Polar auxin transport dynamics of primary and secondary vein patterning in dicot leaves

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ABSTRACT

The growth regulator auxin plays a central role in the phyllotaxy, shape and venation patterns of leaves. The auxin spatial localization underlying these phenomena involves polar auxin transport (PAT) at the cellular level, particularly the preferential allocation of PINFORMED (PIN) efflux proteins to certain areas of the plasma membrane. Two general mechanisms have been studied: an up-the-gradient (UTG) allocation dependent on neighbouring-cell auxin concentrations, and a with-the-flux (WTF) allocation dependent on the flow of auxin across walls. We have developed a combined UTG+WTF model to quantify the observed auxin flows both towards (UTG) and away from (WTF) auxin maxima during primary and secondary vein patterning in leaves. The model simulates intracellular and membrane kinetics and intercellular transport, and is solved for a 2D leaf of several hundred cells. In addition to normal development, modelling of increasing PAT inhibition generates, as observed experimentally: a switch from several distinct vein initiation sites to many less distinct sites; a delay in vein canalization; inhibited connection of new veins to old; and finally loss of patterning in the margin, loss of vein extension and confinement of auxin to the margin. The model generates the observed formation of discrete auxin maxima at leaf vein sources and shows the dependence of secondary vein patterning on the efficacy of auxin flux through cells. Simulations of vein patterning and leaf growth further indicate that growth itself may bridge the spatial scale from the cell–cell resolution of the PIN-auxin dynamics to vein patterns on the whole-leaf scale.

KEYWORDS: Arabidopsis; canalization; leaf; plant growth; polar auxin transport; vein.

1. INTRODUCTION

Vascular tissue in plants forms in complex spatial patterns that ensure water and solute transport throughout the plant. Newly developing veins coordinate with existing vasculature to create a plant-wide transport network: as each leaf is initiated, its new venation needs to connect to the central vasculature of the main stem, and within each developing leaf later-formed veins connect to earlier-formed veins. Plants such as Arabidopsis thaliana have reticulate patterning whereby the primary midvein forms first, followed by secondary veins that form in a basipetal (distal to proximal) sequence (e.g. Fig. 1A and B; Mattsson et al. 1999, 2003). Tertiary veins later form within older leaf areoles or extend from these secondary veins towards the leaf margin. Vein development can be experimentally manipulated, providing an opportunity to study the mechanisms underlying these patterning processes. This paper integrates experimental data into a quantitative model for primary and secondary leaf venation.

Vascular development depends on accumulation of the endogenous phytohormone auxin (indole acetic acid, IAA): (i) exogenous application of IAA induces vascular strand formation in wounded dicot stems (Jacobs 1952; Sachs 1968); (ii) IAA can help induce xylem differentiation in suspension culture cells (reviewed in Fukuda 1997); and (iii) IAA overproduction in transgenic plants results in increased secondary vascular growth (Klee et al. 1987). Synthetic auxin response marker expression suggests that auxin accumulates to high levels in developing procambial cells and disappears in more mature veins (Fig. 1C; Mattsson et al. 2003). A cell's ability to respond to auxin signals is also crucial for proper vascular patterning. Defects in auxin response genes such as MONOPTEROS, AUXIN-RESISTANT1,
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AUXIN-RESISTANT $^6$ TRANSPORT INHIBITOR RESPONSE $^1$ and AUXIN SIGNALING F-BOX $^2$ result in discontinuous, fragmented vascular strands in Arabidopsis leaves (Hardtke and Berleth 1998; Berleth et al. 2000; Hobbie et al. 2000; see review in Biedroń and Banasiak 2018; Verna et al. 2019). Taken together, these results suggest that correct auxin accumulation and perception within developing procambial cells are necessary for continuous vascular strand formation. Indole acetic acid is produced in young leaves and transported basally. Some YUCCA auxin biosynthesis genes, as well as some of their SHORT INTERNODES/STYLISH regulators, are expressed in the margins of

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**Figure 1.** Successive formation of new veins (red) in successively older leaf primordia (A), showing only the lower branches of secondary veins, similar to modelled images in later figures. Dark-field images of vasculature (B, D) and bright-field images showing DR5::GUS auxin response expression (C, E) in Arabidopsis leaves from plants grown without (B, C) or with (D, E) NPA treatment. Midvein (1°), secondary (2°) and tertiary (3°) veins are labelled in ‘B’. (F) Schematic representation of WTF PIN1 allocation. The PIN1 of cell i (green) is allocated to the membrane facing cell j according to the auxin (a) flux ($\phi$) from cell i to cell j; and similarly for all neighbours of cell i. (G) Schematic representation of UTG PIN1 allocation. Cell i allocates PIN1 towards cell j, $p_{ij}$, according to the concentration of auxin in cell j, $a_j$; and similarly for cell j towards cell i ($p_{ji}$). The double-headed arrow represents concentration-dependent, Fickian diffusion, which is in both WTF and UTG models. Figures were obtained with permission from the following: B, D (Mattsson et al. 1999); C, E (Wenzel et al. 2007); F (Bennett et al. 2014); G (Fujita and Kawaguchi 2018).
Arabidopsis leaves, and single or multiple gene knockouts can result in vascular defects (Cheng et al. 2006, 2007; Wang et al. 2011; Baylis et al. 2013; Zhang et al. 2020). Chemical inhibition of auxin transport during Arabidopsis leaf development results in thicker and more numerous secondary veins, more parallel strands in the midvein region and progressive restriction of vascularization to the leaf margin with increasing inhibitor concentration (Fig. 1D; Mattsson et al. 1999, 2003; Sieberth 1999). At high inhibitor concentrations, auxin response expression becomes localized to the margin (Fig. 1E; Mattsson et al. 2003; Wenzel et al. 2007), corresponding to increased marginal relative to internal vascularization. According to the auxin canalization hypothesis, a broad region of auxin becomes restricted into a narrow file (‘canal’) of provascular cells that efficiently conduct IAA (Sachs 1981). The transport inhibitor-induced overgrowth of primary and secondary veins suggests that diminished IAA drainage capacity disrupts the canalization mechanism, resulting in broader regions with high enough auxin levels in cells to differentiate -naphthylphthalamic acid (NPA) treatment causes overgrowth of primary and secondary veins towards the existing shoot vasculature affects the patterning and size of vascular strands.

The PINFORMED (PIN) family of cell efflux transporters are closely linked with auxin patterning (e.g. Gälweiler et al. 1998). In Arabidopsis, PIN1 in particular is a major determinant in vascular patterning. pin1 mutants have similar leaf vascular phenotypes to plants grown in the presence of auxin transport inhibitors (Mattsson et al. 1999, 2003), indicating that auxin efflux through PIN1 is a significant component in vein patterning and canalization. At the scale of the leaf, PIN1 expression is closely correlated with presumed auxin distribution, becoming restricted from broad regions to narrow procambial strands in normal development and losing that restriction when auxin transport inhibitors are applied (Scarpella et al. 2006; Wenzel et al. 2007). At the cellular scale, PIN1 forms distinct polarized distributions (in normal development), with more PIN1 allocated to particular sides of a cell than others (Scarpella et al. 2006; Wenzel et al. 2007). This facilitates polar auxin transport (PAT) between cells, in contrast to isotropic transport (e.g. simple diffusion). During formation of primary and secondary veins, early PIN1 alignment indicates convergence points (CPs) of flow towards auxin maxima at the leaf margin; later PIN1 alignment indicates auxin flow from the CPs into the leaf interior along the developing procambial strand regions (Scarpella et al. 2006; Wenzel et al. 2007; Bayer et al. 2009).

Mutations in pin genes alter vascular patterns, but have not been observed to eliminate vein initiation and inward extension. For example, 50% of pin1 mutants showed non-wild-type venation patterns, but all mutants (including quadruple pin1,3,4,7) formed secondary veins (Govindaraju et al. 2020). Stronger phenotypes, affecting vein extension, can be induced by high doses of auxin transport inhibitors, reducing inward extension and restricting vasculature and auxin response maxima predominantly to the leaf margin (Fig. 1E; Mattsson et al. 1999, 2003). This phenomenon may involve intracellular inhibitor effects on vesicle transport (Dhonukshe et al. 2008), and suggests a broader context of auxin drainage from the margin, of which PIN is a component.

Several lines of evidence indicate that internal leaf cells, and not the epidermis alone, are required for auxin localization and vein formation in developing leaf primordia. Laser ablation of subepidermal cells in developing leaf primordia widens the DR5 (auxin response marker) and PIN1 expression domains, indicating an essential role of these tissues in auxin localization and transport (Deb et al. 2015). Normal venation patterns can be observed in pin1 or pin1,3,4,7 mutants that have PIN1 expressed only in procambial tissues, but not with epidermal-only PIN1 expression (Govindaraju et al. 2020). Likewise, activation of the auxin biosynthesis gene YUCCA1 in internal leaf margin cells, but not epidermal-only cells, was necessary to promote leaf blade growth (Zhang et al. 2020). Secondary veins originate subepidermally in cuc2 mutants that lack epidermal PIN1 CPs (Bilsborough et al. 2011). Also, 1-Naphthylphthalamic acid (NPA) treatment causes marginal vascular hypertropy and presumed auxin distribution in the margin subepidermal tissues (Fig. 1D and E). There may be a developmental aspect: PIN1 CPs appear to be more epidermal early and more internal later (Wenzel et al. 2007; Govindaraju et al. 2020). It makes sense that even if PIN1 may direct auxin flow from the epidermis to internal tissues, procambial strand formation will only occur if functional PIN1 expression occurs in the ‘end-of-the-line’ procambial cells. Epidermal PIN1 function may also depend on intrinsic elements in PIN1: Bilsborough et al. (2011) rescued wild-type venation patterns in pin1 with epidermally localized genomic PIN1; Govindaraju et al. (2020) found no rescue (pin1 or pin1,3,4,7) using an epidermally localized PIN1 coding sequence. Regardless of the precise role of the epidermis, overall the results suggest that internal margin cells are crucial for leaf blade growth and vein formation. We are therefore confident that our current venation model based on transport of auxin from the leaf margin is supported by experimental evidence.

Theoretical work has provided some understanding of the auxin transport mechanisms involved in venation. Sachs (1969, 1981, 1989, 2003) proposed that canalization depended on the tendency for auxin flow to self-enhance. Mitchison (1980, 1981) formalized this mathematically, by having the intercellular auxin flux (ϕ) feedback on and enhance either auxin diffusivity between cells, or affect allocation of transporters between cells. The former option, facilitated diffusion, has not been supported experimentally (but could potentially be relevant for transport through plasmodesmata, as recently discussed by Ravichandran et al. 2020); the latter option fits well with the role of PIN1 allocation in the polar movement of auxin (Smith and Bayer 2009). This can be represented schematically as PIN1 allocated from the cytosol of cell i (green dot, Fig. 1F) to sectors of the cell membrane facing a neighbouring cell j (p, green bar, Fig. 1F) in proportion to the auxin flux from i to j (ϕ, Fig. 1F). Cell i has multiple neighbour cells j in a tissue. If any particular ϕ is larger than the others for cell i (e.g. due to a larger auxin concentration difference and simple diffusion), flow will tend to self-enhance from i to that j. This class of models, termed with-the-flux (WTF; see Bennett et al. 2014) due to the mechanism of PIN1 allocation, has been successful in modelling canalization as well as vein extension (e.g. Feugier et al. 2005; Stoma et al. 2008; Faricot and Yuan 2013; Walker et al. 2013). In particular, a non-linear (e.g. ϕ3) dependence is important in WTF-driven canalization (e.g. Rolland-Lagan and Prusinkiewicz 2005; also see O’Connor et al. 2014, for an application with different PINs in monocots). Such modelling is at the
resolution of cell–cell exchange, with the efflux from \( i \) to \( j \) equalling the influx to \( j \) from \( i \). While this is likely a simplification, for example neglecting dynamics in the apoplast between cells (e.g. Wabnik et al. 2010; also see review in Runions et al. 2014), the cell–cell resolution can generate pattern at multicellular scales, match observations of PIN1 distributions on the membrane and predict response to disruption of PAT. Modelling has focused on efflux transporters such as PIN, which are likely critical in actively moving protonated auxin out of cells, whereas import can occur passively (Bennett et al. 2014). With-the-flux models generally address the PAT associated with PIN1 alignment and auxin flow away from higher auxin concentration cells and towards lower auxin concentration cells, such as the flow from established margin CPs towards the leaf interior.

During the initial formation of CPs in leaf margins, however, PIN1 alignment is observed towards high auxin concentration areas and away from lower concentration areas. Such up-the-gradient (UTG) alignment is also seen towards CPs during leaf initiation on the shoot apical meristem (SAM). (In the terminology of auxin flowing towards a ‘sink’, the sink is low auxin concentration in WTF and high concentration in UTG.) Up-the-gradient models, in which PIN1 is allocated to sectors of the cell membrane according to the neighbouring-cell auxin concentration (Fig. 1G), have been successfully used to generate SAM phyllotactic patterns (Jönsson et al. 2006; Smith et al. 2006) and leaf marginal CPs (Bilsborough et al. 2011). Up-the-gradient-only mechanisms have not been as successful in generating extending veins: Merks et al. (2007) generated ‘travelling waves’ of auxin, which resulted in longer-lived PIN1 polarization tracks. However, these tracks were low in auxin, unlike experimental observations of steady high auxin concentration in veins (e.g. Fig. 1C).

Both WTF and UTG PIN1 alignments are observed during the developmental sequence of: (1) diffuse marginal expression (pre-CP); (2) CP formation; (3) vein initiation from the CP into the leaf interior; (4) vein extension and canalization; and (5) internal alignment towards a high-auxin vein. With-the-flux-only models have been successful in describing 4 and 5, and UTG-only mechanisms have been successful at 1 and 2. Neither WTF-only nor UTG-only models have been successful at all steps, especially the change in alignment direction at step 3. For this reason, Bayer et al. (2009) introduced a combined UTG+WTF (dual-polarization) PAT model for midvein formation. At low auxin concentration, the model is primarily UTG, and forms the midvein CP in the distal margin (steps 1 and 2); while at high auxin concentration, the model switches (step 3) to WTF and extends the midvein into the proximal interior (step 4). This model successfully matched the observed auxin distributions and PIN1 polarizations for the single midvein. In particular, the midvein had high auxin concentration (as seen with WTF models; unlike the Merks et al. (2007) UTG-only model) and generated the observed interior polarization towards the high-auxin CP and vein (step 5). An earlier version of the model that segregated UTG to the margin and WTF to the interior (Smith 2011; Smith and Bayer 2009) could not produce the interior UTG polarization (step 5), nor the PIN1 polarization into and out of CPs (step 3). While not a dual-polarization model (i.e. UTG and WTF in all cells), it will be discussed in Section 4 regarding growth and secondary vein initiation. More recently, Cieslak et al. (2015) developed a model in which auxin influx and efflux are treated separately. This can produce dual-polarization flow (towards CP and away from CP), for instance by an auxin concentration dependence of a decay rate for influx–efflux tally molecules. This model has been applied to venation in 3D (Hartmann et al. 2019). Consistent with auxin concentration-dependent UTG to WTF switching in the models, PINOID appears to be involved in CP formation at incipient floral primordia (Friml et al. 2004), and the subsequent switch to inward basipetal WTF flow can involve the MACCHI-BOU 4/ENHANCER OF PINOID family genes, which are activated by the MONOPTEROS auxin response transcription factor (Furutani et al. 2014).

Dual-polarization (UTG+WTF) models have focused on PIN1-auxin convergence and extension in the formation of single or non-interacting veins (Bayer et al. 2009; Cieslak et al. 2015; Hartmann et al. 2019). There remain a number of open questions, especially pertaining to formation of the multi-vein secondary network in dicot leaves. These include: (a) what dynamic constraints (relative rates of reaction and transport) produce dual PIN1 polarization (convergence and extension); (b) what are the spatial scales of PIN1-auxin patterning, and are additional mechanisms needed for leaf-scale patterning; (c) how are secondaries initiated, and how is this controlled temporally and spatially; (d) how do secondaries interact with and connect to the prior vasculature to form the leaf network; and (e) what is the role of localized auxin production, particularly in the margin vs. the interior? Normal and transport-inhibited development provide rich phenotypes against which to build and test quantitative models to address these questions.

Building on the dual-polarization approaches, we present a model for primary and secondary vein patterning in dicot leaves like Arabidopsis. The model generates the PIN1-auxin distributions corresponding to single-vein initiation, extension and canalization, as well as the synchronized and sequential initiation of secondary veins, control of their extension direction and connection of the secondaries to the midvein. We discuss features of vein patterning that depend on UTG or WTF aspects of PAT, and features that suggest additional dynamics. In particular, we find that dual-polarization PAT displays relatively short-scale vein extension and propose that intrinsic leaf growth helps to expand early veins to the scale of larger leaves. For inhibited auxin transport, the model generates the observed more uniform PIN1 distributions in the margin, more secondary veins, and disrupted vein connections and extension. Questions (a–e) above are discussed in terms of the results in Sections 4.1–4.5, respectively.

2. Model and Methods

The model solves a system of equations (Section 2.1) in a 2D sheet of cells (Section 2.2). Auxin is synthesized in the outer cells (representing the epidermal and subepidermal margin), with successive activation of distal to proximal zones representing the developmental competence for auxin synthesis and vein initiation (Section 2.3). Polar auxin transport is modelled via the PIN1 transporter; the effect of auxin transport inhibitors is modelled via decrease of transmission through PIN1 (strong decrease approximates broader PAT factors affecting vein extension, Fig. 1E). Growth is simulated by expansion and division in the margin (Section 2.4).
2.1 PAT mechanism

Our cellular-resolution dual-polarization mechanism combines components from prior single- and dual-polarization models (Rolland-Lagan and Prusinkiewicz 2005; Jönsson et al. 2006; Merks et al. 2007; Bayer et al. 2009). For cell \(i\), the rate equations for \(A\) (auxin), \(P\) (cytoplasmatic PIN1), and \(P_i\) (PIN1 in membrane towards neighbour cell \(j\)) are:

\[
\frac{dA_i}{dt} = auxpr · A_{prec} - auxdec · A_i + T \sum_j \left[ \frac{P_i A_j}{1 + A_i} - \frac{P_j A_i}{1 + A_j} \right] + D \sum_j L_{ij}(A_j - A_i)
\]

\[
\frac{dP_{ij}}{dt} = \frac{k_{aux} f(A_j)}{1 + A_j} + \frac{P_i}{1 + A_i} (k_{UTG} \phi^2 + k_{WTF} \phi) - k_{d}(P_{ij})
\]

\[
\frac{dP_i}{dt} = pinpr · A_i - pindec · P_i - \sum_j \frac{dP_{ij}}{dt}
\]

In equation (1), auxin is created from a precursor \(A_{prec}\) and decays; the transport terms are as in Merks et al. 2007 (an adaptation from Jönsson et al. 2006), with the \(D\) term representing Fickian diffusion and the \(T\) term representing PAT through membrane-bound PIN1. Equation (2) represents the PIN1 allocation dynamics between cytoplasm and membrane: the first term is the UTG allocation, from Merks et al. 2007, with \(f(A) = 100A/(100+A)\); the second term is the WTF allocation, adapted from Rolland-Lagan and Prusinkiewicz 2005. The total flux \(\phi\) in equation (2) is computed from the sum of the \(T\) and \(D\) terms in equation (1) at the start of each time step. With-the-flux allocation occurs for \(\phi > 0\) from cell \(i\) to \(j\). In contrast to the Bayer et al. (2009) model, equation (2) calculates \(P\) dynamically (rather than an instantaneous re-partition of \(P\) to \(P_i\) in each time step); and UTG and WTF are continuously running (rather than a specified auxin-threshold concentration above which UTG is off). Depending on auxin concentrations in the \(i\) and \(j\) cells, equation (2) will operate in a chiefly UTG or chiefly WTF mode. In equation (3), intracellular PIN1 is produced in proportion to auxin (Vieten et al. 2005; Merks et al. 2007), decays, and is allocated to the membrane.

Equations (1–3) generate converging and extending PIN1-auxin patterns in a cellular-resolution PAT model. A parameter set was selected (Table 1) that generates all normal transport simulations shown (Figs 2, 4, and 6), without any parameter shifts for \(t > 0\). Reductions of \(T\) for transport-inhibited simulations (Figs 3 and 5) are noted in the text. There are a number of constraints on the relative parameter values to successfully generate venation patterns; these are summarized in Table 2.

2.2 Software

The model was implemented in the cell-based software package VirtualLeaf, version 1.0.2 (Merks et al. 2011; https://code.google.com/archive/p/virtualleaf/, compiled in Windows with Qt 4.6.6). The software solves polar and diffusive transport (\(T\) and \(D\) terms in equation (1)); tracks \(P\) contributions to \(P\) (last term of equation (3)); and allows for any specification of the remaining reaction terms (i.e. is not constrained to a particular model). The model specification (UTG=WTF.cpp) and a leaf geometry and parameters (Fig4A.xml) can be downloaded from https://davidhollowayresearch.weebly.com/software.html. VirtualLeaf solves equations (1–3) (Runge-Kutta, adaptive step size), and allows for cell area increase and division (cells divide when their area has doubled and cells shape minimize overall elastic energy). Leaves composed of two-dimensional cells were generated by cell expansion and division from a 4-cell initial shape, producing grids of realistically irregular cell size and shape. Cases in which the division algorithm created an unrealistic triangular cell shape and therefore a discontinuity in marginal transport were excluded. Figures 2, 3, and 6 simulations (equations (1–3) in a non-growing leaf) are on a 128-cell leaf, while Figs 4 and 5 simulations (equations (1–3) in a growing leaf) started from a 32-cell leaf. These match cell numbers of the early leaf primordia and in the early marginal vein initiation zones.

2.3 Auxin production zones

\(A, P, P\) are initially zero on the starting leaf shape. Developmentally, primary and secondary veins are initiated from successive zones of auxin synthesis in the leaf margin (represented as a single layer of cells in VirtualLeaf, but could represent epidermal and subepidermal cells biologically). This developmental succession is represented by a pre-pattern in \(A_{prec}\) from which auxin is synthesized for \(A_{prec} > 0\) (equation (1)). At \(t = 0\), \(A_{prec}\) is set to 0.5 in the apical-most margin cells (Zone 1, Z1, Fig. 2), the auxin source for the midvein; −1 in the neighbouring

| Table 1. Model parameters and definitions. |
|------------------------------------------|
| Parameter            | Definition                  | Value* |
|----------------------|-----------------------------|--------|
| \(A, A\)             | [Auxin] in cell \(i\) or \(j\) |        |
| \(A_{prec}\)         | Auxin precursor             |        |
| auxdec               | Auxin decay rate constant   | 0.5    |
| auxpr                | Auxin production rate constant | 30   |
| \(C\)                | Margin-interior exchange efficiency | 0.833 |
| cells \(i\) and \(j\) | Adjacent (neighbouring) cells |        |
| \(D\)                | Diffusive transport constant | 5e-4   |
| \(k_{UTG}\)          | UTG allocation rate constant | 4e-3   |
| \(k_{WTF}\)          | Linear WTF allocation rate constant | 2e-2 |
| \(k_{WTF}^2\)        | Quadratic WTF allocation rate constant | 5e-5 |
| \(k_{d}\)            | PIN1 membrane detachment rate constant | 4e-3 |
| \(L_{ij}\)           | Cell wall length between cells \(i\) and \(j\) |        |
| \(P_i\)              | Cytoplasmic [PIN1] in cell \(i\) |        |
| \(P_{ij}\)           | [PIN1] in membrane of cell \(i\) towards cell \(j\) |        |
| pindec               | PIN1 decay constant         | 1e-2   |
| pinpr                | PIN1 production constant    | 5e-3   |
| \(t\)                | Computational time           |        |
| \(T\)                | Transmissivity of auxin through PIN1 | 3 (non-inhibited) |
| UTG                  | Up-the-gradient PIN1 allocation |        |
| WTF                  | With-the-flux PIN1 allocation |        |
| \(G_1\)              | Cell growth in auxin-producing cells | See Table 3 |
| \(G_2\)              | Cell growth just proximal to \(G_1\) region | See Table 3 |
| \(G_3\)              | Cell growth constant of all other cells | See Table 3 |

*In computational time (s), concentration and distance units.
zones to the left and right (Z2-left indicated on Fig. 2); and stepping down by 1 in each subsequent zone towards the leaf base (e.g. \(A_{p_{\text{prec}}} = -2\) initially in Z3, \(A_{p_{\text{prec}}} = 0\) in interior cells for the results shown (Figs 2–6). \(A_{p_{\text{prec}}}\) increases in the margin by 0.001 s\(^{-1}\), activating zones in an apical to basal temporal sequence as \(A_{p_{\text{prec}}}\) becomes positive. Auxin is only synthesized for \(A_{p_{\text{prec}}} > 0\) (i.e. \(A_{p_{\text{prec}}}\) is a concentration in the equation (1) kinetics when it is positive; otherwise, it is a marker for zone identity and the developmental delay of zone activation). In fixed (non-growing) leaves (Figs 2 and 3), all zones are five cells wide, corresponding to pre-CP PIN1 localization zone sizes (Scarpella et al. 2006; auxin activation of PIN1, see equation (3) discussion and citations above). In growing leaves (Figs 4 and 5), the first (apical-most) zone is five cells wide, the proximal zones start as 1–2 cells each, generating approximately five cells through growth by the time the zone reaches \(A_{p_{\text{prec}}} > 0\). To model the observed independence of zones (limit the effect of PIN1 polarization in earlier zones on later zones), \(P_y\) is not allocated at zone boundaries. Tissue layers in the leaf are modelled as in Bayer et al. (2009); in exchanges between margin and interior cells \(T\) is multiplied by a factor \(C\). This sharpens patterns but is not necessary: \(C = 1\) simulations are qualitatively similar to those shown. Cells at the base of the leaf (blue walls in figures) represent the pre-existing vasculature of the rest of the plant: these have constant auxin production (0.01 s\(^{-1}\), instead of the first term in equation (1)) and slightly decreased decay rates (\(auxdec = 0.1\) s\(^{-1}\)) to maintain a steady-state auxin concentration. Reduced \(P_y\) in these cells (\(pindec = 100\) s\(^{-1}\)) prevents flow reversal (i.e. prevents the basal sink cells from generating veins into the leaf).

2.4 Growth rates

In simulations with growth (Figs 4 and 5), margin cells increase in area according to their developmental stage, as defined by their \(A_{p_{\text{prec}}}\) value. For auxin-producing cells (\(A_{p_{\text{prec}}} > 0\)), area increases according to parameter \(G_i\) in each time step; cells just proximal to this (0 > \(A_{p_{\text{prec}}} > -1\)) expand by parameter \(G_j\); all other cells expand by parameter \(G_k\) (Table 3). These differential rates represent the greater proximal and lateral than distal expansion observed in leaf growth (e.g. Kierzkowski et al. 2019) and generate an elongated (rather than round) mature leaf shape. This model of growth can be identified with the recent discovery of the control of leaf shape via WOX-YUCCA control of auxin biosynthesis and growth in the margin (Zhang et al. 2020), with auxin biosynthesis state (\(A_{p_{\text{prec}}}\)) determining a growth rate (\(G\)).

3. RESULTS

3.1 Dynamics of normal vein patterning

3.1.1 Midvein formation.

3.1.1.1 WTF and UTG aspects of single-vein formation. Starting from the initial zone of auxin-producing cells at the apical margin (Z1, Fig. 2A), the model produces a broad auxin maximum towards which PIN1 is UTG-polarized (Fig. 2A and B; as also reported in Bayer et al. 2009). The 5-cell width of this zone corresponds to observations (Scarpella et al. 2006, Fig. 5A). The approximately 2-cell depth of auxin penetration and PIN1 activation depends on auxin diffusivity (\(D\)), transport through PIN1 (\(T\)) and the relative efficiency of transport between layers (\(C\)). At initially low auxin, the mechanism is in a chiefly UTG mode. PIN1 allocation and auxin flux amplify small inhomogeneities between cells, self-organizing a CP in the margin (Fig. 2C; Fig. 2D, blue circle; Fig. 2D’, mature CP indicated by red asterisk). As auxin becomes high in the CP, the concentration difference with internal cells increases. This increases diffusive flux and favours a chiefly WTF mode of allocation preferentially across the inner wall, which is less UTG-polarized into the CP than across the marginal walls (Fig. 2D and D’). This initiates vein extension into the interior. With-the-flux polarizes and extends the early midvein (localized PIN1-auxin) from the apical source towards the leaf base and the existing vasculature of the plant (represented by the blue-walled ‘sink’ cells, Fig. 2C arrows), as well as maintaining high auxin concentration in the midvein, as observed experimentally. The flow in the vein is down the concentration gradient, from a steady peak at the CP; it is not a travelling wave, as in prior UTG-only models (Merks et al. 2007). Sink cells can be modelled as either high auxin concentration (shown, representing high auxin vasculature in the main plant), with UTG-dependent basal connection of the midvein; or sink cells can be low auxin (with higher auxin degradation than the rest of the leaf), with WTF-dependent midvein to sink connection.

With the dual-polarization model canalization, the narrowing of the initial broad inflow into a narrow file of auxin-transporting cells (e.g. Fig. 2C) can involve both WTF and UTG factors: \(\phi^*\) WTF allocation alone (\(k_{w_{\phi}}\) term in equation (2)) has been shown to produce canalization (Mitchison 1980; Rolland-Lagan and Prusinkiewicz 2005); and with a high-auxin midvein (absent in the UTG-only Merks et al. (2007) model), UTG allocation can produce channeling UTG into the midvein. Tests with \(k_{w_{\phi}} = 0\) (eliminating WTF \(\phi^*\) but retaining WTF \(\phi\) and UTG terms in equation (2)) show that UTG is sufficient for canalization, but these show poorer left–right symmetry of the secondary vein pattern (Section 3.1.2) than with high auxin \(\phi^*\) as well (i.e. display more variability in vein initiation and extension from left and right zones with the same \(A_{p_{\text{prec}}}\). Without sufficient UTG allocation, lateral diffusion and WTF allocation would create polarization away from a high-auxin vein. The observed interior cell polarization towards high-auxin CPs and veins (Bayer et al. 2009, Figs 2 and 5; Wenzel et al. 2007, Fig. 4) reflects UTG allocation (Fig. 2A and C; Bayer et al. 2009, Fig. 7). Up-the-gradient and WTF contributions to single-vein development are summarized in Table 4.

3.1.1.2 Control of vein directionality. The wall-length dependence of flow (\(L\), term, equation (1)) can favour vein extension crossing longer walls rather than shorter ones. In computations with realistic non-uniform distributions of cell sizes and shapes, this can produce short-scale turnings of the vein which, if strong enough, can cause failure to connect to the sink (midvein to basal plant vasculature, or secondary to midvein), unlike the experimentally observed relatively direct and straight extension from auxin sources to sinks. (As a test, removal of wall-length-dependent flux, which gives incorrect diffusive transport and essentially turns cells into isotropic points, creates straight veins. Similarly, Walker et al. (2013) reported straight veins in
Figure 2. Computed results for primary and secondary vein patterning on a leaf of 128 cells. In each frame, apical (distal) is up and basal (proximal) is down. (A) Initiation of the primary vein (midvein), \( t = 2 \) computational minutes. Colour code: green intensity, auxin concentration; red intensity, PIN1 concentration; white arrows, net direction of auxin flux. Auxin produced in

Apical auxin maximum formation
\( t = 2 \)

1º midvein formation
\( t = 150 \)

First 2º initiated
\( t = 180 \)

First 2º canalized
\( t = 320 \)

Second 2º initiated
\( t = 345 \)

Second 2º canalized
\( t = 630 \)
To counter these short-scale effects, previous implementations of UTG+WTF models hypothesized a vein attraction factor (VAF), which diffuses from established veins, setting up long-range gradients to guide new vein extension (Bayer et al. 2009; Hartmann et al. 2019). As long as auxin diffusive transport ($D_a$) is low relative to PAT ($T$), we have found the VAF to be unnecessary in creating source-to-sink veins longer than those published previously. The simulation in Fig. 2, with no VAF, shows a source-to-sink transit of 15 cells. Bayer et al. (2009) showed a 12-cell transit, using a VAF diffusivity more than 100 times that of auxin; and Hartmann et al. (2019) showed a 6-cell transit using a VAF diffusivity five times that of auxin. Given that auxin is a small molecule...
A role for growth in venation patterns. (A) Initial patterning starts at the 32-cell stage ($t = 2$ min). Polar auxin transport dynamic parameters are as in Fig. 2. Cell growth rates given in Table 3. (B) By $t = 105$ min, the first CP has initiated the midvein and this has extended to the basal sink. The midvein is established over a shorter distance scale than in the fixed-size leaf in Fig. 2. (C) By $t = 180$ min, cell expansion in the margin creates space for new initiation zones. (D) By $t = 360$ min, continued expansion creates successively proximal initiation zones. (E) By $t = 630$ min, with continued expansion, the number of secondary veins is not limited by the initial leaf size, as in the fixed-size Fig. 2 simulation. Panels drawn to scale.

(VAF $= 175$), with an estimated diffusivity around $7 \times 10^{-6}$ cm$^2$ s$^{-1}$ (Goldsmith et al. 1981), about that of sucrose in water, such VAF diffusivities are unrealistically fast, particularly to transit larger more mature leaves. We do find that simulations on larger leaves (twice the number of cells than Fig. 2) frequently fail to connect source to sink, indicating a limitation on the length scale for veins formed solely by a cellular-scale
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UTG+WTF PIN allocation mechanism. Figure 4 shows the possible contribution of tissue growth in the formation of longer and straighter veins. High auxin in sinks (e.g., older vasculature) can itself be an attractant (via UTG), but we find, for the highest auxin diffusivities compatible with observations (in particular, compatible with the ‘lock-in’ of pattern to the margin when PAT is strongly disrupted, Fig. 1E),
that attraction is only effective (in the sense of deflecting a vein’s trajectory) over one to two cells. This suggests that established veins are not primarily long-range attractors for a PAT mechanism, but are termini at which new veins can join the leaf’s vascular network.

### 3.1.2 Secondary vein network, multiple veins

Applying a dual-polarization mechanism to the multiple veins of the secondary network requires a model for sequential initiation. With the $A_{\text{prec}}$ dynamics of Section 2.3 we can address left–right synchronization of pairs of secondaries, directionality of secondaries and connection of the secondaries to the midvein (and to older secondaries, in the case of branching).

Similar to midvein formation, in auxin-producing zones ($A_{\text{prec}} > 0$) UTG creates a CP and WTF initiates inflow and extends the PIN1-auxin pattern inwards. The initial broad inflow (Fig. 2E) canalizes to a narrow file of cells, with interior cells aligned towards the new secondary vein (Fig. 2F), as observed (Wenzel et al. 2007, Fig. 4). Up-the-gradient allocation facilitates connection of the extending secondary vein tip to the higher auxin concentration midvein, to create a functional vascular network (Fig. 2F, blue circle).

The process—broad initiation zone (UTG-polarized), broad inflow (WTF), canalization (UTG or WTF) and joining to the midvein (UTG)—repeats to form subsequent secondaries (Fig. 2G and H; cf. Fig. 1A). As with midvein formation, the model forms these patterns with no specific attraction factor to veins (e.g. VAF), no specified source cell within the auxin-competent zones (UTG self-organizes the source location) and no initial $P_{\text{cy}}$ polarity.

Figure 6. Secondary vein branching. In many dicot leaves (e.g. *Arabidopsis*), the initial secondary vein goes directly to the midvein (A, third top panel), then an upper loop branches to the distal midvein (A, fourth top panel). Branching continues in subsequent secondary veins, but with the upper loop connecting to the prior secondary rather than the distal midvein (A, lower row). (B) Polarization corresponding to the subsequent secondary type of branching in the model: during early ‘cloud’ inflow, flows can separate into towards the midvein and towards the prior secondary (yellow arrows indicate strong PIN1 towards both of these targets; middle-right area of leaf shown, $t = 375$ min; same parameters as Fig. 2). In these cases, cells are net polarized away from the CP source, as indicated in A (black arrows). (C) Later ($t = 600$ min), cells become canalized towards a single target. (D)

For the first, distal-most secondary, Scarpella et al. (2006) discussed upper branch formation UTG-oriented towards the lower branch and towards the distal midvein, meeting in a bidirectional cell (at ‘ULD’). While some bidirectionality can be observed for margin-CP sourced flow (blue arrows, B, PIN1 in this central cell is towards both secondaries), this is transient: canalization favours unidirectional polarization towards the major secondary branch at later times, breaking branched connections between secondaries (C; blue circle around cells with coloured arrows in B; B and C not shown at same scale). A from Wenzel et al. (2007) with permission; D from Scarpella et al. (2006) with permission.
Table 2. Relative parameter constraints.

| Parameter                  | Too low                                                                 | Too high                                                                 |
|----------------------------|--------------------------------------------------------------------------|--------------------------------------------------------------------------|
| $k_{1}$                     | PIN1 polarization too persistent: primary interferes with secondary patterning | PIN1 cannot polarize                                                      |
| pinpr:pindec ratio          | Slow PIN1 upregulation with auxin inflow                                 | PIN1 too persistent: patterns slow to remodel                             |
| $k_{2}:k_{w1}$ ratio        | Weak CP; poor PIN1 alignment towards vein                                 | Curtailed vein extension                                                  |
| $D:T$ ratio                 | Auxin too constrained with strong NPA; WTF flow does not start           | Straighter veins, but overrides UTG; auxin fills smaller leaves           |

Table 3. Growth rates$^a$.

| $t$          | $G_1$ | $G_2$ | $G_3$ |
|--------------|-------|-------|-------|
| $t = 0$ min  | 1.2   | 1.8   | 1     |
| $t = 180$ min| 0     | 1.4   | 2.5   |
| $t = 360$ min| 0     | 1     | 1     |

$^a$Parameters switch to stated values at given time $t$.

3.2 Inhibiting PAT

Chemical inhibition of PAT (such as with NPA) provides a concentration-dependent series of phenotypic effects to test and constrain the quantitative model.

3.2.1 Moderate PAT inhibition. Moderate NPA concentrations can induce a number of effects on venation. These include an increased number of veins initiated, delayed canalization, broader vascular bundles and disrupted vein extension including disrupted connection to the shoot vasculature (Fig. 1D; Mattsson et al. 1999). 1-N-naphthylphthalamic acid’s effect on PAT is modelled by a decrease in transmissivity through PIN1. A moderate decrease of the $T$ parameter (from 3.0 to 0.55) produces some of the observed NPA effects, particularly disruption of vein extension and of the connection of new veins to old (Fig. 3B). Decreased throughput also increases auxin concentration in the veins (compare the more intense green in Fig. 3B to the normal flow case in Fig. 3A), corresponding to observations with the DRS auxin reporter (Mattsson et al. 2003, Fig. 4) and of increased vasculature (Fig. 1D; Mattsson et al. 1999).

3.2.2 Strong PAT disruption. At high NPA concentrations, vein extension becomes strongly affected, with vasculature (Mattsson et al. 1999, Fig. 4) and auxin response (Fig. 1E) constrained to the margin where auxin is synthesized. Lower $T$ generates this effect in computations. At $T = 0.4$, more CPs form in the margin than normal, but do not initiate veins (Fig. 3C). At $T = 0.1$, marginal UTG patterning is eliminated, giving uniform auxin and PIN1 distributions (Fig. 3D). Polar auxin transport is too weak in this case for UTG-dependent CPs or WTF-driven inflow.

3.3 A role for growth

While the PAT dynamics of PIN1-auxin patterning can be modelled in fixed geometries, leaves grow significantly during formation of the vascular network. Not only must patterning dynamics found in fixed geometries be robust to leaf growth, incorporating growth dynamics into the model can potentially account for additional features in vein development.

3.3.1 Normal patterning. In Fig. 4, the same PAT model as above (e.g. Fig. 2) is run on a growing leaf (Table 3 parameters). The smaller initial shape (32 cells vs. 128 in Fig. 2) corresponds more closely to the early primordium geometry when the midvein source (CP) is forming (Scarpella et al. 2006). The CP forms (Fig. 4A), extends the midvein across the growing leaf and connects to the basal plant vasculature (Fig. 4B). Growth augments PAT vein extension: once the midvein is connected to the basal sink (Fig. 4B), the midvein is propagated over large changes in size (to Fig. 4E) without losing that connection. By contrast, PAT-alone vein extension on large fixed-size leaves (twice the area of Fig. 2) shows curvy midveins that can fail to connect to the basal sink. This suggests that while PAT may be a central mechanism for symmetry breaking, pattern initiation and short-range vein extension, growth itself may play a role in expanding early short-range patterns to the scale of the mature leaf, specifically in forming straighter veins than PAT alone and insuring connectivity in the vascular network.

The growth rates in Table 3 represent basal-dominant leaf elongation. As subsequent secondary zones become auxin-producing on this geometry, left–right pairs of secondary veins initiate from mid to basal locations and flow inwards to join the midvein. This results in an extending ‘ladder’ of an unlimited number of secondaries—closer to the observed sequence (Scarpella et al. 2006, Fig. 5; Wenzel et al. 2007, Fig. 7; cf. Fig. 1A) and in contrast to the ‘sweeping down the clock face’ of a limited number of secondaries on fixed geometries (Fig. 2). This indicates that a mechanism combining PAT patterning and growth is not limited by leaf size or number of cells. The features of PAT patterning from Fig. 2 are robust to growth; and incorporation of growth to the model more closely matches observations in terms of leaf size changes, longer veins, connections to sinks, elongated leaf shapes, number of secondaries and proximal initiation zones.

3.3.2 PAT inhibition. On the fixed leaf, simulation of NPA treatment by reducing $T$ produced some of the experimentally observed features (Fig. 3). However, increased numbers of CPs were not readily generated for $T$ levels that still supported vein extension (Fig. 3B). With growth, cell division can create temporary disruptions of PAT in the auxin-producing zones of the margin (while PIN1 accumulates along new anticlinal walls). With normal $T$, a central CP can remain...
dominant, pulling inflow from the whole veins, such that single veins are initiated from each zone (Fig. 4). With reduced $T$, however, the dominance (i.e. length scale) of the central CP is diminished, and transient disruptions, such as cell division, can aid the formation of new supernumerary CPs and veins (Fig. 5A), as observed with NPA treatment (e.g. Mattsson et al. 2003, Fig. 4; Scarpella et al. 2006, Fig. 4).

Secondary veins (Fig. 5B–D) show similarly disrupted extension as in Fig. 3 (fixed size). With the same growth rates as Fig. 4 (for the overall elongated shape), the secondary zones are similarly 5–6 cells, and tend to form single CPs. Increasing the growth (Fig. 5E; growth = 1.4 rather than 0 in the first 2° zone from $t = 180$ to 300) can generate multiple CPs in a secondary zone. Figure 5F and G demonstrates the loss of extension and subsequent ‘lock-in’ of auxin to the margin in low-$T$ (high-NPA) conditions, similar to the fixed-size results (Fig. 3C and D). Early veins appear diffuse and less canalized in NPA-treated leaves than normal, but canalization does eventually occur in these conditions (Scarpella et al. 2006, Fig. 4; Wenzel et al. 2007, Fig. 7). Similarly, canalization appears delayed in the reduced-$T$ computations. Compare Fig. 5B midveins ($T = 1.0$) to Fig. 4C midvein ($T = 3.0$): at $t = 180$, the reduced $T$ (Fig. 5B) is not canalized (unlike Fig. 4C), but it still canalizes by a later time (Fig. 5D).

The reduced-$T$ results suggest that the increased number of CPs and associated veins observed in NPA-treated leaves may at least partially reflect the effects of growth and cell division during reduced-PAT vein patterning.

### 3.4 Secondary vein branching

In many species such as Arabidopsis, secondary veins form a closed loop with a lower and upper branch. In computations, the initial broad inflow can resolve into branched veins (Fig. 6B). The overall polarization in these cases is WFT away from the CP source, branching towards sinks on the primary midvein and older secondary lower branch (Fig. 6B, yellow arrows indicate strong PIN1 alignment from the CP direction and towards both of the sinks; early UTG attachment can be seen at the sinks). This matches the type of polarization discussed by Wenzel et al. (2007) with respect to formation of the lower and upper branches for successive sets of secondaries (Fig. 6A). The branch in Fig. 6B is transient: the canalization needed to narrow veins also eventually selects against veins with weaker flow (Fig. 6C). Persistence of such branches may suggest a maturing factor during vascularization that locks in early branch polarization.

Scarpella et al. (2006) proposed that veins could join via extending UTG alignment from the existing veins until they met at a bidirectional cell (Fig. 6D, shown for the upper branch of the distal-most secondary). Bidirectional cells were reported by both Scarpella et al. (2006) and Wenzel et al. (2007). This type of vein joining has now been observed in many cases in higher-order veins (tertiary and above; Marcos and Berleth 2014). Bidirectional cells are also seen with the model (Fig. 6B, blue arrows), but as a transient stage: as the lower branch becomes dominant through canalization, the upper branch loses WFT flow towards the distal secondary, and polarity flips to UTG towards the lower branch (Fig. 6C). That is, former upper branch cells become interior cells UTG-aligned to the remaining lower branch.

The distinction between the alignments in Fig. 6A–C and Fig. 6D is that the former describes auxin flow from the margin, while the latter suggests an interior source for auxin (at the non-transient bidirectional cell at least, to supply auxin in such a bipolarized vein). The current model is for the margin-sourced primary and secondary veins (whose inward extension can be disrupted by NPA). Blade-derived branches, which chiefly form at a later stage, likely have a distinct mechanism involving auxin production in the interior of the leaf. Simulations with such an auxin production did enhance UTG alignment and attachment within the leaf, but inhibited WFT primary and secondary vein extension. This may suggest a developmental sequence, in which the leaf blade becomes increasingly auxin-producing, forming bidirectional loops and tertiaries after the secondaries have extended from the margin.

## 4. Discussion

### 4.1 Dual-polarization PAT mechanism

Bayer et al. (2009) introduced UTG+WFT dual-polarization to account for convergence and extension of auxin flows in leaf vein development. Similar to Bayer et al. (2009), equations (1–3) specify a cellular-resolution net flux dual-polarization model for PIN1-auxin dynamics which generates these flows; unlike the previous model, PIN1 exchange between cytosol and membrane is modelled as a continuous process (rather than re-partitioning $P$ to $Pd$ in each time step), and convergence–extension switching depends on the relative rates of the first two terms in equation (2), without a specified auxin threshold. Cieslak et al. (2015) formulated a dual-polarization mechanism including extracellular space and independent influx and efflux. Neither prior model was applied to sequential secondary formation or vein interaction. We have used equations (1–3) to create a quantitative framework for the development of the primary and secondary leaf vein network. The model generates normal venation patterns, as well as many of the observed responses to PAT disruption by inhibitors such as NPA. Parameter variation and selection (Tables 1 and 2) indicate aspects of the developmental process that are likely due to particular aspects of the PIN1-auxin dynamics, including UTG or WFT allocation (Table 4), as well as aspects that are likely outside these dynamics, such as the contribution of growth to the venation pattern. This model can be used to predict how auxin dynamics influence different venation patterns.

### 4.2 Essential role of growth in setting spatial scales for auxin-mediated venation patterning

All implementations of dual-polarization PIN1-auxin mechanisms have displayed challenges in producing long straight veins on the scale
of the leaf in realistic tissues with irregular cell shapes (Bayer et al. 2009; Hartmann et al. 2019; present model). The 15-cell midvein in Fig. 2 is nearing an upper limit for successfully transiting a leaf from source to basal sink. Increased down-the-concentration-gradient (diffusive) auxin transport (D or $k_{\text{DM}}$ parameters) can potentially extend the range of PAT patterning. However, D is constrained by (i) not flooding small young leaves with auxin and (ii) maintaining the narrow marginal distribution of auxin under strong NPA treatment; and $k_{\text{DM}}$ is constrained by maintaining a WTF-UTG ratio sufficient for both convergent and extensive flow. Bayer et al. (2009) proposed a non-auxin VAF emanating from established vasculature (e.g. the basal sink) to overcome the bias of WTF flow for longer walls ($L_v$). Implementation of the Cieslak et al. (2015) model in an irregular tissue (Hartmann et al. 2019) likewise used the VAF. With low diffusive transport (D) relative to PAT (T), we found the VAF unnecessary to generate source-to-sink transits longer than previously published.

Given the apparent short length scale of dual-polarization PAT, we propose that growth itself is integral to creating long straight veins across the scale of the mature leaf. While inhibition experiments show that PAT is critical in CP formation and vein inflow, cellular-resolution simulations indicate PIN1-auxin PAT is short-scale, with directionality (or attraction to established vasculature) on the order of several cells. We propose that PAT must be considered within the context of leaf development. That is, PAT can play a critical role in patterning (CP formation) and flow (auxin inflow, canalization, transit across small leaves, vein–vein connection), but these short-scale patterns are then expanded by growth to produce the venation of large mature leaves. For example, after the midvein connects across the relatively short CP-to-sink distance in the early leaf (Fig. 4B), growth can extend the midvein indefinitely as the leaf elongates (Fig. 4E). This effect was also seen with WTF veins in the segregated model of Smith (2011; also Smith and Bayer 2009). Likewise, new proximal secondaries can form relatively close to the midvein, and subsequently be extended by growth. Finally, to the extent that diffusion may play a role in leaf-scale vein orientation, it would be far more effective, especially at biochemical diffusivity values, on smaller than larger leaves.

The role of growth on vein patterning could potentially be tested. For instance, experiments could test whether driving faster than normal growth increased non-connections to sinks, or whether ectopically initiated veins on large slow-growing leaves could form straight transects. Such experiments could be complicated though by existing vein patterns and developmental stage (e.g. early vs. late auxin production).

### 4.3 Generation of the secondary vein network

To generate multiple veins and begin studying secondary network patterning, we combined the convergence–extension dual-polarization dynamics (equations (1–3)) with a model for successive apical to basal (distal to proximal) activation of auxin-synthesizing zones in the margin (Section 2.3). This corresponds to the observed developmental sequence of auxin activity (Mattsson et al. 2003; Wenzel et al. 2007), and generates multiple sets of secondaries to study left–right branching and interaction. In the model, each zone is already defined in the $t = 0$ leaf; this is followed by successive activation via $A_{\text{syn}}$ increase and zone expansion due to growth. Biologically, there are likely additional dynamics involved in differentiating auxin-producing zones from undifferentiated tissue at the leaf base.

Prior work provides some suggestions. Scarpella et al. (2006) noted the parallels between the successive initiation of leaf primordia from the SAM and the successive initiation of secondary veins within leaves; and indeed a similar combination of growth and UTG patterning can simulate the emergence both of regularly spaced leaf primordia on the SAM apex (Jonsson et al. 2006; Smith et al. 2006) and of CPs at the proximal leaf margin (Smith 2011; and Smith and Bayer 2009). Jonsson et al. modelled a peripheral zone of auxin synthesis in the SAM; the developmental sequence of auxin competence in the leaf margin suggests a series of these zones generated proximally, perhaps with a finite lifetime for synthesis, as indicated by the decay of marginal source intensity after a vein is established (Mattsson et al. 2003; Scarpella et al. 2006; Wenzel et al. 2007). Smith’s (2011; and Smith and Bayer 2009) work suggests zone spacing could involve UTG as well as growth, but this model invokes an auxin-threshold differentiation that would have to be reconciled with dual-polarization dynamics in all cells to generate PIN1 convergence and extension. The association of zone emergence with proximal leaf growth driving the distal–proximal succession of secondary vein initiation (e.g. Fig. 4) reinforces the central role of growth in venation patterning.

### 4.4 Multi-vein modelling enables dissection of a broad range of PAT-inhibited phenotypes

The application of a dual-polarization PAT model to secondary venation is new, and therefore provides a new quantitative framework, calibrated to finer features of NPA phenotypes, than could be provided by prior models. Increasing PAT inhibition shows a succession of effects on the primary and secondary vein network. Low inhibition exhibits delayed canalization and supernumerary veins; moderate inhibition shows altered or disrupted vein connections; and strong inhibition shows reduced or lost extension. Polar auxin transport inhibition in a dual-polarization mechanism was previously simulated in the single-vein model of Bayer et al. (2009). This showed partial effects at the low and high end of the responses: broadening of the midvein PIN1 distribution at low inhibition (disrupted canalization), and loss of the CP and lost extension at stronger inhibition. The multi-vein model allows us to address responses in the middle of the range (supernumerary veins and altered vein connections), as well as more fully characterizing the weakest and strongest responses (delayed canalization and reduced extension).

#### 4.4.1 Delayed canalization and vein extension

While PAT inhibition can lead to PIN1 broadening (Bayer et al. 2009 simulations), in the leaf this is transient and canalization does eventually occur (e.g. Scarpella et al. 2006, Fig. 5). A simulation that does not eventually canalize indicates an overwhelmed canalization mechanism; for example, reduction of UTG ($k_{\text{UTG}}$ in equation (2)) can produce such results. The broad marginal PIN1 expression in the Bayer et al. (2009) results for strong PAT inhibition (lost vein extension) indicates high auxin diffusion (D). Lowering this to match the observed margin ‘lock-in’ of auxin for strong PAT inhibition (Figs 1E and 3D; Mattsson et al. 2003,
Fig. 4) increases the UTG-WTF ratio, and results in veins with the observed developmental sequence of early poor canalization (Fig. 5B) and later sharp canalization (Fig. 5D). This suggests that if auxin diffusive transport could be experimentally increased, vein canalization could be reduced.

For strong PAT inhibition, affecting extension enough to constrain vascularization to the margin, our model predicts a progression from non-extending supernumerary CPs (Fig. 3C) to a uniform margin (Fig. 3D) as inhibition increases. This is suggested by auxin activity patterns in Mattsson et al. (2003).

4.4.2 Supernumerary veins, and bundling vs. broadening (effects on vein connections). The parameter conditions to form a CP in an auxin-synthesis zone do not tend to produce multiple CPs at moderate PAT inhibition where vein extension is maintained (multiple CPs do form at higher inhibition, Fig. 3C). Growth, however, can promote CP splitting at moderate PAT inhibition. With the accompanying cell division, anticlinal divisions can temporarily disrupt marginal flow (as PIN1 forms towards the new wall) and allow inflow of a new vein (Fig. 5A); i.e. interrupting flow to the central CP (already weakened by PAT inhibition) can cause ‘leaks’ from the margin that initiate new veins. This suggests that experimental reduction of cell division might produce leaves resistant to forming supernumerary veins upon PAT inhibition.

With moderate PAT inhibition, the vascularized midvein (as distinct from the early transient uncanalized PIN1 distribution) can appear broadened, but is actually a bundle of multiple canalized veins (Fig. 1D; Mattsson et al. 1999). Polar auxin transport inhibition induced supernumerary veins approach but fail to join the midvein. Rather, they turn proximal wards and extend parallel to the midvein, resulting in a bundle of parallel veins in the central region of the leaf. These ‘turnings’ are also observed in pin1 mutants. This indicates an effect on the PAT-dependent mechanism connecting the secondaries to the midvein. Simulating the patterning effects of these altered vein interactions requires a multi-vein model. With the regular model (Table 1 parameters running continuously), we observed somewhat impaired secondary to midvein connections at reduced T (e.g. Fig. 3B, purple circle). More distinct proximal turnings could be induced at reduced T by strongly decreasing UTG (kₚ) after CP formation and vein initiation. In these conditions, the model is primarily WTF as the secondaries approach the midvein; reduced T may reduce canalization towards the midvein enough to favour diffusion towards low auxin in the basal part of the leaf. This suggests a mechanism in which UTG vein connection is more strongly affected by PAT inhibition than is WTF vein extension. Such differential response might stem from a differential response for different proteins of the PIN family, a PAT inhibition effect on factors in the interior of the leaf that weaken UTG, or a PAT inhibition effect on timing such that UTG PIN1 alignment towards the midvein is not complete as the secondary approaches. High-resolution videos of PIN1–GFP could be instructive for whether alignment is UTG towards a secondary tip during extension, then flips to UTG towards the midvein in the final approach—suggesting a UTG connection mechanism throughout the process; or, whether alignment is WTF out from the extending secondary tip for a significant portion of its transit towards the

midvein, as suggested by the ‘turning’ simulations in which UTG is dropped during the extension phase.

4.5 Vein branching and the location of auxin production

Auxin inflow from a marginal CP and its associated PIN1 alignment can bifurcate, with a lower branch connecting to the midvein and an upper branch connecting to the older distal secondary (Fig. 6B). PIN1 polarity in these cases is always away from the CP source in the margin. Canalization can make the forked flow transient, eventually favouring a single dominant vein. These characteristics of margin-CP initiated veins are distinct from interior veins (generally tertiary and later) that bridge two older veins, in which both termini have PIN1 polarized towards the older veins. Bridging veins have bidirectional cells, implying interior auxin sources. Our modelling indicates that interior auxin production can impede inflow of the main secondary from margin to midvein. This suggests a potential temporal regulation of auxin production locales, with interior production increasing later in development. Localized manipulation of auxin synthesis could be a means of distinguishing the two types of vein, with at one extreme margin-only synthesis producing only secondary-type veins and branches PIN1-polarized away from the margin, and at the other extreme, interior-only synthesis generating tertiary-type veins PIN1-polarized away from bidirectional cells. The different types of veins may be manifestations of the UTG+WTF allocation dynamics’ response to developmentally varying auxin-source levels and distributions.

4.6 Conclusions

The molecular evidence for PAT, the ability to manipulate it, e.g. via NPA or in various mutant backgrounds, and the rich pattern forming dynamics characterized through simulations and mathematical analysis have created a large body of research over the past several decades. In leaf venation there are two aspects of PAT, WTF and UTG PIN1 allocation, which can be inferred from observed auxin concentrations and PIN1 polarization. Patterning in multiple pin type knockouts (or intermediate NPA treatment) suggests both PAT and non-PAT aspects of venation (Verna et al. 2019). Further identification of UTG and WTF contributions to vein patterning will be aided by experiments targeting PIN1 allocation, and understanding the role of PIN-facilitated transport within the context of other factors, such as diffusion or transport through plasmodesmata (Ravichandran et al. 2020). Non-PIN factors could potentially be involved in the observed straightness of veins.

Polar auxin transport tends to have a very local character, based on intracellular kinetics and cell-to-cell transport. Leaf venation can also be addressed at the much larger leaf-wide scale. This includes physics-based models in which venation reflects the stress patterns in the leaf (e.g. Couder et al. 2002; Bar-Sinai et al. 2016), and also higher-level chemical models, such as Runions et al. (2017). This latter paper posits a ‘drainage efficiency’ for margin-produced auxin, which controls the secondary to midvein contact angle, and is highly successful, when coupled to growth, in generating leaf-scale morphogenesis and species-specific shapes. A great challenge in modelling is to bridge these scales, so that we can understand the molecular dynamics at the cellular level that generate leaf-scale patterns, and that underline the differences between species
shapes. We have characterized the UTG and WTF PAT dynamics indicated by normal and NPA-treated leaf vein development. We discussed the limitations for diffusion to establish long length scales, and suggest that growth of the leaf itself may be a scale-bridging mechanism for transducing the several cell scale to leaf-scale patterns.

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CONFLICT OF INTEREST
None declared.

CONTRIBUTIONS BY THE AUTHORS
C.L.W. and D.M.H. conceived the project; D.M.H. developed and coded the mathematical model and ran the simulations; C.L.W. and D.M.H. wrote the manuscript.

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