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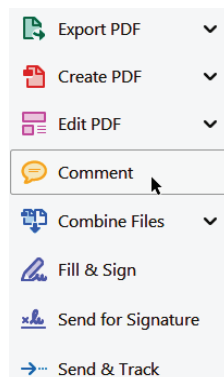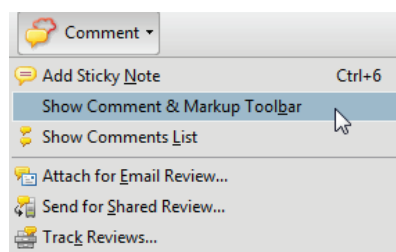

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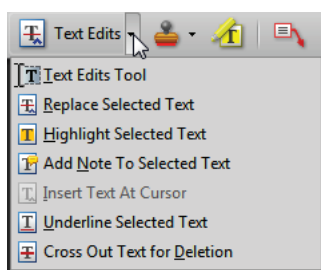
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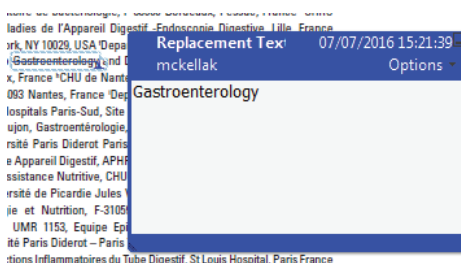
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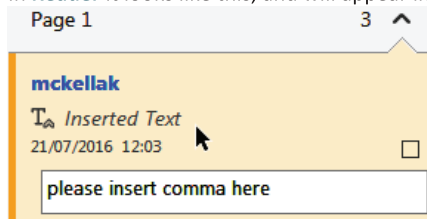
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OPINION PAPER

Theoretical approaches to understanding root vascular patterning: a consensus between recent models

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Abstract

The root vascular tissues provide an excellent system for studying organ patterning, as the specification of these tissues signals a transition from radial symmetry to bisymmetric patterns. The patterning process is controlled by the combined action of hormonal signaling/transport pathways, transcription factors, and miRNA that operate through a series of non-linear pathways to drive pattern formation collectively. With the discovery of multiple components and feedback loops controlling patterning, it has become increasingly difficult to understand how these interactions act in unison to determine pattern formation in multicellular tissues. Three independent mathematical models of root vascular patterning have been formulated in the last few years, providing an excellent example of how theoretical approaches can complement experimental studies to provide new insights into complex systems. In many aspects these models support each other; however, each study also provides its own novel findings and unique viewpoints. Here we reconcile these models by identifying the commonalities and exploring the differences between them by testing how transferable findings are between models. New simulations herein support the hypothesis that an asymmetry in auxin input can direct the formation of vascular pattern. We show that the xylem axis can act as a sole source of cytokinin and specify the correct pattern, but also that broader patterns of cytokinin production are also able to pattern the root. By comparing the three modeling approaches, we gain further insight into vascular patterning and identify several key areas for experimental investigation.

Key words: Auxin, cytokinin, mathematical modeling, organ patterning, systems biology, vascular development.

Introduction

Over the last few years there has been considerable insight into the molecular mechanisms controlling the specification of the root vascular pattern. In Arabidopsis embryos, the vascular cylinder forms from a group of four provascular initial cells (Scheres *et al.*, 1994) (Fig. 1). As the embryo develops, these provascular initials proliferate through a sequence
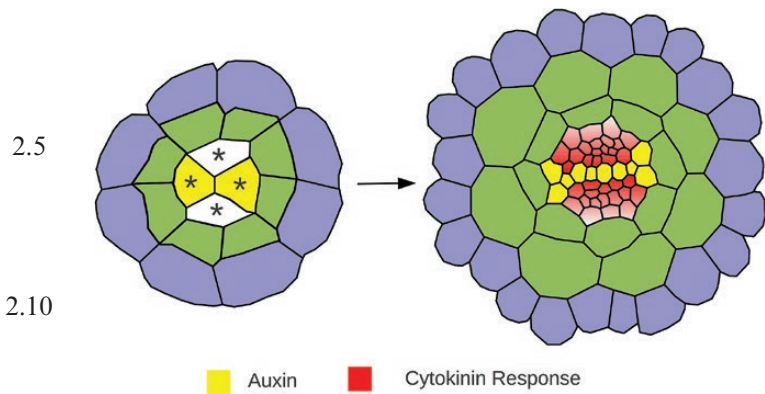
of highly regulated cell divisions to produce a vascular cylinder of ~40 cells by the time the seed germinates. In addition to cell proliferation, cell specification is critical to establish the xylem and phloem cell lineages. These tissues go on to form the main conduits for long-distance transport of water, nutrients, and signaling molecules within the plant. As the xylem and phloem initials differentiate, a bisymmetric pattern becomes apparent, and this is defined by a central axis of xylem cells flanked by two domains of pluripotent procambial cells and two phloem poles.

Experimental studies have shown that the two hormones, auxin and cytokinin, are essential in mediating both the cell proliferation and specification processes. The auxin response factor MONOPTEROS (MP/ARF5) is a central regulator of vascular formation, and mutants lacking this gene show defects in the formative divisions that create the vascular cylinder (Hardtke and Berleth, 1998). Amongst other targets, MP promotes the expression of a basic helix–loop–helix transcription factor, TARGET OF MONOPTEROS 5 (TMO5) (Schlereth *et al.*, 2010). Together with its homologs, TMO5 forms heterodimers with the LONESOME HIGHWAY (LHW) group of helix–loop–helix transcription factors to determine the frequency and orientation of cell divisions within the vascular cylinder (De Rybel *et al.*, 2013; Katayama *et al.*, 2016).

Auxin and cytokinin also play a crucial role in regulating patterning, and the bisymmetric vascular pattern is the outcome of an initial bisymmetry in the signaling domains of these two hormones. Auxin response is highest in a central line of cells that will go on to become the xylem axis, while cytokinin signaling peaks in the two domains flanking this axis (Mähönen *et al.*, 2006; Bishopp *et al.*, 2011*a*) (Fig. 1). Mutants severely impaired in either auxin or cytokinin response lack bisymmetry and display a radially symmetric vascular pattern (Mähönen *et al.*, 2006; Bishopp *et al.*, 2011*a*).

These distinct boundaries in the domains of hormonal signaling are maintained by two key interactions. High auxin response directly promotes transcription of the cytokinin inhibitor *ARABIDOPSIS HISTINE PHOSPHOTRANSFERASE 6* (*AHP6*) (Bishopp *et al.*, 2011*a*). In contrast, cytokinin signaling modulates the activity of a group of auxin transfer proteins known as PINFORMED proteins (PINs) (Ruzicka *et al.*, 2009; Bishopp *et al.*, 2011*a*; Pernisova *et al.*, 2016), although the molecular mechanisms governing the control of PINs by cytokinin are not completely understood. Cytokinin indirectly regulates PIN7 transcription, and modulates both the expression (Ruzicka *et al.*, 2009) and the subcellular localization of PIN1 (Bishopp *et al.*, 2011*a*; Marhavý *et al.*, 2011, 2014).

A second patterning process controls the disposition of the two cell types which make up the xylem axis. Protoxylem forms first at the marginal positions of the axis. It is characterized by the helical deposition of lignin that allows the cells to elongate as the root grows. Once the cells have completed elongation, larger metaxylem cells with a pitted secondary wall structure form in the center of the axis. This patterning of the axis is controlled by an additional group of transcription factors. The transcription factor SHORT ROOT (SHR) is expressed within the stele and moves to the endodermis where it forms a complex with SCARECROW (SCR) (Cui *et al.*, 2007). The SHR:SCR complex induces the expression of miRNA165/6, which moves into the vascular cylinder and targets the class III HD-ZIP transcription factors, including *PHABULOSA* (*PHB*), for degradation (Carlsbecker *et al.*, 2010). Collectively, these HD-ZIP genes determine proto- versus metaxylem identity in a dose-dependent manner, and also interfere with the hormonal patterning mechanism by restricting *AHP6* expression (Carlsbecker *et al.*, 2010).

Why model biological systems?

Molecular research has traditionally focused on individual gene products. However, these products often undergo a complex series of interactions, often in non-linear pathways with multiple feedbacks occurring at both the cellular and tissue scales. Mathematical modeling provides a framework to formalize these interactions and understand how they can generate pattern in both time and space. While mathematical models can serve to 'document' molecular processes and test the plausibility of interactive networks by recapitulating observed patterns of expression, they have a more powerful role in challenging experimental assumptions and identifying gaps in our knowledge to direct future theoretical and experimental work.

Previous models of hormone action in the root tip

There have been models of auxin transport for several decades (Mitchison, 1980), but only more recently has auxin transport been considered in multicellular models at the organ scale. In order to explore the transport dynamics of auxin

within the root, a number of independent models of auxin transport have been generated based on structured grids of rectangular cells (Swarup *et al.*, 2005; Grieneisen *et al.*, 2007; Mironova *et al.*, 2010). In Grieneisen *et al*. (2007), multiple auxin transporter types are placed within the cells based on experimental observations, while in Mironova *et al*. (2010), a single PIN type is modeled with its synthesis and degradation controlled by auxin. Both these models are able to generate an auxin maximum correctly at the root quiescent center. More recent auxin transport models have used realistic root geometries (Band *et al.*, 2014) and new sensor lines (Brunoud *et al.*, 2012) to incorporate a more detailed understanding of where auxin is localized within the root. While PIN levels change in response to perturbations in the mature root, PIN polarity seems to be fixed; therefore, most models of auxin action in the root are not concerned with the establishment and regulation of PIN polarity. These studies have shown that, in addition to the PIN proteins controlling auxin efflux, a group of auxin importers (AUX/LAX) is also required to recreate the pattern of auxin seen at the root tip. In general, these models have all focused on the longitudinal flow of auxin; while there have been some models considering the radial flow of auxin in outer tissues (Swarup *et al.*, 2005; Laskowski *et al.*, 2008; Péret *et al.*, 2013), these have not studied radial auxin flow through the vascular tissues. Other studies have considered radial pattering of the shoot using models formulated with a one-dimensional ring of cells (Ibañes *et al*., 2009; Fàbregas *et al*., 2015). There have also been models which consider the crosstalk between auxin and cytokinin, initially within the context of a single cell, but later in a one-dimensional line of cells (Muraro *et al.*, 2011, 2013).

Modeling root vascular patterning

In the last 2 years, there have been three independent publications modeling root vascular patterning in Arabidopsis. At first glance, these models might seem redundant, but each model asks different questions and provides novel insights into the system. In this paper, we explore the commonalities between these models, as well as investigating their differences. We also run new simulations to test whether the findings of specific models are supported by the different modeling approaches. Finally, we discuss specific areas where there is as yet no clear consensus and highlight areas where future experimental programs may provide new insights.

The first of the three publications considered here (Muraro *et al.*, 2014) uses both a two-cell template and a multicellular geometry to identify a minimal gene regulatory network involved in establishing and maintaining vascular pattern. The second (De Rybel *et al.*, 2014) builds upon this patterning mechanism to explore how the root vascular pattern is established and develops during embryogenesis. Importantly, it considers both cell growth and division and provides new data showing how auxin and cytokinin interact. The final publication (el-Showk *et al.*, 2015) focuses on auxin transport in a spatially realistic model incorporating hormonal regulation of the auxin transporters. Hereafter, the three models are

referred to as the Minimal Framework model (Muraro *et al.*, 2014), the Growing Root model (De Rybel *et al.* 2014), and the Auxin Flux model (el-Showk *et al.* 2015). A summary of the network configurations in the different models is given in Fig. 2.

Model construction

Mathematical models will always be an abstraction of complex biological systems. There is never a clear answer as to how detailed to make them, and different teams will always take different approaches regarding how much information to include, depending on the question being addressed. A summary of the different network configurations is given in Fig. 2.

The Minimal Framework model seeks to understand the interaction between molecular components and how these interact as a network to control pattern. To this end, it includes each key class of molecule modeled explicitly. This has the advantage of providing greater insight into the molecular circuitry, and indeed this model has led to new insights into the patterning of AHP6 by a PHB–miR165/6 module that has not been considered in the other models.

In contrast, the Auxin Flux model addresses a different question, asking how the hormonal activation of auxin transport is regulated in a spatial context. In terms of spatial structure, this is the most comprehensive of the three models, as it offers the most detail in terms of compartmentalization of cells by considering the apoplast as a separate compartment. In contrast, the molecular network in this model is designed using the most conservative approach, making the most parsimonious model of vascular development possible. Essentially, the model uses as few molecular components as possible while maintaining the ability to address the biological question. In this model, no distinction is made between hormone levels and hormone signaling output. Some key components, such as AHP6, are not modeled as discrete components; instead AHP6 is handled via a generic repression of cytokinin in response to auxin.

The Growing Root model asks how hormones control tissue development, and as such it is essential to use a growing template to investigate the effects on both cell growth/proliferation and cell patterning. Since a new role for auxin-mediated cytokinin biosynthesis is an essential finding of this study, the authors investigate this by modeling cytokinin levels and cytokinin response separately, enabling the two quantities to be differentiated. However, they take a more parsimonious approach to some elements of their model where modeling would be unlikely to provide critical insights, applying a generic repression of cytokinin signaling by auxin (similar to the Auxin Flux model) and simplifying chains of interaction.

The three models used different modeling platforms: the Minimal Framework model was built using OpenAlea (Pradal *et al*., 2008), the Growing Root model used VirtualLeaf (Merks *et al.*, 2011), and the Auxin Flux model was a Cellular Potts model. The first two approaches are the most similar, as they are both vertex based. Geometrically, such models
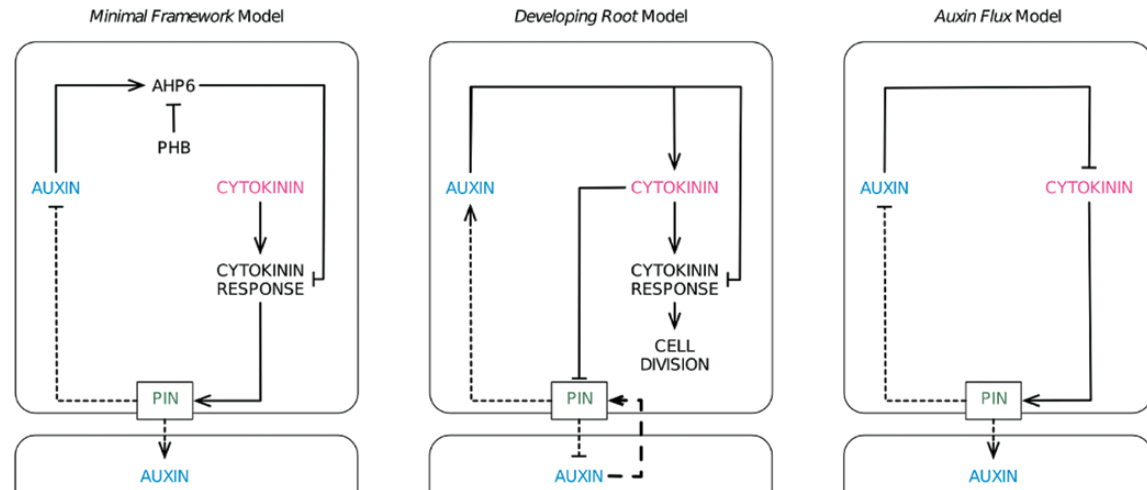
Fig. 2. Schematic diagrams showing the network configurations of the three vascular patterning models. These have been re-arranged from the original figures to aid comparison between models. Activation or repression is shown with solid lines. Dashed lines indicate transport of auxin into and out of the cell, with the arrowhead indicating whether it promotes or inhibits auxin accumulation within that cell. The long dashed lines indicate a mechanism by which PIN proteins are polarized within a cell in a manner dependent on the concentration of auxin within neighboring cells (see text). Although only two cells are shown, these models are all embedded within multicellular templates.

are comprised of polygons, with each polygon representing a distinct cell. A set of ordinary differential equations (ODEs) determines the molecular processes occurring within each cell, and components can move between cells based on a set of terms in the ODEs governing the fluxes across membranes. Cellular Potts models differ in that cells are comprised of a number of pixels or voxels arranged in a grid and thus have internal space; in addition, the apoplast is explicitly included in the model. While the vertex-based approaches simulate movement of molecules purely as permeability across membranes, the Cellular Potts model, in contrast, also allows the investigation of diffusion within a cell and in the apoplast. Simulating the diffusion within cells has previously been shown to be important in templates with larger cells, such as those considering root bending or lateral root initiation (Laskowski *et al.*, 2008). In general, vertex-based and Cellular Potts models have similar capabilities in a static setting; the major differences emerge when they are used to model cellular growth. This discussion is not within the scope of this paper and is covered in detail elsewhere (Prusinkiewicz and Runions, 2012; Liedekerke *et al.*, 2015).

A minimal molecular framework for vascular patterning

The Minimal Framework model (Muraro *et al.*, 2014) investigated the feasibility of an auxin–cytokinin mechanism as a control of tissue-specific patterning, first in a two-cell system but later in a multicellular template. The two-cell system was based on a pair of identical cells with a shared interface through which auxin and cytokinin could diffuse or, in the case of auxin, be transported through polar auxin transport. Within each cell, a series of equations calculated how the various components (auxin, cytokinin, AHP6, and PIN7) interacted to determine the steady-state solutions for each cell. For simulations run with extremely high/low levels of either auxin or cytokinin, only one possible steady-state solution existed,

both cells having similar outputs. For example, extremely high auxin levels resulted in both cells expressing high levels of AHP6 and having a negligible cytokinin response. However, for a large subset of intermediate conditions, multiple steady-state solutions existed in which one cell had high AHP6 and the other high PIN7. The presence of multiple steady-state solutions suggests that the system can act in a 'switch-like' manner to determine discrete domains of gene activity, and reinforces the concept that the auxin–cytokinin interaction can act as a patterning mechanism. However, this two-cell approach does not address how these patterns would look in a realistic tissue.

To introduce this model into a multicellular template, a series of simulations were run in which the expression/localization of PINs were fixed based on experimental observations and were not regulated by the model in order to explore genetic redundancy between the PINs. In an effort to simplify an otherwise complex network, the model later included only a single PIN type whose activity was based upon that of PIN7. During this work, the authors assumed a flat field of both cytokinin and auxin production, although subsequent work has shown this not to be the case. In order to direct pattern formation, an initial asymmetry was required; this was supplied through an initial pre-pattern in PIN7 expression. An experimentally defined network of auxin and cytokinin regulation, including the regulation of AHP6 by PHB (Carlsbecker *et al.*, 2010), was not sufficient to recreate the stable domains of gene expression as seen in roots. However, the authors were able to reproduce the observed patterns of gene expression stably by making two changes to this network configuration. The first involved altering the way in which PHB and miRNA165/6 interact. The introduction of a mutual degradation between these two components was required in order to produce stable gradients that could restrict AHP6 sufficiently to the marginal positions. The second change involved the incorporation of an additional, as yet unidentified, inhibitor of cytokinin (termed CKIN).

This was required alongside AHP6 to restrict cytokinin response, and therefore PIN7, in the central parts of the xylem axis. At the time, the authors proposed that this component could target either cytokinin biosynthesis or signaling; however, subsequent studies suggest that the former is unlikely.

The revised network could reproduce a stable vascular pattern, but it required an initial asymmetry in PIN7. To test the robustness of this system, the output from a previous simulation was used as a set of initial conditions that closely resembled the pattern of gene expression seen in wild-type roots. Simulations were then run to steady state in a system in which every cell had the potential to express PIN7. These simulations revealed that the initial vascular pattern was maintained, suggesting that the network provides a robust mechanism for maintaining pattern around an initial asymmetry, even though it does not generate the initial asymmetry or address its possible causes.

Early events specifying the xylem axis

This question was addressed in the Growing Root model (De Rybel *et al.* 2014) by investigating how the xylem axis was specified during embryogenesis. The model incorporated both growth and patterning within a dynamic array of cells. This study identified a crucial new interaction through which auxin promotes the transcription of the *LONELY GUY 4* (*LOG4*) gene via the TMO5/LHW dimer (De Rybel *et al.*, 2014). LOG4 is a crucial enzyme involved in the final stages of the cytokinin biosynthesis pathway and is believed to be the rate-limiting step controlling cytokinin homeostasis (Kuroha *et al.*, 2009). LOG4 is expressed in all four of the vascular initials, but in the growing root it is expressed throughout the xylem axis, suggesting that the xylem axis acts as a source of cytokinin (De Rybel *et al.*, 2014). Although in this model both growth and patterning are regulated, it is likely that these activities are achieved through two independent cytokinin responses: while cytokinin signaling promotes periclinal cell division, PIN localization is regulated via cytokinin directly, in what the model assumes to be an independent cytokinin response pathway. The PIN dynamics differ from those used in the Minimal Framework and Auxin Flux models; cytokinin mediates the inhibition of PIN1 localization, as observed during lateral root formation (Marhavý *et al.*, 2011), rather than inducing expression of PIN7 (Bishopp *et al.*, 2011*a*). Furthermore, PIN1 is also polarized in response to auxin gradients, as in some other simulations in different developmental contexts (Jönsson *et al.*, 2006). In this model, xylem cells are capable of producing cytokinin via TMO5/LHW-dependent activation of LOG4. As a result of the mutual interaction between cell growth and the reaction network, the production of cytokinin is constrained to the developing xylem axis in model simulations. As a result of high auxin levels in the xylem axis, cytokinin signaling is inhibited in these cells, which results in suppression of periclinal cell division.

The model itself comprised a combination of two interconnected feed-forward loops that controlled both growth and patterning (de Rybel *et al.*, 2014). The first loop considered cytokinin rather than cytokinin response to control PIN regulation and cell growth. The second incorporated the interaction between auxin and cytokinin response to control periclinal cell divisions. When applied to a template consisting of four provascular initial cells, these interconnected loops were sufficient to recapitulate both the growth and patterning processes necessary to create an axis of high auxin response in a growing template (de Rybel *et al.*, 2014). However, this required two additional inputs within the initial four-cell template (de Rybel *et al.*, 2014). The first was a bias in which auxin was elevated in two source cells representing the convergence points of the cotyledons. Secondly, the two source cells had to be connected by a small bridge (see Fig. 1), an assumption that subsequent experimental analyses have shown to be valid. Not only can these simulations recreate experimental observations based on limited prior information, but the simulations also showed gradients in both cytokinin and cytokinin response, with the highest cytokinin response in the cells adjacent to the xylem axis. While some cytokinin markers are not sensitive enough to reflect this gradient, re-analysis of others has shown such a gradient (de Rybel *et al.*, 2014).

A parsimonious model of auxin fluxes

The Auxin Flux model (el-Showk *et al.* 2015) delves much deeper into the concentration and flux patterns of auxin. While the previous models only considered a single PIN protein, this model included PIN1, 3, and 7, together with a combined role for PIN2 and the PGPs. It also incorporated a generic auxin importer to account for AUX1, LAX1, and LAX2. The Auxin Flux model incorporates cytokinin-mediated up-regulation of *PIN7* in a similar way to the Minimal Framework model. However, the role of AHP6 is simplified in this model; instead of explicitly modeling AHP6, the linear chain between auxin, AHP6, and the repression of cytokinin-mediated PIN activation is simplified to a generic repression of PIN7 and PIN1 by auxin.

Using a series of so-called 'static simulations', in which the auxin transporters were localized as observed experimentally but were not regulated by the hormones, the model was able to recreate patterns of auxin response similar to those seen experimentally in wild-type roots. However, static simulations run in the *woodenleg* (*wol*) mutant (Mähönen *et al.*, 2000), which almost completely lacks cytokinin signaling response, did not recreate the auxin response patterns observed in mutant plants; hormonal regulation of transporters in 'dynamic simulations' was required to recapitulate the correct pattern. Further analysis revealed that the difference between the static and dynamic simulations resulted from differences in the diffusion of auxin through the apoplast. The authors also simulated the outcome of a mutation in the auxin importers. While auxin still accumulated in the xylem axis in these mutations, the concentration was much lower. This led the authors to suggest that xylem specification might be unstable in plants with impaired auxin transport, a finding they

confirmed experimentally, as *aux1 lax1 lax2* triple mutant plants were found to have unstable pattern formation.

Although the Auxin Flux model focused on vascular patterning in the root tip, it provided unexpected insights into the process of lateral root priming. Lateral roots originate from the pericycle cells flanking the xylem poles (Lavenus *et al.*, 2016). In the model, certain subcellular arrangements of PIN1 generate an auxin flux circuit that not only allows the xylem pole pericycle cells to accumulate auxin at the expense of the xylem axis, but also ensures that two the poles compete against each other for auxin. While this dynamic provides a potential mechanism to prime lateral roots, future work is needed to assess experimentally the subcellular status of PIN1 and to evaluate the auxin flux circuit in simulations of a growing, three-dimensional root.

What initial conditions are required to set vascular pattern?

All three models require an initial asymmetry in order to establish the vascular pattern, but each addressed the asymmetry differently. In both the Minimal Framework and Auxin Flux models, this initial asymmetry was generated by an initial pre-placement of PINs. Furthermore, in the case of the Minimal Framework model, this initial asymmetry is required only transiently; once established, the system is able to maintain a stable pattern, even after the asymmetry is removed. The Growing Root model, in contrast, uses a persistent asymmetry in auxin input to drive pattern formation. Two of the four vascular cells continuously receive higher auxin input than other cells in the vascular cylinder, based on the observation that symmetry breakage first occurs in the apical part of the embryo and leads to an asymmetric production and transport of auxin at the incipient cotyledon, as

cells immediately subtending the cotyledons have been shown to have higher auxin response (De Rybel *et al.*, 2014), while mutants with altered numbers of cotyledons have been shown to generate roots with irregular numbers of xylem poles (Help *et al.*, 2011). A key question, which we now investigate with a new set of simulations, is whether a transient asymmetry in auxin input can be used to drive patterning in the Minimal Framework model.

To test this, we reran the Minimal Framework model using the original parameters and allowing each component (including PIN7) to be expressed in any cell, but with an initial condition of high auxin at both protoxylem cells and all four xylem-pole pericycle cells, where high AHP6 expression has been observed. The production rate of auxin is uniform throughout the tissue. We made one additional change to the model (see Supplementary Model S1 at *JXB* online). In the original model, we had a hypothetical component termed 'CKIN' that acted redundantly to AHP6 to inhibit cytokinin in the metaxylem. We changed this from a repressor of cytokinin levels to a repressor of cytokinin response, in keeping with the subsequent discovery of the xylem as a source of cytokinin. With these changes, the resulting steady-state pattern of AHP6 expression closely resembles the initial conditions, with only a minor shift in the position of the xylem axis (Fig. 3). These simulations support the idea that an asymmetry in auxin drives pattern formation in the root but suggest that such an asymmetry is required only transiently. While in the embryo and primary root of Arabidopsis the continuous transport of auxin from the cotyledons/pre-existing vascular is likely to provide a continuous asymmetry in auxin input, as incorporated within the Growing Root model, this is not necessarily the case in newly formed roots (such as lateral or crown roots) or during pattern specification in other plant species with three or more vascular poles. To investigate the
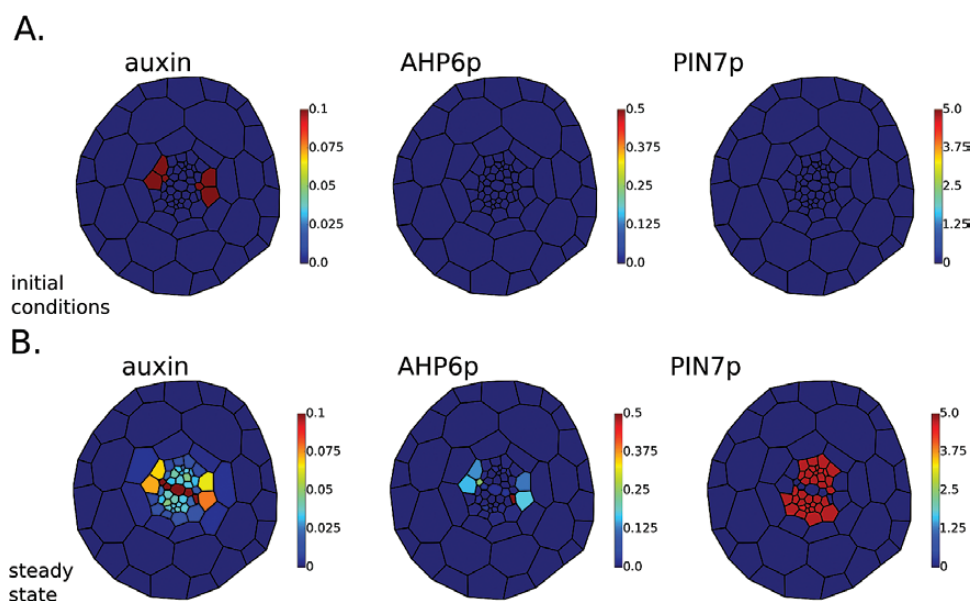
Fig. 3. Initial conditions (A) and steady-state values (B) of a simulation using the Minimal Framework model with the previously published parameter values. Rather than pre-patterning via PIN7 localization as in Muraro *et al*. (2014), the model is primed by having high auxin in the protoxylem and xylem pole pericycle as an initial condition, after which the production rate of auxin is equal in all cells. The model is able to pattern two poles of AHP6 at either end of the xylem axis, with only a small change to the initial pattern provided. Color ranges show arbitrary non-dimensional values.

importance of a continuous asymmetric auxin input for the functionality of the Growing Root model, we ran model simulations using a transient asymmetric auxin input; the model does not produce the correct pattern of auxin and cytokinin. However, when run in a static template (Fig. 4A), the same simulation produces the correct cytokinin and auxin patterns. The results from the Growing Root model suggest that continuous asymmetric auxin input is necessary during establishment of the vasculature, while this input is not required in later stages and to maintain auxin and cytokinin signaling domains in mature roots.

We believe that the observation that transient changes in auxin can be propagated as stable changes in vascular pattern will allow future models to address the patterning process in other species.

The xylem axis as a source of cytokinin

Although there are multiple markers for observing cytokinin response at a cellular/tissue scale, there are no methods for imaging the location of cytokinin itself at this resolution. Although the Minimal Framework and Auxin Flux models could produce stable patterning with a homogenous field of cytokinin production in each cell, experimental results published with the Growing Root model showed that the xylem serves as the major source of cytokinin. One output of the Growing Root model is that it is possible to create a gradient of cytokinin by driving cytokinin synthesis in the xylem axis only. Does such a gradient exist in plants and is it required for patterning? Analyses of independent cytokinin-responsive marker genes suggest that a gradient in cytokinin response does occur, but current technology does not allow us to know whether this is mirrored by a gradient in cytokinin itself. The presence of a cytokinin gradient in these tissues is an issue where there are different viewpoints between the authors.

The Growing Root model investigates both cell division and patterning through regulation of the PINs. Although cytokinin regulates both of these processes, it is likely that it does so using different downstream regulatory components; cytokinin-mediated patterning and cell proliferation are therefore handled separately in the model. As little is known about the mechanism through which cytokinin regulates PIN1, this was modeled as a direct interaction between cytokinin and PIN1 based on data from lateral root organogenesis (Marhavý *et al.*, 2011). This simplification was introduced in order to reduce unnecessary parameters, although it has subsequently been shown that this interaction is dependent on cytokinin signaling rather than being a direct activity modulated by the hormone itself (Marhavý *et al.*, 2014). Within the Growing Root model, a gradient of cytokinin promotes increased
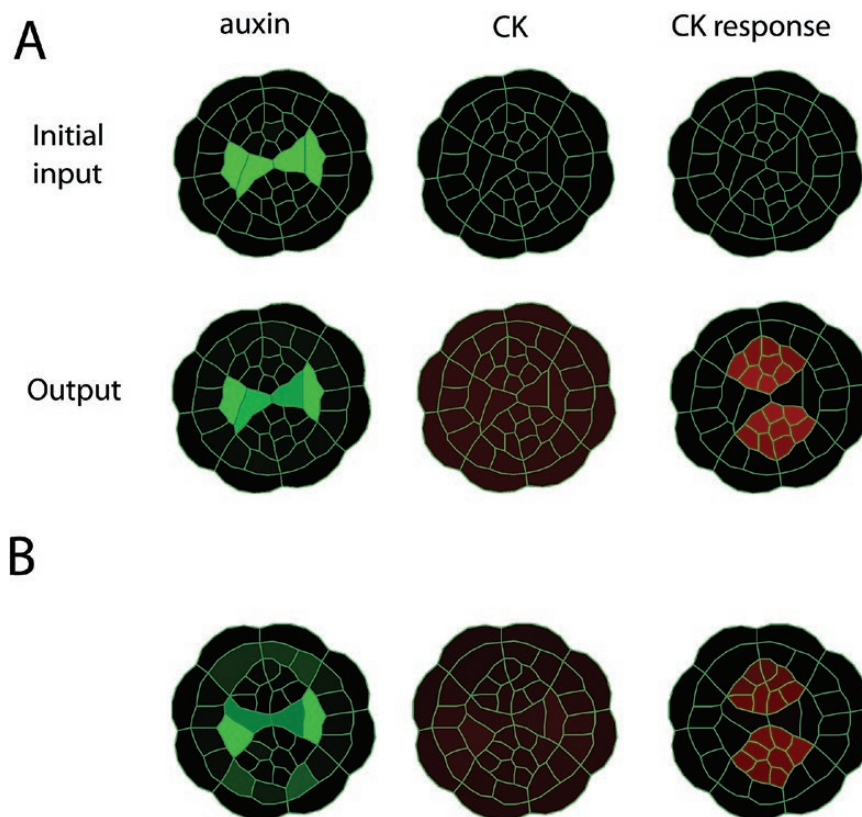

Fig. 4. Alternative simulations of the Growing Root model. (A) Results of a simulation of the growing root model in a static template with a transient high auxin input within the xylem axis. The initial asymmetric transient auxin input is sufficient to establish the correct patterns of auxin and cytokinin (CK) within the template. The transient auxin input is implemented as an initial auxin concentration of 10 µmol µm^{-2} in the xylem cells. (B) Output of a simulation using the growing model where CK localization on the cell wall is up-regulated via CK signaling. The correct patterns of auxin and CK signaling is obtained, while CK forms a homogeneous gradient throughout the cellular template. Parameters used in this simulation are listed in Supplementary Table S1.

polarization of PINs away from the xylem axis. Here we test whether a gradient of cytokinin is an absolute requirement for this model.

By running simulations in the Growing Root model with increased cytokinin diffusion, we were able to observe that the desired model output can be achieved with much shallower gradients than were previously published. In the most extreme simulations, cytokinin diffusion could be increased up to 40-fold with near-homogeneous levels and maintain correct patterning. Nevertheless, to specify pattern correctly, there is a requirement for cytokinin levels to be higher in the xylem than in adjacent cambial cells. The idea that only a shallow gradient of cytokinin is required is appealing, as other sources of cytokinin exist, such as phloem (Bishopp *et al.*, 2011*b*), although the majority of LOG activity is within the xylem axis (De Rybel *et al.*, 2014). In order to explore whether it is the regulation of PINs that is responsible for the requirement for a cytokinin gradient, we ran new simulations using the Growing Root model, in which PIN localization is promoted by cytokinin (Supplementary Model S2). These simulations resulted in correct patterning even when the rate of cytokinin diffusion was raised so high that it produced a homogeneous field of cytokinin (Fig. 4B).

In reality, multiple modes of cytokinin-mediated PIN activity probably co-exist in plants, and further experimental analyses documenting the exact interaction between cytokinin and individual PINs in the given developmental context is needed. Collectively, these simulations show that a shallow gradient of cytokinin is required in simulations incorporating PIN1-like regulation, as this appears to be the most important PIN during embryonic root formation (Friml *et al.*, 2003); it is possible that there are different requirements for cytokinin gradients during the formation of embryonic and mature roots. These studies highlight the need for detailed analyses analyzing exactly how each PIN responds to cytokinin in specific tissue types. Whilst this is feasible for the growing root, investigating this process during embryogenesis would represent a significant technical challenge.

A gradient of any molecule across a multicellular tissue is possible providing it is synthesized (either exclusively or at higher levels) in a group of source cells and that it moves between cells (e.g. via diffusion). The slope of the gradient results exclusively from the balance between the rates of diffusion and degradation. A molecule that diffuses very quickly or degrades very slowly will form a shallow gradient or become homogenously distributed throughout the tissue; a slower rate of diffusion or a faster rate of degradation will form a steeper gradient. The identification of the xylem axis as a key source for cytokinin provides a group of source cells, but what kind of gradient forms around this source?

Unfortunately, as we are unable to visualize individual molecules, the degradation rate and diffusion coefficient of cytokinin are both unknown. In the Auxin Flux paper, the authors argue that the parameters required to generate an informative gradient of cytokinin in tissues the size of an Arabidopsis root are unrealistic. Since all three models only include passive movement of cytokinin via diffusion, the choice of these parameters is critical for determining the shape of the resulting gradient; however, problems arise because these parameters are simply unknown.

Can a gradient of cytokinin exist?

In the Minimal Framework and Growing Root models, movement of individual molecules is governed only by permeability across a membrane. In the Auxin Flux model, movement also occurs via diffusion within cells and in the apoplast, arguably giving a more realistic modeling of diffusion. This is of particular importance for hormonal signaling since, at least over a short range, signaling molecules are thought to propagate faster apoplasticaly than symplasticaly (Robert and Friml, 2009). The Auxin Flux model uses the same diffusion coefficient for cytokinin and auxin based on the rationale that they are similar sized molecules. Although the parameter for auxin diffusion has been used in other computational models and is based on experimental values, these were not measured in plants but were generated using a polar membrane created between egg lecithin and decane (Gutknecht and Walter, 1980). Within this system, diffusion was dependent on pH, and the rate of auxin flux across the membrane was increased through conversion of auxin to an ionized form at the membrane surface. Whilst these may represent the 'best estimates' of cytokinin diffusion, they are open to debate, and only direct measurements will be able to provide irrefutable parameters.

Since an informative gradient would require that the cytokinin diffusion or degradation rates differ by several orders of magnitude from those used in the Auxin Flux model, the authors carried out an experiment to establish a lower limit on the diffusion rate of cytokinin in plants. They treated roots with exogenous cytokinin and measured the change in cytokinin response within the root; cytokinin response increased in both the outer and inner layers of the root within 6 h, suggesting that cytokinin can traverse the radius of the Arabidopsis root in a matter of hours at most (el-Showk *et al.*, 2015). This is a rate of cytokinin movement which is incompatible with the formation of an informative cytokinin gradient via diffusion unless the degradation rate is also orders of magnitude higher; however, such rapid degradation would also place limits on how far cytokinin could travel and would affect the time scales of regulatory networks. The authors therefore suggest that other mechanisms, such as active cytokinin transport or uneven expression of the cytokinin perception machinery, might be responsible for the observed cytokinin signaling patterns.

Although the models use a different system for dealing with diffusion, the Auxin Flux model was able to reproduce the gradient formed in the Growing Root model with an appropriate choice of parameters. We therefore endeavored to evaluate the questions of whether the xylem axis can act as a sole source of cytokinin and whether an informative cytokinin gradient can exist using the Minimal Framework model.

To reconcile these findings and test whether the xylem axis could function as the sole source of cytokinin, we ran new simulations in the Minimal Framework model with cytokinin production restricted to the xylem axis. As before, we provide

an initial asymmetry in PIN localization and then test the robustness of any pattern by removing this restriction on PIN placement. Using the original model parameters produces a sharp gradient of cytokinin away from the xylem axis, resulting in very low PIN7 expression except in the protoxylem, and AHP6 expression spreads throughout the pericycle and adjacent cells in the stele (Fig. 5A).

As discussed above, while the Auxin Flux model uses a diffusion coefficient in a Cellular Potts model to simulate cytokinin movement within and between cells, the other models only simulate movement from cell to cell using a permeability parameter. Based on an approximate average width of the cell layers in the cross-section of 30 μm, we estimate that, for a

given cytokinin production and degradation rate, the cytokinin distribution in the Auxin Flux model with $D_{ck}=600$ μm^2 s^{-1} can be reproduced in the other two models with $P_{ck}=20$μm s^{-1}. Similarly, we predict the results with permeability $P_{ck}=10$ μm s^{-1} used in the Minimal Framework model can be roughly reproduced in the Auxin Flux with a diffusion coefficient of 300 μm^2 s^{-1}. Though this diffusion coefficient is of the same order of magnitude to that used in the Auxin Flux model, cytokinin degradation (d_{ck}), the other key parameter in determining the sharpness of any cytokinin gradient, is much higher in the Minimal Framework model than in both the Growing Root and Auxin Flux models, resulting in the sharp gradient in (Fig. 5A). Although the exact data
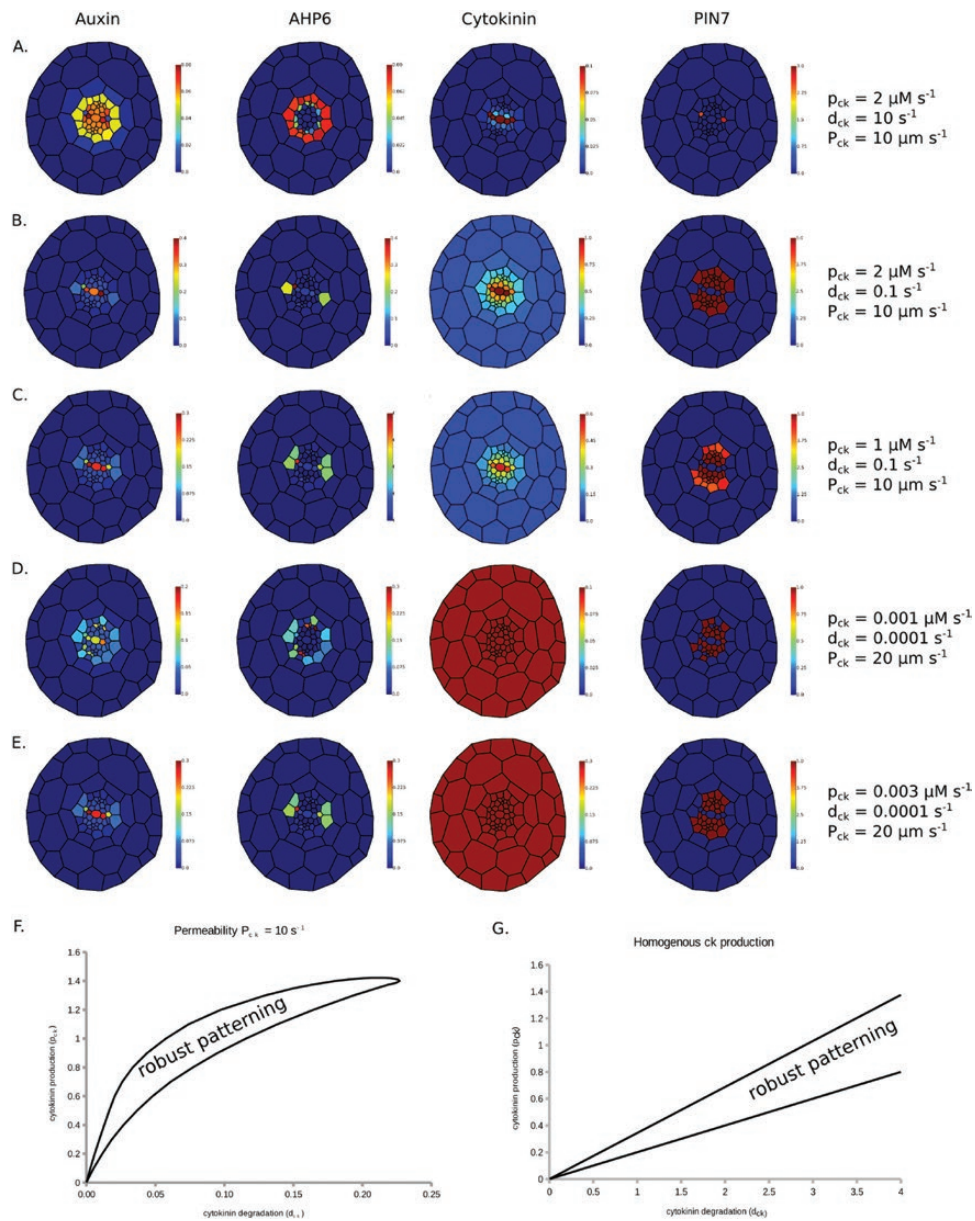

Fig. 5. Output from the Minimal Framework model with cytokinin production limited to the proto- and metaxylem cells, for a range of parameter values. (A) Parameters as in Muraro *et al*. (2014). (B) as (A) with lower cytokinin degradation. (C) as (B) with lower cytokinin production. (D) Parameters as in El Showk *et al.* (2015). (E) as (D) with higher cytokinin production. Only (C) and (E) are able to pattern the tissue correctly. Color ranges show arbitrary non-dimensional values.(F and G) Two-parameter plots for cytokinin production and degradation showing the region of parameter space for which robust patterning is maintained. (F) Cytokinin permeability as in the Minimal Framework model with production restricted to the xylem axis. (G) Cytokinin production homogeneous throughout the tissue.

regarding the turnover of cytokinin are not available, reducing this in line with the other models (from 10 s^{-1} to 0.1 s^{-1}), so that it degrades over the time scale of minutes rather than seconds, results in improved patterning of the xylem axis (Fig. 5B). A further adjustment, so that the level of cytokinin in the procambium regions is closer to that in the published model, results in the correct patterning of the root vascular cylinder and the formation of a gradient of cytokinin peaking within the xylem axis (Fig. 5C).

Using the parameters from the Auxin Flux model (cytokinin production p$_{ck}$=0.001 arbitrary units s^{-1}, degradation d$_{ck}$=0.0001 s^{-1}, estimated permeability P$_{ck}$=20 μm s^{-1}) in the Minimal Framework model with cytokinin production restricted to the xylem results in little or no gradient in cytokinin, but also no regular pattern due to insufficient overall levels of cytokinin (Fig. 5D). However, when raising the level of cytokinin production to p$_{ck}$=0.003 arbitrary units s^{-1}, while there is still no cytokinin gradient, the overall level of cytokinin is raised sufficiently so that the correct pattern is generated and maintained robustly (Fig. 5E).

As these new simulations required a change of parameters, we tested the sensitivity of the three key parameters relating to cytokinin activity: cytokinin production (p$_{ck}$), cytokinin degradation (d$_{ck}$), and cytokinin permeability (P$_{ck}$). Using the value of P$_{ck}$ from the Minimal Framework model (P$_{ck}$=10 μm s^{-1}) as a starting point, we found ranges of the other two parameters, p$_{ck}$ and d$_{ck}$, for which the desired pattern is maintained robustly. To assess whether the pattern is formed correctly, we use k-means clustering to categorize cells into two clusters based on the level of AHP6 protein. If and only if the cluster of cells with the highest AHP6 level is exactly equal to the set of six cells comprising the two protoxylem and four xylem pole pericycle cells do we conclude that the model has patterned the tissue correctly.

Plotting the region of two-parameter space for which the desired pattern is formed (Fig. 5F) shows that with the permeability P$_{ck}$=10 μm s^{-1} no pattern can be formed for degradation rates above around d$_{ck}$=0.22 s^{-1}. Below this degradation rate, there always exists a range for which patterning occurs. This shows that while the model is able to form a pattern with very shallow or intermediate cytokinin gradients, when the cytokinin degradation rate is too high, the cytokinin gradient away from the xylem axis becomes too steep to be able to maintain a stable pattern. We also note that for a given degradation rate below the threshold value, there is only a relatively narrow range of cytokinin production that will support a pattern. This parameter does not affect the cytokinin gradient, but instead determines the overall level of cytokinin in the tissue. Since PIN7 is sensitive to the level of cytokinin, if production is too high, PIN7 expression dominates throughout the stele, while if it is too low AHP6 expression dominates instead. The narrow range of viability to form the correct pattern further illustrates the importance of the regulation of cytokinin production as shown by De Rybel *et al.* (2014). Similarly shaped regions of parameter spaces, but with different numerical ranges, are produced when repeating the exercise using the permeability from the Growing Root model (P$_{ck}$=0.1 μm min^{-1}) and the estimated representative permeability from the Auxin Flux model (P$_{ck}$=20 μm s^{-1}).

If cytokinin is produced evenly throughout the tissue, rather than just in the xylem axis, it is still possible to produce the desired pattern of *AHP6* expression. Plotting the region of two-parameter space for which the desired pattern occurs as before, we see that while for a given cytokinin degradation rate there is a corresponding range of production rates for which there is the correct, stable pattern, there is no upper limit on the degradation rate, as is the case when production is limited to the xylem (Fig. 5G).

Together these simulations suggest that, while restricting cytokinin production to the xylem axis is a plausible method for vascular patterning, at least in the Minimal Framework and Growing Root models, it is not an absolute requirement for the patterning process. Furthermore, these results suggest that the overall cytokinin level as well as cytokinin distribution can act as the driving force for this patterning process.

Conclusion

The organization of root vascular tissues provides a fascinating model to investigate how patterns form in multicellular structures, and the surge in research in vascular development is testament to this. Experimental studies have identified the key components involved in this process and determined how they interact. More recently, theoretical studies have investigated these non-linear interactions and feedback mechanisms and revealed how they collectively determine patterning output. While each of the models incorporates known values where possible, all models by necessity rely on parameter estimation. To address the uncertainty of these parameters, each of the models has made an exploration of the parameter space that allows patterning.

While there are some areas where the authors favor alternative hypotheses, on most issues the three models are largely in agreement. All three models support the interaction of auxin and cytokinin as a plausible system for generating pattern. The exact wiring of this system varies, and this largely depends upon the different questions that each model pursues. In this paper, we ran new simulations to test the findings of the different models. Although the three models use quite different platforms, it is reassuring to see that several of the findings are transferable across these platforms. Collectively, we feel that our modeling efforts support the hypothesis that an asymmetry in auxin input can direct the formation of vascular pattern. We concur that the correct pattern can be generated using the xylem axis as the sole source of cytokinin, but other sources of cytokinin probably exist, and our simulations herein show that broader patterns of cytokinin production are also able to pattern the root. We do not have a consensus of whether there is a meaningful gradient of cytokinin present in the root. However, we show that a cytokinin gradient is required only with certain configurations of PIN dynamics, and even then correct patterning can be generated from a very shallow gradient. Moreover, the theoretical dispute about the formation and presence of a cytokinin gradient presents a challenge to be resolved by experiments; we hope that future work will either show that the parameters

relevant to cytokinin diffusion are in the range to form an informative gradient or demonstrate that cytokinin is patterned by an alternative mechanism. We have also highlighted several other key areas that need further research, namely further insight into how cytokinin regulates PIN activity and the factor(s) that operate alongside AHP6 to limit auxin response in the xylem axis.

The three independent modeling approaches together offer considerable insight into the patterning process. All of the models support each other in some aspects; but at the same time each model provides new insights into the network that are unique to that model. In a few areas, they find disagreements. By comparing the three modeling approaches, we are able to focus future experimentation on aspects where the modeling has indicated that there is additional complexity. We believe that the approach of integrating multiple independent models of root vascular patterning serves as an exemplar for understanding other developmental processes in plants.

Supplementary data

Supplementary data are available at *JXB* online.
Supplementary Model S1. Minimal framework model/
Supplementary Model S2. Growing root model.
Table S1. Parameters used for the simulation in Fig. 4.

Acknowledgements

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