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REVIEW

Self-organizing periodicity in development: organ positioning in plants

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ABSTRACT

Periodic patterns during development often occur spontaneously through a process of self-organization. While reaction-diffusion mechanisms are often invoked, other types of mechanisms that involve cell-cell interactions and mechanical buckling have also been identified. Phyllotaxis, or the positioning of plant organs, has emerged as an excellent model system to study the self-organization of periodic patterns. At the macro scale, the regular spacing of organs on the growing plant shoot gives rise to the typical spiral and whorled arrangements of plant organs found in nature. In turn, this spacing relies on complex patterns of cell polarity that involve feedback between a signaling molecule – the plant hormone auxin – and its polar, cell-to-cell transport. Here, we review recent progress in understanding phyllotaxis and plant cell polarity and highlight the development of new tools that can help address the remaining gaps in our understanding.

KEY WORDS: *Arabidopsis*, Auxin, Cell polarity, Periodicity, Phyllotaxis, Plant

Introduction

Periodic patterns are common in complex organisms. Although sometimes specified by positional information, often these patterns are generated spontaneously (Marcon and Sharpe, 2012). This latter class of phenomena can involve local activation and long-range inhibition, leading to instabilities that break symmetry with a characteristic length. If the dimensions of the tissue are relatively large then a repetitive pattern can arise. So far, three classes of mechanisms that underlie such spontaneous periodic patterning have been recognized: reaction-diffusion mechanisms, mechanisms based on cell-cell interactions, and mechanisms involving mechanical buckling (Hiscock and Megason, 2015). Examples of processes that involve such mechanisms include the periodic patterning of vertebrate digits (Raspopovic et al., 2014), the formation of sensory organ patterns in *Drosophila* (Corson et al., 2017), and the creation of villi in the gut (Shyer et al., 2013), respectively. Another example of periodic patterning in development is phyllotaxis (derived from the ancient Greek words *phyllon*, meaning leaf, and *taxis*, meaning order or arrangement), which is defined as the regular arrangement of lateral organs such as leaves and flowers around the plant stem. The stunning phyllotactic patterns found in nature (Fig. 1A,B), which include whorled, spiral, distichous (alternate) and decussate (opposite) arrangements, have long fascinated scientists, artists and mathematicians (Jean and Barabé, 1998). Such patterns are now known to follow necessarily from two simple features that are characteristic of plant organ formation. First, organs form continuously with a periodic spacing, and second, they form within a restricted but gradually expanding generative region (Fig. 1C) that encircles the plant shoot apex. After forming, these organs are displaced out of this generative region, thus opening up more space for new organs to form (Douady and Couder, 1992; Levitov, 1991; Mitchison, 1977). But how do plants achieve periodic spacing within the generative region? Does it involve the mechanisms, as mentioned above, that are known to operate in animal systems? In this Review, we discuss our current understanding of phyllotaxis, including evidence that it is self-organizing and that the underlying molecular mechanism is likely to be different to those so far found in animal systems.

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Local accumulation of the plant hormone auxin directs organogenesis

Early last century, it was found that when the plant hormone auxin is applied to the shoot apical meristem (SAM) and young leaves of *Lupinus albus* and *Epibolium* the leaves that subsequently develop are larger than those in the wild type and are often fused at their margin, indicating that auxin positively regulates leaf growth (Snow and Snow, 1937). Unlike most signaling molecules, auxin is transported in a polar fashion via a family of polarly localized, membrane-bound efflux carriers called PIN proteins. Auxin influx carriers also facilitate auxin uptake, although auxin can passively diffuse into cells to a significant degree. When auxin efflux is disrupted, either chemically or in *Arabidopsis* plants mutant for the auxin efflux carrier PIN-FORMED 1 (PIN1), the plants that develop form leaves that are mispositioned and often fused, and flowers fail to form altogether (Okada et al., 1991). Flower formation can be rescued by local application of auxin to the SAM, with flowers forming at locations around the circumference corresponding to where the auxin is applied, although position along the radial axis appears fixed (Fig. 2A-F). These findings reveal that the distribution of auxin in the shoot is likely to determine where organs form, and that polar auxin transport plays a crucial role in shaping this distribution (Reinhardt et al., 2000). They also demonstrate that only the periphery of the meristem is competent to respond to auxin (discussed further below). If the activity of auxin influx carriers is disrupted in addition to auxin efflux, local auxin application gives rise to tissue outgrowth all along the circumference, indicating that auxin influx carriers function to reduce auxin diffusion (Stieger et al., 2002; Reinhardt et al., 2003).

Another *Arabidopsis* mutant that fails to produce flowers is the *pinoid* (*pid*) mutant (Bennett et al., 1995; Okada et al., 1991). PID encodes a serine/threonine kinase that targets the PIN1 protein (Christensen et al., 2000; Friml et al., 2004; Michniewicz et al., 2007) and, as in the case of *pin1* mutants, the local application of auxin to *pid* meristems can rescue organogenesis (Fig. 2G-I). However, unlike the *pin1* phenotype, the organs that subsequently develop form with a regular spacing, regardless of the pattern of applied auxin, implying that PIN1 (despite the loss of PID function)

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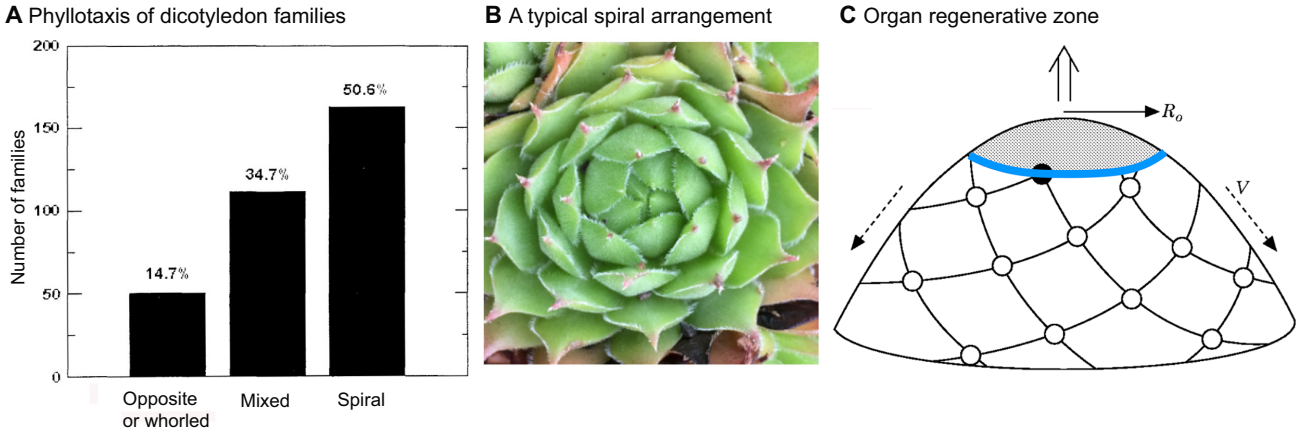

Fig. 1. Spiral phyllotactic patterns are common in nature. (A) The percentage of dicotyledonous families exhibiting opposite or whorled leaf phyllotaxis only, mixed (including whorled, opposite and spiral) phyllotaxis, and spiral phyllotaxis only. Adapted and modified from *Symmetry in Plants*, Jean and Barabè (1998), ©1998 World Scientific. (B) Typical spiral arrangement of leaves. (C) The output of a computer simulation that shows that organs form in a specialized organ generative zone (blue) located at a radial distance (*R*) from the tip of the shoot meristem. *V*, velocity with which the initiated primordia drift away from the organ initiation zone. Adapted and modified with permission from Douady and Couder (1996).

is able to spontaneously redistribute auxin to generate a characteristic spacing (Reinhardt et al., 2003). This spontaneous patterning is reminiscent of the creation of regularly spaced floral organs from flower meristems as well as the formation of oppositely positioned cotyledons during plant embryogenesis. Together, these findings indicate that organogenesis in plants involves a polar transport system that spontaneously generates patterns of auxin accumulation that are periodic.

Models that might explain cell polarity patterns in the shoot

Immunolocalization studies of the PIN1 protein, as well as live-imaging studies of GFP-labeled PIN1 reporters, reveal that PIN1

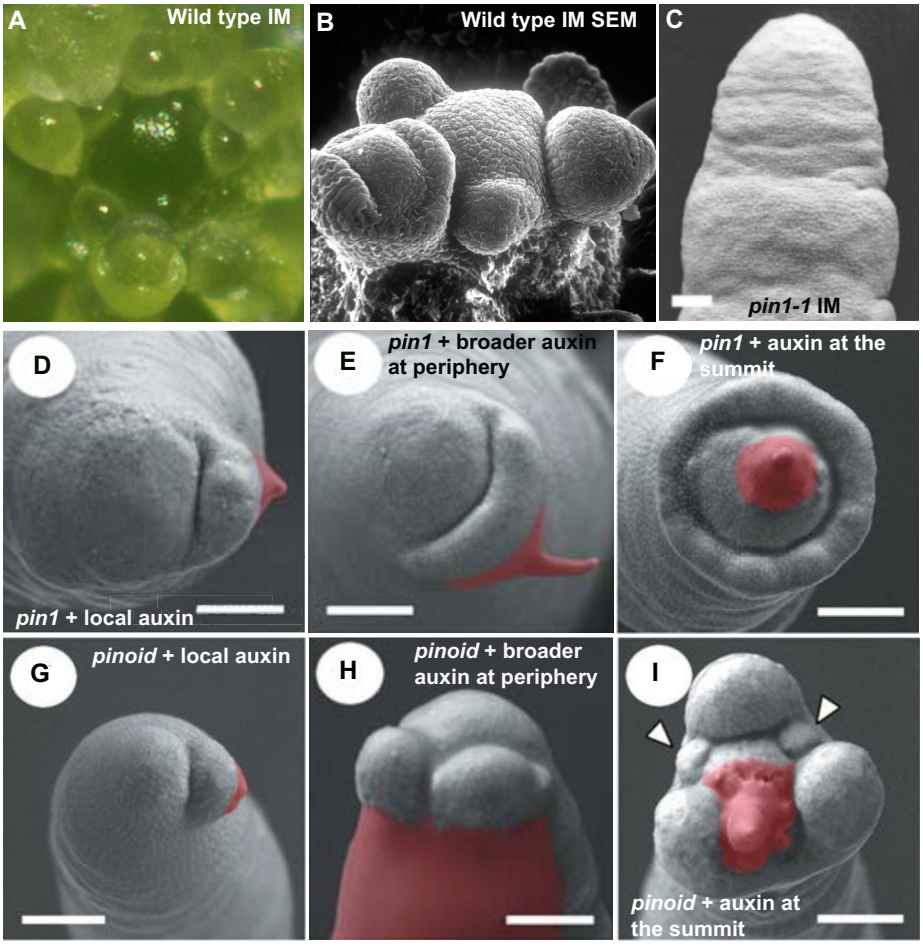

Fig. 2. Local auxin accumulation is required for localized organogenesis. (A,B) Photograph (A) and a scanning electron microscopy (SEM) image (B) of the inflorescence meristem (IM) of a wild-type *Arabidopsis* plant. Note the lateral organs (flower primordia) originating at the periphery of the meristem. (C) SEM image of a *pin1-1* mutant IM lacking organs at the flanks. (D) SEM of a *pin1* mutant IM after local auxin (1 mM IAA) application (red) at the periphery. Note the formation of a localized outgrowth at the site of local auxin application. (E) SEM of a *pin1* mutant IM after slightly broader auxin application at the meristem periphery, resulting in a broader organ than that shown in D. (F) SEM of a *pin1* mutant IM after auxin application at the meristem summit, resulting in a ring-shaped organ initiating along the periphery of the meristem. (G) SEM of a *pinoid* mutant IM after local auxin (1 mM IAA) application (red) at the periphery, showing a localized organ primordia originating at the site of auxin application, similar to that in D. (H) SEM of a *pinoid* mutant IM after slightly broader auxin application at the periphery, resulting in two separated organ primordia (compare with E). (I) SEM of a *pinoid* mutant IM after auxin application at the meristem summit. Note the separated organ primordia (arrowheads) originating from the periphery of the meristem (compare with F). Scale bars: 100 µm in C-I. B is adapted and modified from Meyerowitz et al. (1991); C is adapted with permission from Reinhardt et al. (2000); D-I are adapted with permission from Reinhardt et al. (2003).

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polarities in the SAM orient in a convergence pattern towards organ initiation sites (Fig. 3), supporting a direct role for PIN1 polarity in concentrating auxin locally to pattern phyllotaxis (Fig. 3E) (Benková et al., 2003; Heisler et al., 2005; Reinhardt et al., 2003; see Bhatia et al., 2016). Although the convergence sites are located in the outer cell layer or epidermis, the cells that orient towards them are located both in the epidermis and below (Fig. 3A-D) (see also Bayer et al., 2009). At a later stage of organ initiation, cells at the center of the convergence pattern in the epidermis, as well as those immediately beneath, start to orient themselves away from the surface cells towards the interior, forming an auxin transport channel associated with the differentiation of vascular tissue (Bayer et al., 2009; Reinhardt et al., 2003). Elsewhere in the epidermis, beyond a certain distance from the primordium site, cells reorient their polarity back towards the meristem and towards adjacent primordia (Heisler et al., 2005) (Fig. 3C). This results in an auxin depletion zone in the vicinity of that primordium (Fig. 3F) (see Bhatia et al., 2016; Vernoux et al., 2011).

These complex and intriguing patterns have inspired, often with the help of mathematical modeling and computer simulations, several hypotheses to explain how PIN1 patterns arise, with many of these assuming some kind of feedback from auxin to its transport. In early studies, Sachs proposed that passive auxin flux down auxin gradients may establish vascular patterns (the reticulated network of cells that transport water and nutrients within internal tissues) through positive feedback on polar transport – somewhat analogous to the creation of a river network (Sachs, 1969, 1981). This idea was taken up by Mitchison and others and was modeled explicitly (Mitchison, 1980; Rolland-Lagan and Prusinkiewicz, 2005), demonstrating that auxin flux can potentially act as a polarity cue in models that recapitulate some aspects of vascular patterning (Fig. 4A, top). Could the convergent patterns of PIN1 polarity associated with organ formation in the SAM epidermis also be instigated by passive flux? Even though this seems unlikely at first, since opposing fluxes would tend to cancel out at a convergence point, several studies have now shown that a flux-based polarity mechanism can indeed create convergence patterns (Abley et al., 2016; Stoma et al., 2008) (Fig. 4A, bottom). The key assumption is that when primordia are specified by high auxin, this promotes the formation of an auxin sink (a region where auxin concentrations remain low). For example, a sink could be formed through the creation of a transport channel that moves auxin internally. Passive auxin flux towards the sink thereby orients PIN1 polarities to create a convergence pattern that results in the depletion of auxin from surrounding cells. Auxin thus reaches the threshold required to specify the next primordium at the position farthest from previous primordia, thereby generating a spiral phyllotaxis pattern (Stoma et al., 2008). However, as mentioned above (see also Bhatia et al., 2016), early convergence patterns not only involve cells in the epidermis but also the cells underneath (Fig. 3D, i1), with transport channels leading internally forming only later (Fig. 3D, P1). Hence, if there is an auxin sink at the center of convergence patterns, it is unlikely to involve auxin transport. Also, an important prediction of this type of model is that auxin levels should drop prior to, or at the

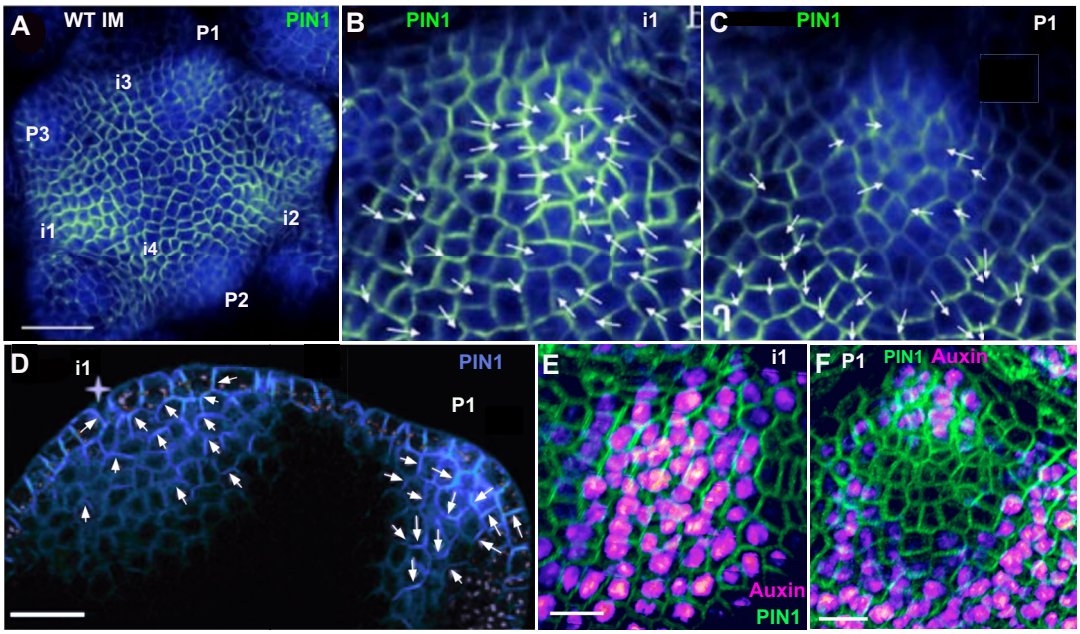

Fig. 3. Dynamic patterns of PIN1 expression, PIN1 polarity and auxin distribution underlie the visible phyllotactic patterns in the SAM. (A) A confocal projection of a wild-type (WT) *Arabidopsis* inflorescence meristem (IM) expressing pPIN1::PIN1-GFP (with a blue to green gradient representing low to high expression). Different primordial stages are labeled from i4 to P3 based on the convention used in Heisler et al. (2005): i, incipient primordium; P, primordium. (B,C) Magnified views of positions i1 (B) and P1 (C) as marked in A. PIN1 is highly expressed and forms a polarity convergence pattern at i1 (B). At stage P1, PIN1 polarities on the adaxial side of the primordium are oriented away from P1. (C) By contrast, cells at the distal tip of the primordium polarize towards the center of the distal tip. (D) A median longitudinal optical reconstruction of a confocal projection of a wild-type tomato vegetative meristem expressing AtPIN1:GFP (with a blue to green gradient representing low to high expression). At position i1, PIN1 in sub-epidermal cell layers displays apical polarization towards a PIN1 convergence point in the epidermis. At position P1, PIN1 in the sub-epidermal cell layers displays a basal polarization in the provasculature or the developing veins. Arrows (B-D) represent PIN1 polarity directions. (E,F) Magnified views of positions i1 (E) and P1 (F) of a separate wild-type *Arabidopsis* IM showing PIN1-GFP expression (green) and predicted auxin distribution (magenta) based on the R2D2 auxin sensor. Note the high auxin concentration at the PIN1 convergence point at position i1 and low auxin concentration on the adaxial side of P1 correlating with PIN1 polarity patterns. Scale bars: 30 µm in A; 20 µm in D; 10 µm in E,F. A-C are adapted and modified with permission from Heisler et al. (2005); D is adapted and modified with permission from Bayer et al. (2009); E and F are adapted from Bhatia et al. (2016).

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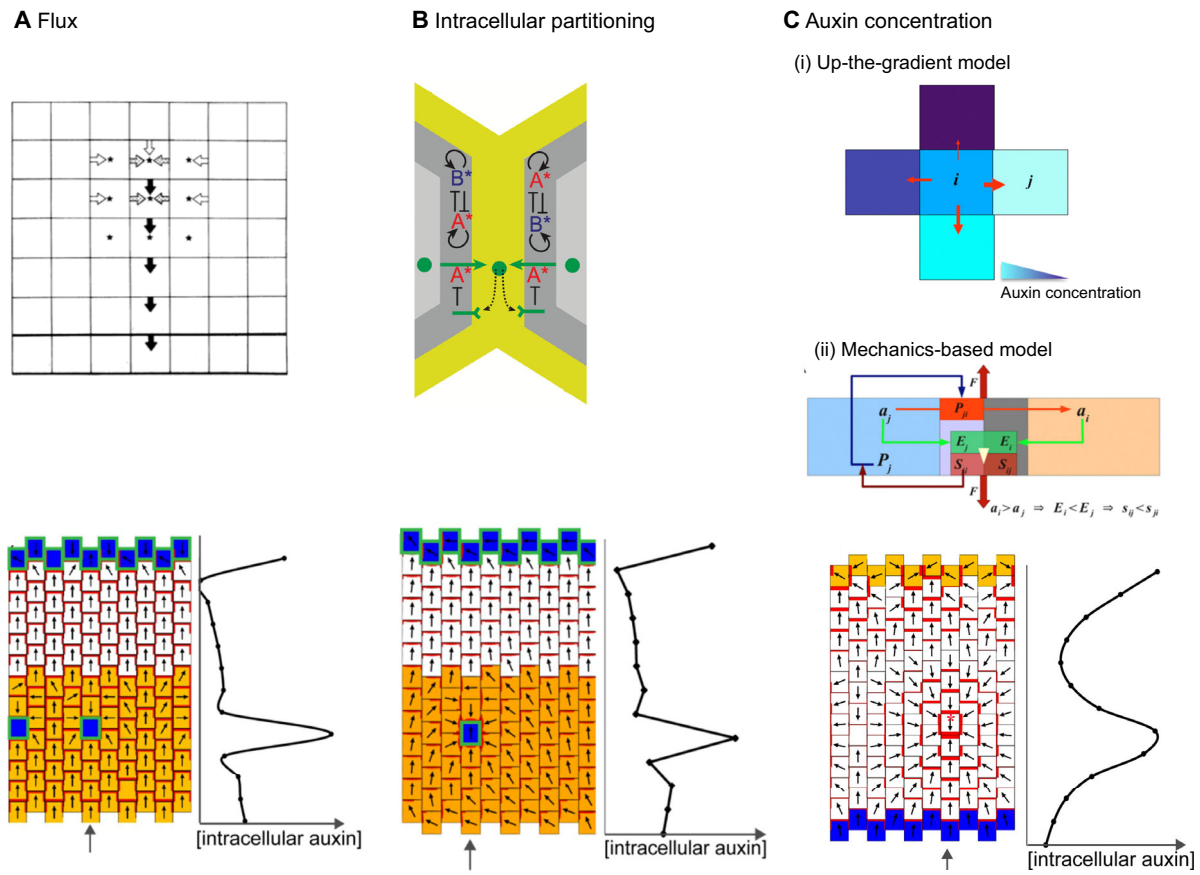

Fig. 4. Proposed models to explain PIN1 polarity. (A) Flux-based, or 'canalization', models were initially developed to explain patterns of vein formation. These models propose a positive-feedback loop between auxin flux and polar transport. (Top) In this simulation, a vein-like channel has formed transporting auxin towards sink cells located at the bottom of the template. (Bottom) Output of a simulation of the flux model in which high auxin levels trigger localized auxin influx and removal (blue cells with green outlines). Such a response to high auxin can lead to a polarity convergence pattern. (B) The intracellular partitioning model does not require the establishment of differential auxin concentrations or auxin flux for cells to acquire a polarity (Abley et al., 2013). (Top) The model assumes that two different polarity components, A and B, can either exist in rapidly diffusible forms in the cytoplasm or can switch to more slowly diffusing membrane-bound states (A* and B*). This leads to their segregation to opposite sides of the cell. To coordinate polarities between cells, extracellular auxin is assumed to reduce the concentration of A* locally. At the same time, A* is proposed to recruit PIN1 to the membrane leading to auxin efflux. (Bottom) Simulation based on this model, showing that PIN1 polarizes towards regions with low extracellular auxin and away from regions with high extracellular auxin (Abley et al., 2013). Convergence patterns can form under similar conditions to the flux model. (C) Concentration-based models have also been put forward. (Top) A model based on gradients of intracellular concentrations of auxin (more popularly called the up-the-gradient model) assumes that PIN1 polarizes at the plasma membrane towards neighboring cells according to how much auxin these cells contain (Jonsson et al., 2006). A more recent model (the mechanics-based model) assumes that auxin levels in neighboring cells are sensed via changes in wall tension, with PIN1 polarizing at the plasma membrane adjacent to more highly stressed walls (Heisler et al., 2010). (Bottom) A simulation based on a concentration-based model shows a PIN1 convergence pattern forming centered at the cell with the highest intracellular auxin concentration (red asterisk; see adjacent graph). A (top) is adapted with permission from Mitchison (1980), B (top) from Abley et al. (2013), and Cii (top) from Heisler et al. (2010). The simulations shown at the bottom of A-C are reproduced from Abley et al. (2016).

time when, a polarity convergence pattern starts to form (Stoma et al., 2008), and this is not seen experimentally (Bhatia et al., 2016; Vernoux et al., 2011). One way around these concerns would be to assume that auxin influx carrier activity and auxin degradation are both triggered by high auxin concentrations, since both processes occurring together may trigger a flux without necessarily lowering auxin concentrations (Abley et al., 2016).

Similar requirements are associated with another model for polarity patterning called the indirect coupling, or intracellular partitioning, model (Abley et al., 2013) (Fig. 4B). This model assumes that cells can polarize without external cues due to mutual antagonism between subcellular components of the polarity machinery. This internally created polarity can then be oriented with the help of external signals, such as auxin, which locally modulate the concentrations of the internal polarity determinants (Abley et al., 2013). In this way, external gradients of auxin can coordinate patterns of PIN1 polarity, including convergence patterns (Fig. 4B), albeit with the same caveats as the flux-based model above (Abley et al., 2016). It is worth noting in regard to these caveats that although auxin influx carriers are known to contribute to the formation of stable phyllotactic patterns, they are not absolutely required for organ formation or for the formation of polarity convergence points, although both processes are disrupted to varying degrees depending on environmental conditions and stage of development (Bainbridge et al., 2008). Hence, rather than acting as central players crucial for the formation of polarity convergence patterns, influx carriers might function more to help maintain high intracellular auxin concentrations in general (Bainbridge et al., 2008) and prevent excessive auxin diffusion (Stieger et al., 2002). Furthermore, no evidence has emerged that auxin degradation is important for phyllotaxis. Most importantly, although both the flux-based and indirect coupling models can

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break symmetry within a tissue, they do not exhibit a characteristic length over which the pattern repeats. Hence, they are sensitive to initial conditions and do not spontaneously generate periodicity. In other words, they cannot explain the generation of a regular pattern from a uniform or irregular starting configuration, as demonstrated in Fig. 2H,I.

Another class of models is based on the assumption that PIN1 polarity within a cell orients towards neighboring cells according to their intracellular auxin concentrations (Jonsson et al., 2006; Smith et al., 2006). According to these concentration-based models (Fig. 4C), the more auxin a cell contains, the more likely its neighbors will transport auxin toward it, up the intracellular auxin gradient in a positive-feedback loop. As auxin concentrations build locally, auxin is depleted from cells nearby, spontaneously giving rise to a characteristic spacing between auxin peaks that depends on the strength of polar versus passive transport [see supporting movie 1 from Jonsson et al. (2006)]. Importantly, this spacing mechanism is insensitive to initial conditions and occurs spontaneously (as long as auxin is present) as there is an inherent instability, similar to that occurring in a reaction-diffusion system. One limitation of these models is that they can only recapitulate convergent polarity patterns and do not explain the gradual change in cell polarity direction toward interior cells below primordia. Also, the auxin peaks and associated polarity patterns can potentially shift with respect to the underlying cells unless additional mechanisms are in place to anchor them, such as auxin influx carrier expression (Heisler and Jonsson, 2006). These caveats suggest that additional mechanisms might be responsible for aspects of polarity patterning underneath primordia (discussed further below) and predict that auxin influx carriers may play an important role in stabilizing polarity convergence patterns, as was found to be the case (Bainbridge et al., 2008).

Addressing some of the limitations of the flux-based and concentration-based models, Bayer et al. (2009) proposed that both types of mechanism may function in a coordinated fashion in all cells, thereby accounting for both phyllotaxis and vascular patterning. The degree to which each mechanism operates is proposed to depend on cellular auxin concentrations, with high concentrations triggering a transition from concentration-based to flux-based polarities. By adding a hypothetical factor that helped polarize PIN1 towards pre-existing transport channels, this model could successfully be used to recapitulate the observed polarity patterns in both the epidermis and the cells underneath it. This study was also notable for providing the first experimental evidence that localized auxin promotes PIN1 polarity convergence patterns (Bayer et al., 2009), although whether this was via auxin flux, intracellular concentration or some other means, remained unclear.

Experimental data supporting at least some aspects of the combined concentration- and flux-based idea came from the discovery of a family of proteins called MACHI-BOU 4 (MAB4) proteins. These proteins, which are polarized and expressed similarly to PIN1, are transcribed in response to auxin and are required in the epidermis for PIN1 to switch from a convergence polarity pattern to polarizing towards internal cells (Furutani et al., 2014). As PIN1 polarity convergence patterns in the epidermis still occur despite the absence of MAB4 function, the implication is that the overall pattern of PIN1 polarity can indeed be separated into at least two distinct modes of behavior, with auxin concentrations being involved in switching from one mode to the other. Nevertheless, whether the polarization of PIN1 promoted by the MAB4 proteins towards internal cells corresponds to polarization according to auxin flux remains to be tested experimentally. Also, there is no evidence yet that the MAB4-based switch applies to cells other than those in the epidermis, although the MAB4 proteins are expressed more broadly. Lastly, the role of MAB4 proteins at the molecular level is still unclear, although they have been proposed to antagonize PID activity (Furutani et al., 2014).

More evidence indicating the existence of distinct regulatory systems governing PIN1 polarity in the epidermis and sub-epidermal tissues comes from a study investigating the role of PIN genes in the grass *Brachypodium* (O'Connor et al., 2014). By examining the sequence of PIN genes in *Arabidopsis* and other members of the Brassicacea, and in angiosperms outside the Brassicacea, the authors first found evidence that the Brassicacea have lost a clade of PIN genes still present in all other angiosperms examined, called the *sister-of-PIN1* (*SoPIN1*) clade. By examining the localization of proteins from both the PIN1 and SoPIN1 clades in *Brachypodium*, the authors discovered that the SoPIN1 protein is localized in convergent polarity patterns centered in the epidermis, similar to PIN1 in the *Arabidopsis* epidermis. However, the two members of the PIN1 clade, PIN1a and PIN1b, are expressed sub-epidermally and are localized basally, similar to the *Arabidopsis* PIN1 patterns associated with sub-epidermal vascular tissues. Hence, PIN1 in *Arabidopsis* seems to be doing the job of two distinct proteins in other species, suggesting the existence of distinct regulatory systems (O'Connor et al., 2014) (Fig. 5).

Convergence patterns are sufficient to give rise to phyllotaxis

As discussed above, different patterns of cell polarity are clearly evident in different cell layers and they are probably regulated by distinct factors. But what is the relative importance of the two types of polarity pattern for phyllotaxis? It was suggested that existing organ primordia might prevent other organs from initiating nearby by transporting auxin through their vasculature strands, thereby depleting auxin in their vicinity (Bayer et al., 2009; Reinhardt et al., 2003). Hence, there has been ongoing interest in understanding the role of vascular tissue-associated PIN1 in phyllotaxis and the relative contribution of influx and efflux carriers to both phyllotaxis and vascular tissue formation. Two studies addressing these issues revealed that epidermal expression of PIN1 in the *pin1* mutant is sufficient to rescue proper phyllotaxis (Bilsborough et al., 2011; Kierzkowski et al., 2013). Surprisingly, it was also found that when PIN1 is absent in the epidermis but present elsewhere, organs continue to form 55% of the time, albeit in irregular positions

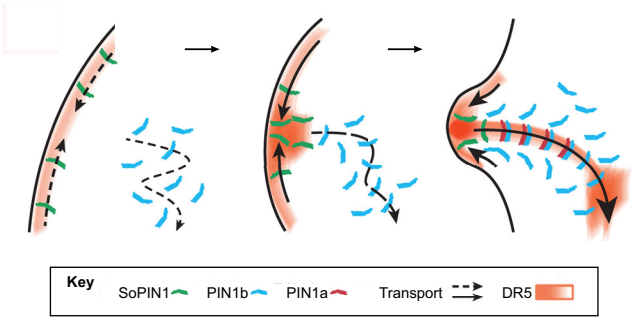

Fig. 5. The roles of SoPIN1 and PIN1 clade members during leaf initiation in *Brachypodium*. Like PIN1 in higher plants, SoPIN1 (green) forms convergent polarity patterns in the epidermis (depicted here via auxin activity reporter DR5, orange). By contrast, PIN1b (blue) polarizes basally in association with the formation of vascular tissue. PIN1a (red) polarizes similarly to PIN1b. Adapted from O'Connor et al. (2014).

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(Kierzkowski et al., 2013). By examining PIN1 polarity in the cell layer underneath the epidermis in these cases, the authors found that PIN1 is polarized towards the epidermis where the organs form, indicating that PIN1 in the sub-epidermal layer may help localize auxin accumulation in the epidermis. Supporting this proposal, it was noted that when PIN1 is absent in both the epidermis and cell layer beneath, no organs form. Also, if PIN1 is only functional in the epidermis and not below, proper phyllotaxis becomes more dependent on the presence of epidermal influx carriers. Thus, PIN1 in both epidermal and sub-epidermal cell layers contributes to localizing auxin in the epidermis in periodic patterns, as do the epidermally expressed auxin influx carriers (Kierzkowski et al., 2013). Evidence that it is specifically convergence-type PIN1 polarity patterns that are crucial, and not the polarity patterns associated with vasculature tissues, comes from a recent study examining the ability of the *Brachypodium* PIN1b and SoPIN1 proteins in rescuing *Arabidopsis pin1* mutants; whereas SoPIN1 can rescue *pin1* mutants and displays convergence patterns similar to those exhibited by PIN1, PIN1b is localized basally in vascular cells and cannot rescue organ formation in the *pin1* mutant (O'Connor et al., 2017).

Overall, these results indicate that it is the localization of PIN1 in convergence patterns that is both necessary and sufficient to mediate regular organ spacing, while polarization towards sub-epidermal cells is required for proper organ growth (Furutani et al., 2014), presumably because growth is normally triggered by auxin in sub-epidermal regions. However, this does not exclude the possibility that other factors in sub-epidermal tissues also play a key role in regulating organ positioning, as discussed further below.

Auxin triggers convergence patterns non-cell-autonomously via its transcriptional targets

If polarity convergence patterns are instrumental in mediating phyllotaxis, how can we experimentally investigate how they are created? To test previously proposed models based on auxin flux or concentration, ideally one would be able to modulate these factors in defined cells and then monitor the response of PIN1 in those cells and surrounding cells, similar to the mosaic approach used to dissect the role of the planar cell polarity (PCP) pathway in *Drosophila* (Vinson and Adler, 1987). However, rather than modulating auxin itself, which may be rapidly redistributed, an alternative is to locally manipulate auxin signaling. Since the auxin receptor TIR1 is localized within cells and is thought to transduce responses to auxin in an auxin concentration-dependent manner (Dharmasiri et al., 2005; Kepinski and Leyser, 2005), this approach was used recently to test a key prediction of the concentration-based models, i.e. that PIN1 polarizes towards cells with relatively high levels of auxin, assuming that signaling levels reflect auxin levels (Bhatia et al., 2016). The authors focused on *MONOPTEROS* [*MP*; also known as *AUXIN RESPONSE FACTOR 5* (*ARF5*)], which encodes an auxin response transcription factor. It had previously been shown that *mp* mutant meristems on their own fail to form flowers (Przemeck et al., 1996) and do not display the typical PIN1 polarity patterns observed in wild-type plants (Reinhardt et al., 2003). Furthermore, unlike *pin1* mutants, exogenous auxin application does not restore flower formation in these mutants, indicating that auxin requires MP for its activity (Reinhardt et al., 2003). By utilizing a clonal mosaic approach to induce MP expression in strong loss-of-function *mp* mutants, it was demonstrated that PIN1 does indeed polarize towards neighboring cells with high auxin signaling (Bhatia et al., 2016), as would be predicted by the concentration-based models (Fig. 6A-D). The orientation of microtubules also changed, reconfiguring to form circumferential patterns around the clones, similar to the microtubule patterns normally seen surrounding organs.

As the concentration-based mechanism is thought to operate primarily in the epidermis, where convergence patterns of polarity are most prominent, the authors also expressed MP solely in the epidermis in the *mp* mutant background (Bhatia et al., 2016). Surprisingly, it was found that this leads to the formation of continuous flanges of organ tissue that spiral around the stem instead of discrete leaves or flowers (Fig. 7A). A closer examination showed that the organ tissue originates from two PIN1 polarity convergence patterns at the apex on opposite sides of the meristem, indicating that epidermal MP is sufficient for the creation of convergence patterns and their spacing (Fig. 7B). However, over time, these convergence patterns were shown to shift circumferentially, leaving organ tissue growing behind them as they moved. Hence, MP is somehow required in sub-epidermal cells to prevent polarity convergence patterns in the epidermis from moving. Underneath the epidermis, there was no evidence of auxin transport channels oriented internally, unlike in the wild type, which is consistent with the known role of MP in promoting the formation of vascular tissue (Przemeck et al., 1996). One hypothesis supported by computer simulations and suggested by previous laser ablation experiments (Deb et al., 2015) is that the shifting of auxin peaks and PIN1 polarity patterns is due to excessive auxin build-up that arises because of the lack of auxin transport to internal tissues (Bhatia et al., 2016). However, the fact that neither PIN1 nor auxin influx carrier activity is required below the epidermis for normal phyllotaxis argues against an auxin transport-related

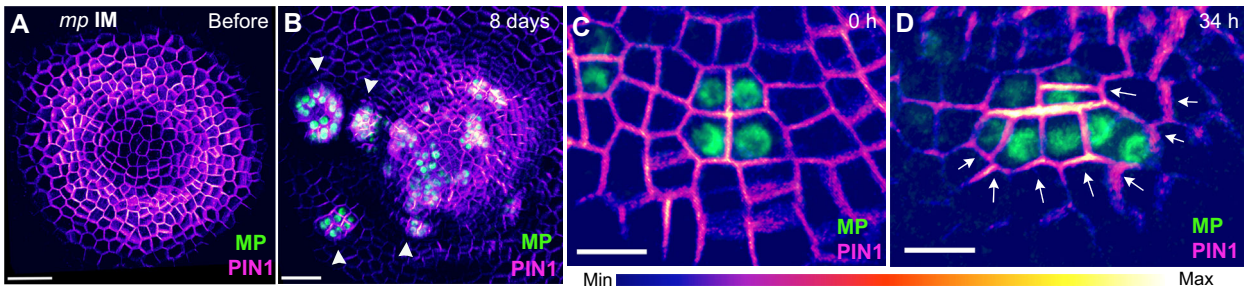

Fig. 6. MONOPTEROS (MP) orients PIN1 polarity non-cell-autonomously. (A,B) Confocal projection of *mpB4149* mutant IM before (A) and 8 days after (B) the induction of MP-YPet clones (green). PIN1 expression is in magenta. Arrowheads indicate organs initiating from cells expressing MP clones. (C) Magnified view of an *mpB4149* mutant apex containing a 4-cell MP-YPet clone (green), showing PIN1-GFP polarity and expression (magenta) 2 days after induction (i.e. when the clone was first visible). (D) Magnified view of the MP-YPet clone shown in C 36 h later, showing an increase in PIN1 expression within the clone and polarization of PIN1-GFP (magenta) in neighboring cells directed towards the clone (arrows). Scale bars: 30 µm A,B; 10 µm in C,D. Adapted from Bhatia et al. (2016).

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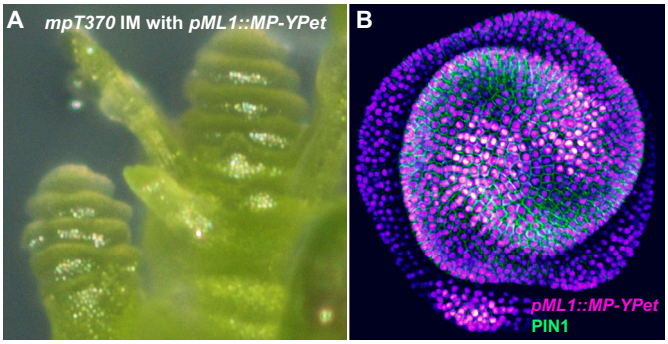

Fig. 7. Restricting MP expression and activity to the epidermal cell layers results in continuous organogenesis. Photograph (A) and confocal projection (B) of an *mp-T370* mutant IM expressing *MP* only in the epidermis (magenta) as well as PIN1-CFP (green). Note two continuous spirals of organ-like tissue originating from the meristem flanks extending down the stem (A).

problem (Kierzkowski et al., 2013), suggesting that other auxin-regulated factors must be required sub-epidermally to anchor PIN1 polarity convergence patterns in the epidermis. It is intriguing to speculate whether lack of sub-epidermal MP activity could explain similar phenotypes exhibited by some spiral cacti, for example Twisted Cereus (*Eulychnia castanea spiralis*).

Mechanical stress as a polarity cue?

If auxin-induced transcriptional activity induces PIN1 polarity convergences non-cell-autonomously, what signals act downstream of auxin in this context? One possibility is that genes encoding auxin influx carriers and auxin degradation enzymes are activated to promote the formation of an auxin sink, as discussed previously in relation to the flux and indirect coupling models. Consistent with this proposal, AUX1 and the related influx carrier LAX2 have been identified as direct targets of MP (Robert et al., 2015). However, as also discussed above, it seems unlikely that influx carriers are absolutely necessary for polarity convergence formation since plants lacking AUX1 and related influx carriers can still produce regularly positioned organs (Bainbridge et al., 2008). What are the alternatives? Clues to answering this question come from a study reporting that microtubule orientations align parallel to mechanical stresses in the cell wall (Hamant et al., 2008). This was shown for meristem tissues not only during normal development but also in response to mechanical perturbations such as laser ablation and mechanical manipulation. An important implication of this finding is that plant cells and tissues actively remodel themselves to resist tensile stresses. This influences morphogenesis by minimizing deformation along maximal tension directions but, at the same time, it promotes growth in orthogonal directions, thereby promoting anisotropic shape changes overall (Bozorg et al., 2014). In regard to cell polarity, in the meristem epidermis and possibly elsewhere, microtubule orientations and PIN1 polarities correlate and are jointly regulated by MP, indicating that PIN1 polarity in the epidermis is also likely to be regulated by mechanical stress (Bhatia et al., 2016; Heisler et al., 2010). Further supporting this proposal, it has been shown that modifications to the status of pectin in the cell wall disrupt PIN1 polarity (Braybrook and Peaucelle, 2013). More recently, mechanical perturbations have been shown to alter the polarity of another protein, BRXL2 (which is involved in the specification of stomata), this time in the context of the leaf epidermis, indicating a role for mechanical stress in regulating polarity more generally (Bringmann and Bergmann, 2017).

Although the evidence suggests a role for mechanical tension in orienting cell polarity, it should be noted that mechanical tension defines an axis, not a direction. How then could mechanical stress define the direction of polarity? One possibility is that the relative magnitude of tensile stress within cell walls provides the directional cue. This idea has been used to explain how auxin in one cell might influence the polarity of adjacent cells in the concentration-based model for phyllotaxis (Heisler et al., 2010). The idea assumes that high intracellular auxin activates cell wall loosening in a cell-autonomous manner; since adjacent cell walls are mechanically coupled by pectin bridges, if one cell wall is loosened then the tensile stress in the adjacent cell's wall will increase. The model proposes that cells target their PIN1 protein towards plasma membrane regions adjacent to more highly stressed walls (Fig. 4C). Hence, the neighboring cell will polarize towards the cell that loosened its walls in response to auxin (Heisler et al., 2010). So, just like the simpler up-the-gradient models proposed earlier (Jonsson et al., 2006; Smith et al., 2006), the mechanics-based model has cells polarizing towards their neighbors that have more auxin (Fig. 4C) and it can generate spacing spontaneously (Heisler et al., 2010). The main difference here is that anything that changes cell wall mechanical properties also has the potential to regulate PIN1 polarity.

Apart from possibly influencing the expression of cell wall-modifying enzymes, auxin may also alter mechanical stresses by disrupting cortical microtubule arrays (Sassi et al., 2014). Examination of cellular microtubule patterns at locations where primordia initiate reveals a local loss of supracellular organization. Furthermore, such disorganization occurs in response to exogenous auxin within 24 h of application. Finally, when the microtubule depolymerizing drug oryzalin is applied locally on *pin1* mutant apices, organ-like outgrowths form, indicating that disruption of microtubule arrays is sufficient to cause organ outgrowth. Given these findings, it was suggested that auxin promotes organ formation by reducing the mechanical anisotropy of cells, thereby promoting establishment of new growth directions (Sassi et al., 2014).

Lastly, while on the subject of mechanics, it should be mentioned that buckling has also been proposed to underlie the periodicity of plant organogenesis, with some models also coupling such buckling with auxin transport (Newell et al., 2008; Shipman and Newell, 2004). However, while auxin distribution patterns are known to play a dominant role in promoting organ outgrowth, such a role for buckling remains to be experimentally demonstrated.

Secondary inhibitory fields

Although studies have indicated that it is the depletion of auxin in cells surrounding primordia that inhibits organs from forming nearby, another type of 'inhibitory field' has been discovered that works in conjunction to promote robustness of the overall phyllotactic pattern. This secondary system is based on the plant hormone cytokinin, which, along with auxin, helps promote organ formation (Yoshida et al., 2011). The gene encoding ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6 (AHP6) was found to be induced in young organ primordia in response to auxin (Besnard et al., 2014). Unlike other phosphotransfer proteins involved in cytokinin signaling, AHP6 acts in a dominant-negative fashion since it lacks an otherwise conserved histidine residue required for the phospho-relay that occurs between cytokinin receptors and their downstream transcription factors (Mahonen et al., 2006). In *ahp6* mutants, close to 30% of organ pairs initiate simultaneously rather than consecutively, although divergence angle and organ spacing are normal (Besnard et al., 2014). To understand how AHP6 might

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prevent such co-initiation, AHP6 protein was visualized by fusing it to YFP, and was found to be present not only where it was transcribed but also in cells surrounding the primordia. This finding suggests that AHP6 might physically move outward from primordia to repress organogenesis in regions nearby. By fusing three tandem copies of YFP to AHP6 instead of one, the authors were able to prevent AHP6 accumulating outside primordia (supporting the movement hypothesis) without disrupting its cell-autonomous activity. In contrast to the 1×YFP fusion, the 3×YFP version did not rescue the phyllotaxis defect, supporting the proposal that AHP6 acts to repress organ initiation in the vicinity of developing primordia due to its diffusion, thereby decreasing the incidence of organ co-initiation (Besnard et al., 2014).

The finding that specific mechanisms exist to promote the robustness of phyllotactic patterns in *Arabidopsis* has recently led to the realization that the variation in pattern found in the *ahp6 Arabidopsis* mutant is actually quite common in various plant species (Refahi et al., 2016). By analyzing these patterns in detail, it was found that a noisy perception of secondary inhibitory fields is likely to underlie this variation. Finally, by introducing stochasticity into a deterministic model for phyllotaxis, computer simulations could be produced that not only captured the observed variability in various plant species but also predicted disruptions that were yet to be found, hence providing testable predictions (Refahi et al., 2016).

Open questions and future challenges

The finding that localized MP expression can act as a polarity cue represents an important advance. Apart from helping to clarify how auxin feeds back on its transport, it shows that polarity can be established by cells 'talking' to each other locally (Fig. 8). In other words, the problem can be broken down by figuring out how cells generate a polarity signal, and how cells receive and respond to such signals. As MP is a transcription factor, the pathway forward in terms of the first task is fairly straightforward, with several MP target genes already identified that may be involved in polarity signaling (Capua and Eshed, 2017; Schlereth et al., 2010; Yamaguchi et al., 2016). Achieving an understanding of how cells perceive and act on such polarity signals might be a harder task. However, the identification of MP target genes that encode proteins involved in sending the polarity signal might help. For instance, if cell wall-modifying enzymes are found to act downstream of MP to polarize neighboring cells, this would further support the proposal that mechanical stresses are the polarizing signal. Since cell walls represent the load-bearing structures within plant tissues, a focus on stress-sensing mechanisms within the cell wall of signal-receiving cells might help identify such stress-sensing molecules.

Despite progress in understanding PIN1 convergence patterns, the finding that MP expression in the epidermis of *mp* mutants is not sufficient to fully rescue wild-type phyllotaxis (unlike computational models that work well with only a single cell layer) makes it clear that there are still aspects of phyllotaxis that we do not understand (Bhatia et al., 2016). Formally, one reason for the incomplete rescue could relate to the promoter that was used to drive MP expression. Rather than its native promoter, MP was driven by the *ML1* promoter, possibly resulting in a broader pattern of activity than should otherwise be present. However, despite the *ML1* promoter, MP remained auxin responsive due to cis-elements within its coding region. Also, even when these cis-elements were mutated and MP was driven from the *UBQ10* promoter, only minor organ fusion defects were observed (Bhatia et al., 2016). Another explanation favored by modeling experiments is that the lack of MP in sub-epidermal regions somehow leads to auxin build-up within the epidermis (as might be expected if an auxin transport channel oriented away from the primordium toward internal tissues is not formed), which in turn causes a continuous drift, laterally, of auxin maxima (Bhatia et al., 2016). This hypothesis should be relatively easy to test by reducing auxin levels using chemical inhibitors for auxin biosynthesis. However, as mentioned earlier, even if true, previous studies indicate that both auxin efflux and influx carriers need only be active in the epidermis (Kierzkowski et al., 2013), indicating that important MP targets in sub-epidermal cells remain to be discovered.

Another key aspect of polarity not addressed by experiments so far is long-range pattering. For example, localized MP activity has been shown to alter the polarity of nearby cells only one to three cells away. Whereas such a small range of influence is consistent with the existence of stress gradients that are likely to exist between neighboring cells at organ inception, it is not intuitively likely that such gradients exist over the much longer distances that these convergence patterns eventually cover, such as the convergence patterns that extend from the base to tip of leaf and petal primordia (Koenig et al., 2009; Sauret-Güeto et al., 2013). Is the initial polarity pattern established by MP actively maintained by MP as cells divide and proliferate to form a large organ, or does auxin-mediated auxin-response factor activity become dispensable at later stages? Could

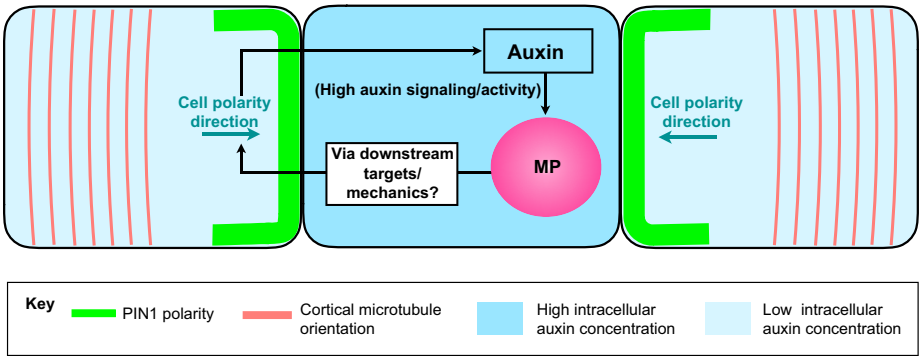

Fig. 8. A feedback loop at the cell-cell communication level generates cell polarity patterns at the tissue level. A positive-feedback loop between auxin abundance and its transport orients PIN1 polarity and microtubule orientations in the neighboring cells non-cell-autonomously, towards the cell with high auxin. This loop acts via a localized auxin transcriptional response mediated by MP activity. This substantiates the proposed up-the-gradient model in organizing complex cell polarity patterns underlying visible phyllotactic patterns. How MP acts to mediate such a response is yet to be discovered; it might act through its downstream targets or via altering cellular mechanics but these hypotheses need to be tested *in vivo*. See Bhatia et al. (2016).

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polarities be maintained passively through inheritance or are they oriented over long distances via a cell-cell coupling or long-range signal gradient that is yet to be identified (Abley et al., 2013)? To distinguish between these and other hypotheses experimentally will require considerable further effort.

Finally, one aspect of phyllotaxis not discussed so far is the confinement of organogenesis to the peripheral zone of the SAM. While auxin may dictate organ position circumferentially around the shoot apex, the question of how organs are positioned along the radial axis of the shoot has only recently been investigated in detail (Caggiano et al., 2017). An important insight revealed by this work is that auxin response is restricted through the function of transcription factors that are involved in specifying leaf dorsoventrality, i.e. the top (dorsal)-bottom (ventral) axis. Dorsally expressed genes encoding class III HD-ZIP transcription factors such as REVOLUTA (REV) (Otsuga et al., 2001) are expressed centrally in the meristem, whereas genes that specify ventral leaf tissues such as *KANADI1* (*KAN1*) (Kerstetter et al., 2001) are expressed more peripherally. Both sets of genes act to repress organ initiation, thereby restricting sites of organogenesis to a narrow boundary region between their expression domains. A simple modification to the up-the-gradient polarity model, in which REV and KAN1 repress the downstream targets of auxin involved in polarizing PIN1, is able to restrict the formation of PIN1 polarity convergence patterns to the shoot periphery (Caggiano et al., 2017).

In summary, there are clearly a number of open questions in the field that need to be addressed. Fortunately, several new tools have recently been made available that should help tackle these challenges. These include drugs that can be used to rapidly deplete auxin from cells or repress auxin signaling (Hayashi et al., 2012; He et al., 2011; Nishimura et al., 2014), dynamic ratiometric sensors for monitoring intracellular auxin levels (Liao et al., 2015), and constitutively active versions of MP that can activate the transcriptional targets of auxin without auxin being present (Ckurshumova et al., 2012). Combined with techniques that allow the modulation of gene expression in clones, such tools should allow a much more detailed dissection of polarity signaling to be undertaken. However, to better understand the role of mechanics in polarity signaling it will also be necessary to build more elaborate 4D models of plant tissues that incorporate mechanics, gene expression and signaling, as well as cell division and growth.

Broader implications

In our view, the evidence so far suggests that, like other periodic patterning systems found in animals, organ spacing in plants occurs spontaneously and with a characteristic spacing. Hence, it seems appropriate that Alan Turing made phyllotaxis a focus of his research towards the end of his life and made considerable progress in applying his reaction-diffusion mechanism towards explaining the associated macroscopic patterns (Turing and Saunders, 1992). However, as discussed, recent work has now shown that, microscopically, the molecular mechanism involves directional transport of a signaling molecule as well as positive feedback from this molecule to its directional transport, with mechanical signals possibly acting to coordinate this behavior between cells. Conceptually, such a framework is interesting to compare with models of mesenchyme aggregation in animal systems where, as in the case of plants, mechanical signals generated by cells are thought to help coordinate cellular behaviors in a positive-feedback loop that results in the creation of periodic patterns. However, in this case, rather than concentrating a signaling molecule such as auxin, the cells themselves self-organize into regularly spaced aggregates due to their collective influence on the extracellular matrix (Oster et al., 1983). For example, such a process has been proposed to underlie patterns of mesenchyme aggregation associated with the regular spacing of hair follicles in skin (Glover et al., 2017; Shyer et al., 2017).

Conclusions

The periodic formation of plant leaves and of floral organs give rise to some of the most striking organismal architectures found in nature. Although not so obvious to the naked eye, the cell polarity patterns that underlie these architectures are certainly no less intriguing, having inspired over the years many computational models that can potentially explain them. Although it has taken some time, we believe the new tools and techniques that have recently become available will finally enable many of the predictions made by the models proposed so far to be conclusively tested. We hope that this Review will help to inspire such efforts and that these experiments will lead to a new generation of ideas to explain phyllotaxis.

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Competing interests

The authors declare no competing or financial interests.

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