expressed in the epidermis (Fig. 4B,H); (3) PIN1::cPIN1:GFP, which is expressed in the leaf inner tissues (Fig. 4C,D,I,J); (4) SHR::cPIN1:GFP, which is only expressed in the vascular tissue (Fig. 4E,K); and (5) SCL32::cPIN1:GFP, which is expressed in the nonvascular inner tissue of the leaf (Fig. 4F,L). We then compared vein patterns of mature first leaves of the resulting backgrounds.

As previously shown (Verna et al., 2019), the vein pattern of *pin3*;*4*;*7* was no different from that of wild type, and none of the *pin1*,*3*;*4*;*7* leaves had a wild-type vein pattern (Fig. 4M-P). The vein patterns of PIN1::gPIN1:GFP;*pin3*;*4*;*7*, ATML1::cPIN1:GFP;*pin3*;*4*;*7*, PIN1::cPIN1:GFP;*pin3*;*4*;*7*, SHR::cPIN1:GFP;*pin3*;*4*;*7*, and SCL32::cPIN1:GFP;*pin3*;*4*;*7* were no different from the wild-type vein pattern (Fig. 4M-P). Both PIN1::gPIN1:GFP and PIN1::cPIN1:GFP normalized the phenotype spectrum of *pin1*,*3*;*4*;*7* vein patterns (Fig. 4M-P; Fig. S4A,C), and SHR::cPIN1:GFP shifted the phenotype spectrum of *pin1*,*3*;*4*;*7* vein patterns toward the wild-type vein pattern to match the phenotype spectrum of *pin1* vein patterns (Fig. 4M-P; Fig. S4D; compare with Fig. 2M-P). By contrast, the vein pattern defects of ATML1::cPIN1:GFP;*pin1*,*3*;*4*;*7* and SCL32::cPIN1:GFP;*pin3*;*4*;*7* were no different from those of *pin1*,*3*;*4*;*7* (Fig. 4M-P; Fig. S4B,E). We observed a similar effect of tissue-specific *PIN1* expression on the component of cotyledon patterning that depends on *PIN1*, *PIN3*, *PIN4* and *PIN7* (Fig. S5).

Therefore, that PIN1 expression in the epidermis is dispensable and that PIN1 expression in the inner tissues of the leaf is sufficient for *PIN1*-dependent vein patterning cannot be accounted for by compensatory functions provided by *PIN3*, *PIN4* and *PIN7*. Such compensatory functions are also unlikely to be provided by the remaining PIN proteins, by the ABCB1 and ABCB19 auxin efflux carriers or by the AUX1/LAX auxin influx carriers because these proteins either are not expressed in the epidermis or lack functions in vein patterning, whether in normally grown wild-type or in auxin-transport-inhibited leaves (Sawchuk et al., 2013; Verna et al., 2015, 2019). As such, we conclude that auxin transport in the epidermis is dispensable for vein patterning. This conclusion is consistent with the observation that *cup-shaped cotyledon2* mutants lack convergent points of epidermal PIN1 polarity and yet have normal vein patterns (Bilsborough et al., 2011).

By contrast, PIN1 expression in inner tissues is required and sufficient for auxin transport-dependent vein patterning. Such function of PIN1 expression seems to mainly depend on PIN1 expression in the vascular tissue. Indeed, only PIN1 expression in the vascular tissue, and not PIN1 expression in the nonvascular inner tissues of the leaf, is required for auxin transport-dependent vein patterning. Furthermore, PIN1 expression in the vascular tissue is sufficient for most of the *PIN1* functions in vein patterning, but PIN1 expression in the nonvascular tissues of the leaf is sufficient for none. Because PIN1 localization is strongly polarized in vascular cells and only weakly polarized, or altogether nonpolarized, in the inner nonvascular cells of the leaf (Scarpella et al., 2006; Wenzel et al., 2007; Bayer et al., 2009; Marcos and Berleth, 2014), our observations also suggest that auxin transport-dependent vein patterning is sink driven. As such, our results are consistent with the conceptual framework of the auxin canalization hypothesis, which proposes formation of vascular strands through the autocatalytic drainage from nonvascular cells of an auxin-dependent inductive signal (Sachs, 1969).

In conclusion, vein patterning hypotheses based on auxin transport from the epidermis (reviewed by Prusinkiewicz and Runions, 2012; Bennett et al., 2014; Runions et al., 2014; Linh et al., 2018) are unsupported by experimental evidence. Our results do not rule out an influence of the epidermis on vein patterning, e.g. through local auxin production (e.g. Abley et al., 2016), but they do exclude the possibility that such influence is brought about by polar auxin transport. Alternatively, patterning of local epidermal features, such as peaks of auxin production or response, and of the processes that depend on those features may be mediated by auxin transport in underlying tissues; there is evidence for such possibility (e.g. Deb et al., 2015) and our results are consistent with that evidence. In the future, it will be interesting to test these and other possibilities, but for now our results refute all the vein patterning hypotheses that depend on auxin transport from the epidermis.

MATERIALS AND METHODS

Notation

In agreement with Crittenden et al. (1996), linked genes [<2500 kb apart, which in *Arabidopsis thaliana* on an average corresponds to ∼10 cM (Lukowitz et al., 2000)] are separated by a comma; unlinked genes are separated by a semicolon.

Plants

The origin and nature of lines, genotyping strategies and oligonucleotide sequences are in Tables S2-S4. Seeds were sterilized and sown as described previously (Sawchuk et al., 2008). Stratified seeds were germinated and seedlings and plants were grown as described previously (Verna et al., 2019). Plants were transformed and representative lines were selected as described by Sawchuk et al. (2008).

Imaging

Developing leaves were mounted and YFP was imaged as described previously (Sawchuk et al., 2013). CFP, YFP and autofluorescence were imaged as described previously (Sawchuk et al., 2013). GFP and autofluorescence were imaged as described previously (Amalraj et al., 2020). Images were stacked, aligned with the Scale Invariant Feature Transform algorithm (Lowe, 2004), and maximum-intensity projection

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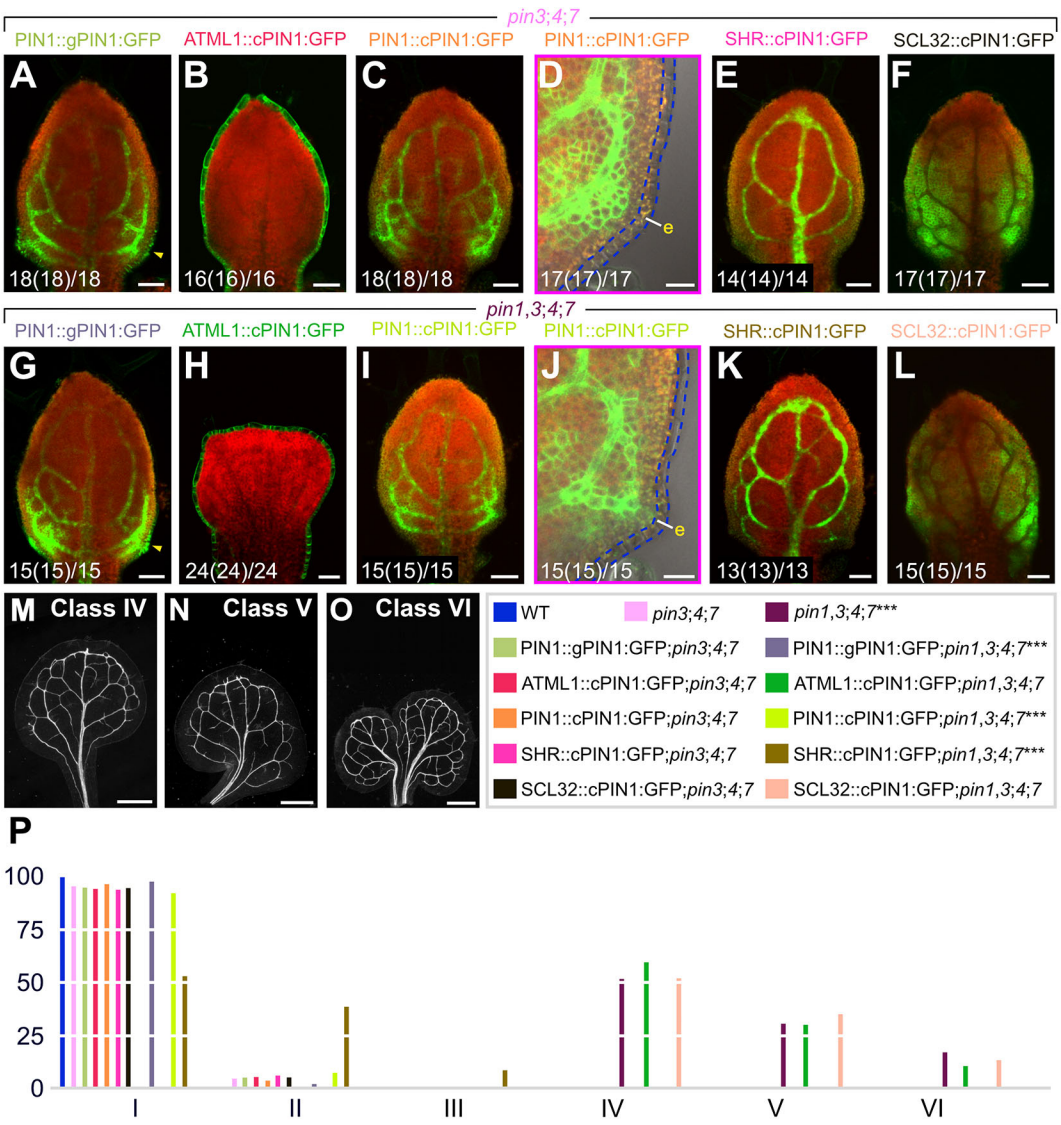

Fig. 4. Tissue-specific PIN1 expression in *PIN1*/*PIN3*/*PIN4*/*PIN7*-dependent vein patterning. (A-L) Confocal laser scanning microscopy with (D,J) or without (A-C,E-I,K,L) transmitted light; first leaves 4 DAG. Green, GFP expression; red, autofluorescence. Yellow arrowheads in A,G indicate epidermal expression. Bottom left: reproducibility index, i.e. number of leaves with the displayed inner-tissue expression (number of leaves with the displayed epidermal expression/number of leaves analyzed). (M-O) Dark-field illumination of mature first leaves illustrating phenotype classes (top right): class IV, I-shaped midvein and thick veins (M); class V, Y-shaped midvein and thick veins (N); class VI, fused leaves with thick veins (O). (P) Percentages of leaves in phenotype classes. Differences between *pin1,3;4;7* and wild type, between PIN1::gPIN1:GFP;*pin1,3;4;7* and *pin1,3;4;7,* between PIN1::cPIN1:GFP;*pin1,3;4;7* and *pin1,3;4;7*, between SHR::cPIN1:GFP;*pin1,3;4;7* and *pin1,3;4;7,* and between SHR::cPIN1:GFP;*pin1,3;4;7* and *pin1,3;4;7* were significant (***P<0.001 by Kruskal-Wallis and Mann-Whitney test with Bonferroni correction). Sample population sizes: wild type 48; *pin3;4;7,* 45; *pin1,3;4;7,* 70; PIN1::gPIN1:GFP;*pin3;4;7,* 60; ATML1::cPIN1:GFP;*pin3;4;7,* 37; PIN1::cPIN1:GFP;*pin3;4;7,* 28; SHR::cPIN1:GFP;*pin3;4;7,* 50; SCL32::cPIN1:GFP;*pin3;4;7,* 38; PIN1::gPIN1:GFP;*pin1,3;4;7,* 45; ATML1::cPIN1:GFP;*pin1,3;4;7,* 57; PIN1::cPIN1:GFP;*pin1,3;4;7,* 53; SHR::cPIN1:GFP;*pin1,3;4;7,* 62; SCL32::cPIN1:GFP;*pin1,3;4;7,* 69. e, epidermis. Scale bars: 60 µm in A-C,E-I,K,L; 20 µm in D,J; 0.75 mm in M-O.

was applied to aligned image stacks in the Fiji distribution (Schindelin et al., 2012) of ImageJ (Schneider et al., 2012; Schindelin et al., 2015; Rueden et al., 2017). Mature leaves were fixed in ethanol:acetic acid (6:1), rehydrated in 70% ethanol and water, and mounted in chloral hydrate:glycerol:water (8:2:1). Mounted leaves were imaged as described previously (Odat et al., 2014). Greyscaled RGB color images were turned into 8-bit images, and image brightness and contrast were adjusted by linear stretching of the histogram in the Fiji distribution of ImageJ.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: P.G., C.V., E.S.; Methodology: P.G., C.V., T.Z., E.S.; Validation: P.G., C.V., T.Z., E.S.; Formal analysis: P.G., C.V., T.Z., E.S.; Investigation: P.G., C.V., T.Z., E.S.; Writing - original draft: P.G., C.V., E.S.; Writing - review & editing: P.G., C.V., T.Z., E.S.; Visualization: P.G., C.V., T.Z., E.S.; Supervision: E.S.; Project administration: E.S.; Funding acquisition: E.S.

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Supplementary information

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https://dev.biologists.org/lookup/doi/10.1242/dev.187666.supplemental

Peer review history

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