From Auxin Transport to Patterning

Marina Oliva, Teva Vernoux, and Jan Traas

Abstract The phytohormone auxin is often observed to be asymmetrically distributed across tissues and regulate crucial events of plant development. These gradients are generated by a network of polarly localized membrane-bound transporters, called PIN-FORMED (PIN) proteins. Understanding how auxin controls patterning in plants comes to define how polar auxin transport is regulated to form gradients and how these gradients will be, in turn, interpreted by cells. The long-standing belief that auxin regulates its own transport via a positive feedback loop has led to the establishment of different theoretical models for auxin-dependent patterning processes that are tested in silico. Most of these simulation models are able to reproduce patterns observed in planta and have been crucial to predict and test the behavior of the PIN network in several developmental processes. Here, we review computational approaches to describe PIN polarization together with recent experimental data on PIN polar targeting, which can explain the patterned distribution of auxin throughout tissues. We also discuss how auxin gradients are perceived by the downstream signaling pathway and transduced to generate specific cellular responses.

1 Introduction: Auxin as a Crucial Morphogenetic Regulator in Plant

How multicellular organisms are able to create de novo patterns is a central question in developmental biology. One way to achieve patterning in developing organisms is through gradients of small molecules that we call morphogens. This term was first introduced by Turing (1952) who was interested in how interacting

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molecules could lead to the formation of patterns across a group of cells in both animal and plants. Morphogens are long-range signaling molecules that induce cellular responses in a dose-dependent manner. In a given tissue, a graded distribution of a morphogen exposes cells to different concentrations of the molecule. Cells exposed to higher levels of morphogen will adopt different fates than cells exposed to lower levels, thereby subdividing tissues into distinct cell types according to their distance from the morphogen source.

In plants sciences the best potential candidates for morphogen-like regulators are phytohormones. The most studied of them is unquestionably auxin. Auxin plays a crucial role in a large range of patterning processes and dose-dependent responses such as apicobasal axis formation (De Smet et al. 2007; Friml et al. 2003), de novo organ positioning (Heisler et al. 2005; Okada et al. 1991; Reinhardt et al. 2003a; Swarup et al. 2008; Vernoux et al. 2000), vascular development (Sachs 1981, 1991; Scarpella et al. 2006), and tropisms (Abas et al. 2006; Band et al. 2012; Friml et al. 2002b; Kleine-Vehn et al. 2010).

To date, evidence shows that auxin fulfills at least some of the criteria associated with morphogens in animals. First of all, its movement and graded distribution seems to be crucial for its action. In fact, it has been shown principally in *Arabidopsis thaliana* that in different developmental contexts, auxin response maxima (visualized by auxin signaling sensors) define both growth direction and tissue polarities (Benkova et al. 2003; Friml et al. 2002a; Sabatini et al. 1999).

Are these concentration gradients important for determining cell fates? Some evidence suggests that this is the case. During vascular tissue differentiation, for instance, local application can trigger distinct development response at specific distances from the point of application (Warren-Wilson et al. 1991, 1994). In the root apex, altering auxin distribution causes pattern reestablishment, and cells close to the newly formed auxin maximum acquire new developmental features (Friml et al. 2002a; Sabatini et al. 1999). In the shoot meristem, perturbing auxin transport also results in dramatic effects on cell fate (Vernoux et al. 2000) and auxin buildup during organ initiation is correlated with patterning. However, whether auxin actually functions as a plant morphogen is still a matter of debate (Benkova et al. 2009; Bhalerao and Bennett 2003) and would require proving that cells can respond directly to this molecule in a concentration-dependent manner. Along this line, we will discuss at the end of this chapter how auxin is perceived and cells elicit specific responses.

A key question for understanding how auxin can control patterning processes in plant is to know how auxin gradients are generated and maintained. In animals it has been proposed that morphogens diffuse from a producing localized source. In this case, local production of morphogen is a key process in determining gradients. For auxin a different scenario seems to apply, as the hormone is actively transported by polarly localized membrane proteins. This is linked to the fact that auxin cannot freely diffuse throughout tissues. According to the chemiosmotic model (Goldsmith et al. 1981; Raven 1975; Rubery and Sheldrake 1974), auxin which is a weak acid is largely protonated at extracellular pH and as such can enter cells by diffusion. Once inside cells, because of the higher pH, the hormone is deprotonated and trapped.

In order for auxin to leave a cell, it requires the activity of efflux carriers located at the plasma membrane, probably asymmetrically since auxin is known for some time to be transported in a polar fashion (Goldsmith 1966; Leopold and Hall 1966). Therefore, one major difference between auxin and animal morphogens as described by Turing is that its distribution involves an active transport. As we will see, this active polar transport is essential for the control of auxin-dependent patterning processes since inactivation of auxin cellular transporters can alter patterning in plants.

In this chapter we will discuss how this active transport in combination with different feedback mechanisms could self-organize to generate patterns of auxin distribution and how they are, in turn, interpreted by the signaling network in terms of cell differentiation.

2 Auxin Transport as a Pattern-Generating Process

2.1 The Basic Cellular Machinery

The chemiosmotic model has largely been confirmed by the discovery of different families of auxin transporters that contribute to either the import or the export of the molecule. Before discussing the processes that lead to the patterned distribution of auxin throughout tissues, we will briefly consider the cellular mechanisms that regulate distribution of the main transporters at the plasma membrane.

2.1.1 Auxin Efflux Carriers

The founding members of the PIN-FORMED (PIN) family of auxin efflux carriers were identified by classical forward genetics (Galweiler et al. 1998; Luschnig et al. 1998; Muller et al. 1998; Wisniewska et al. 2006). Most PIN family members are localized at the plasma membrane and distributed in a polar fashion in cells. They play an essential role in controlling the establishment of auxin gradients throughout plant development, and strong evidence exists that their polar localization in cells determines the direction of auxin fluxes (Wisniewska et al. 2006).

In this context membrane transport plays a central role. The colocalization of PIN proteins with an endocytic tracer has shown that PINs are continuously internalized (Paciorek et al. 2005). Evidences for recycling of PINs at the plasma membrane have also been provided by drug treatments. The fungal toxin brefeldin A (BFA), known to block trafficking from the endoplasmic reticulum to the plasma membrane, causes an aggregation of endosomes and internalized endocytic cargos into the so-called BFA compartments. It has been shown that PINs are found in BFA bodies after treatment and this effect is reversible, providing indirect evidence of PIN recycling (Geldner et al. 2001). Clathrin-dependent PIN cycling between the plasma membrane and

internal compartment has been demonstrated directly later on, by using a green-to-red photo-convertible EosFP fluorescent reporter (Dhonukshe et al. 2007).

How do cells address the transporters to a restricted portion of the plasma membrane? Although the question is far from being solved, several mechanisms essential for targeting PINs to correct membranes have been identified. Both the actin and microtubular cytoskeleton play important roles in guiding trafficking vesicles within cells. By using BFA and drugs that depolymerize actin filaments, it was demonstrated that the PIN recycling back to plasma membrane is actin dependent (Geldner et al. 2001; Kleine-Vehn et al. 2006, 2008). Although PIN movements do not seem to be directly controlled by microtubules, except during cell division (Geldner et al. 2001), there is a significant correlation between their localization in various tissues. PIN proteins are predominantly localized on membranes that are parallel to the main microtubule orientation in roots, in the shoot apical meristem, and in the leaf epidermis (Heisler et al. 2010). Interestingly, PIN remains restricted to certain membranes when microtubules are depolymerized, while microtubule reorients in a coordinated manner, even when auxin transport is inhibited. This indicates that their localizations might be regulated by a common upstream factor. Good candidates for such upstream regulators that control both PIN localization and cytoskeleton organization in cells are members of the Rho-GTPase (Rho in Plants or ROP) family. Indeed, dominant-negative mutants of ROP2 show that, at least in epidermal leaf cells, PIN1 localization is dependent on ROP2 signaling, which is also crucial (together with ROP6 signaling) in controlling cytoskeleton organization in these interdigitated cells (Fu et al. 2002, 2005; Xu et al. 2010). An additional element required for PIN localization is the cell wall. Removing the cell wall by enzymatic digestion, for example, causes a loss of PIN polarity and even leads to a complete retrieval of PIN from the plasma membrane (Boutte et al. 2006), while genetic and pharmacological interference with the cellulose synthesis perturbs PIN localization (Feraru et al. 2011).

Posttranslational modifications in the PIN proteins themselves also function in their localization. In this context, a striking role for PIN1 phosphorylation has been demonstrated. Mutants of the serine/threonine kinase PINOID (PID) produce pin-like inflorescences (Christensen et al. 2000), and in this mutant, PIN1 polarization switches from apical to basal membranes (Friml et al. 2004). Further studies have shown that the kinase PID and the phosphatase PP2A act antagonistically to control PIN polarization in different cell types (Michniewicz et al. 2007). Three serine residues conserved within the PIN1 hydrophilic loop are directly phosphorylated by PID (Huang et al. 2010). Neither the loss of phosphorylation nor phosphomimicking versions of PIN1—generated by mutating the three serine residues—were able to rescue the defects of *pin1* mutant highlighting the importance of the dynamics of PINs' reversible phosphorylation for proper localization (Huang et al. 2010).

A scenario thus emerges where cytoskeleton-based membrane traffic and interactions with the cortical microtubules and cell wall lead to specific localizations of PIN at the plasma membrane (Fig. 1). This leads to another question, that is, how is this basic cellular machinery coordinated throughout

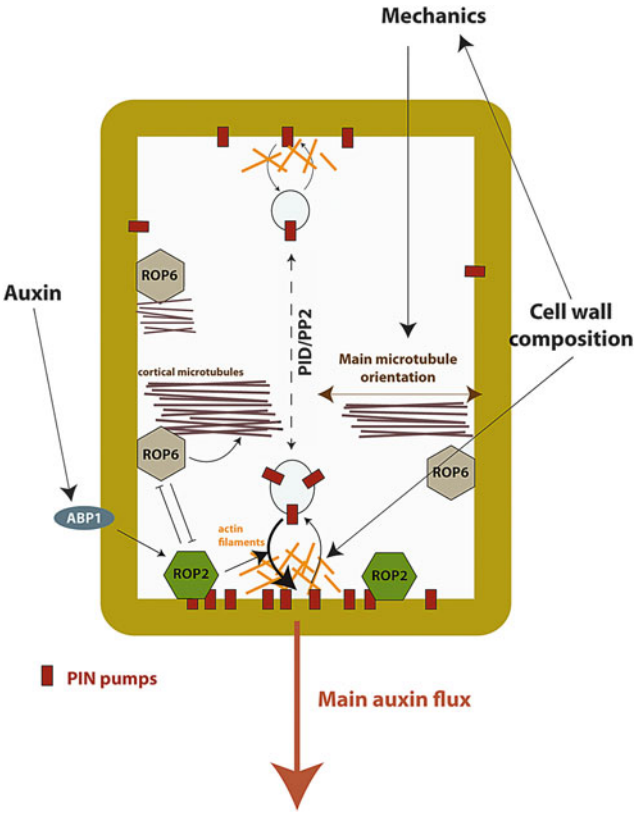

Fig. 1 Regulation of PIN distribution at the plasma membrane. Auxin can enter cells both by diffusion or auxin influx carrier. Once inside cells, auxin is trapped and needs PIN efflux transporters to leave cells. Interestingly, most of the time PIN proteins are polarized at the plasma membrane in order to direct auxin fluxes. PINs have been demonstrated to continuously cycle between internal compartments and the plasma membrane. Different factors have been demonstrated to control and influence PIN polarization at the plasma membrane. First, cytoskeleton seems to be essential to address the transporters to a restricted portion of the plasma membrane: actin is important for the recycling back to plasma membrane of PIN vesicles and PINs have been shown to be mainly addressed to the membranes parallel to the main microtubule orientation. This organization might be triggered by members of the ROP family. ROP2 would address PINs to the plasma membrane in an actin-dependent manner while ROP6 would promote microtubule attachment. Since both classes of ROP proteins mutually inhibit each other, this would lead to alternate microtubule-rich and PIN-/actin-rich domains. Others factors such as auxin or mechanical tensions would then contribute to this polar organization by promoting, respectively, PIN recycling at the plasma membrane (via an ABP1-dependent activation of ROP2) or microtubule organization. Posttranslational modifications could then help in targeting PINs to the right actin-rich portions. In fact, it has been shown that the PINOID (PID) kinase and the PP2A phosphatase act antagonistically to control PIN polarization and PID mutations can trigger switches from basal to apical membranes

tissues to generate auxin fluxes and auxin distributions. Indeed, the generation of particular patterns of auxin distribution in tissues somehow implies that cells have to coordinate PIN polarities. We will address these questions in Sect. 2.2.

2.1.2 Auxin Influx Carriers and Other Regulators of Polar Auxin Transport

Genetic studies have also led to the discovery of auxin influx carriers of the AUX1/LIKE_AUX1 (LAX) family. The *auxin resistant 1* (*aux1*) mutant was isolated from a screen for auxin-resistant and agravitropic mutants. Positional cloning demonstrated that *AUX1* encodes an H+−symport plasma membrane permease (Bennett et al. 1996). Studies on the effects of exogenous auxins on root growth in both wild-type and *aux1* seedlings have demonstrated that *aux1* mutant was much more resistant to the membrane-impermeable synthetic auxin 2,4-D than to the membrane-permeable 1-NAA (Maher and Martindale 1980). In fact, the root agravitropic phenotype could be rescued by supplying roots with 1-NAA, whereas 2,4-D did not show this effect (Marchant et al. 1999; Yamamoto and Yamamoto 1998). Besides, it has been shown that *aux1* root tips accumulate 50 % less radiolabeled IAA or 2,4-D than the wild-type root tips do (Marchant et al. 1999; Rahman et al. 2001). Taken together these data strongly supports a role for AUX1 in auxin uptake. This hypothesis was confirmed by heterologous expression of AUX1 in *Xenopus laevis* oocyte cells which demonstrated a specific, high-affinity, pH-dependent auxin uptake entirely dependent on AUX1 (Yang et al. 2006).

In addition to AUX1, three LAX genes have been identified in *Arabidopsis* (Parry et al. 2001). They function similarly as auxin influx carriers (Peret et al. 2012) and play partially redundant role with AUX1 during development (Bainbridge et al. 2008; Peret et al. 2012). The AUX/LAX proteins are mainly asymmetrically localized on plasma membrane, except for AUX1 in the phloem in *Arabidopsis* root tips (Swarup et al. 2001). AUX1 membrane-targeting mechanism is also different from the PIN mechanisms since AUX1 and PIN proteins accumulate in different subsets of vesicles and their trafficking show different sensitivities to various inhibitors (Dharmasiri et al. 2006; Kleine-Vehn et al. 2006).

Members of the P-glycoprotein subfamily of ATP-binding cassette (ABC) proteins (PGPs) are also involved in both the cellular efflux and influx of auxin (Cho et al. 2007; Geisler et al. 2005; Petrasek et al. 2006; Santelia et al. 2005). PGPs are important regulators of plant development since their mutant exhibit obvious developmental defects (Noh et al. 2001). However, PGP mutants exhibit weak auxin-related phenotypes, thereby leaving open the question of whether auxin transport represents their primary physiological function (Santelia et al. 2005; Terasaka et al. 2005).

2.2 *From Transport to Pattern: A Key Role for Modeling*

2.2.1 Auxin Distributions in Different Organs

The difficulty to obtain a detailed picture of local concentrations for small molecule-like auxin has been partly compensated by the development of auxin-inducible markers, the most widely used being the one driven by the DR5 promoter (Ulmasov et al. 1997) and, more recently, the DII-VENUS sensor (Brunoud et al. 2012). Both markers were designed from knowledge on the auxin signaling pathway but depend on different steps of this pathway that we present further in Sect. 3. DR5 reports transcriptional activation in response to auxin, whereas DII-VENUS monitors the degradation of Aux/IAAs, the first step of the auxin signaling pathway, thus relating more directly to auxin concentrations. Together with high-resolution mass spectrometry (Novak et al. 2012), these markers have provided a detailed picture of auxin distribution in different parts of the plant. In this context three organs, the root apex, the shoot apex, and the developing leaves, have received particular attention.

At the root apex, the DR5 auxin-responsive promoter is activated in a restricted group of cells comprising columella and the quiescent center. Inhibition of polar auxin transport by chemicals is able to shift and expand DR5 activity domain at the root tip, and treatment with exogenous 2,4-D auxin activates homogeneously DR5 in the tissue (Sabatini et al. 1999). Thus, in the root meristem, DR5 was suggested to report an auxin maximum in a restricted group of cells. This auxin maximum has been recently confirmed using DII-VENUS (Brunoud et al. 2012).

Analysis of PIN polarities in the root suggests the existence of three regions with specific polarities: basal polarity in vasculature and cortex, homogeneous distribution of PINs on membranes in columella cells, and apical polarity in the epidermis in addition to a lateral polarity at the transition between the meristem and the elongation zone (Blilou et al. 2005). The topography of the PIN network was suggested to create a reflux loop maintaining the auxin maximum at the root meristem, a hypothesis supported by simulations of auxin transport on a simplified root structure (Grieneisen et al. 2007). More recently, a model using this topography could also lead to auxin accumulation at the start of the elongation zone, a prediction confirmed using DII-VENUS (Brunoud et al. 2012; Santuari et al. 2011). These two studies strongly support the idea that the auxin distribution in the root apex results mostly from the polar auxin transport activity.

At the shoot apical meristem, the importance of patterned auxin distribution in organ formation became evident from experiments where the PIN machinery is impaired. Indeed, inhibition of polar auxin transport genetically (*pin1* mutant) or chemically (NPA treatment) completely abolishes organ initiation at the shoot apical meristem (Okada et al. 1991; Reinhardt et al. 2000, 2003b). In both cases, local application of exogenous auxin can restore organogenesis in a position-specific manner, suggesting that auxin is an instructive signal in this process and that local auxin maxima are required to induce the formation of a new organ. Using the

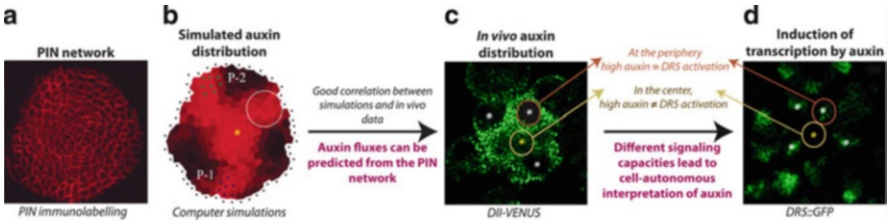

Fig. 2 From transport to patterning: the example of the shoot apical meristem. (**a**, **b**) Simulations of auxin distribution in the shoot apical meristem can be inferred from the topography of the PIN1 network in the structure (adapted from Barbier de Reuille et al. 2006). They suggest that auxin is accumulated in young primordia (visualized by *green* and *blue dots*) but also in the meristem center (*yellow asterisk*). (**c**) In order to confirm these predictions, the pattern of DII-VENUS—a sensor that monitors Aux/IAA degradation which is directly related to auxin levels (see Fig. 3)—is analyzed at the shoot apex. Auxin is shown to accumulate both in incipient organs (*white asterisks*) and at the meristem summit (*yellow asterisk*) as predicted by simulation studies, demonstrating that PIN1 proteins effectively control auxin distribution in the structure (adapted from Vernoux et al. 2011). (**d**) To test whether cells can perceive these auxin gradients and respond accordingly in order to create patterns, activation of the DR5 auxin-inducible promoter is analyzed. It has been shown that at the periphery of the meristem, DR5 is upregulated in sites where auxin is accumulated [which corresponds to young organs, visualized by *white asterisks*, also seen in (**c**)], whereas in the center of the structure it is not the case. In fact, in the center of the meristem (*yellow asterisk*), auxin levels are relatively high (**c**) but DR5 is not activated (adapted from Vernoux et al. 2011). These results suggest that the center and the periphery of the shoot apical meristem have different sensitivities to auxin and that the auxin signaling pathway limits gene activation in response to auxin in the meristem center. Thus, local differences in competence and tissue-specific response (mediated by the downstreaming signaling pathway) appear crucial to understand auxin-mediated pattern formation

synthetic auxin-responsive DR5 promoter, it has been shown that auxin effectively accumulates in young initia, forming spots that display a phyllotactic arrangement (Benkova et al. 2003; Smith et al. 2006). Based on these data and on PIN1 polarization patterns at the shoot apex, Reinhardt (2003a, b) proposed a model to explain regulation of phyllotaxis based on polar auxin transport. Auxin would be transported apically towards developing primordia that would act as auxin sinks by transporting the hormone in provascular tissues. As a result, surrounding tissues would then be depleted of auxin, preventing new organs from being formed too close to the previous ones. This hypothesis is supported both by simulations of auxin distribution based on the topography of the PIN1 network in the shoot apical meristem (Barbier de Reuille et al. 2006) and more recently by DII-VENUS pattern in this tissue (Brunoud et al. 2012) (Fig. 2). These data also demonstrated that the existence of a maximum of auxin at the center of the shoot meristem, and similarly to the root, suggests that the PIN1 network is controlling auxin distribution in the shoot.

Similar conclusions came from the analysis of auxin distribution during vein formation in young leaves. Indeed, DR5 expression marks the future site of leaf veins prior to the expression of the early pre-procambium marker (Mattsson et al. 2003; Scarpella et al. 2004, 2006). This early DR5 expression occurs concomitantly with PIN1 expression, and both are considered the earliest known markers for vascular initiation (Scarpella et al. 2006).

2.2.2 Different Feedback Models for Auxin Transport

As we have seen, auxin and its transport have been intimately linked to patterning processes. How do neighboring cells communicate to coordinate auxin transport? How are PIN transporters aligned in supracellular patterns? These questions are not new. Over the last 50 years, different concepts were proposed to explain how directed fluxes of auxin are generated. Since the precise cellular processes are only understood in a very partial manner, these concepts remain abstract, although they provide a general framework for analyzing the processes that lead to particular auxin distributions.

The Canalization Hypothesis: Transporting Auxin with the Flux

Sachs (1969, 1975, 1981) was the first to hypothesize that auxin could regulate its own transport, years before the discovery of PIN proteins. He was interested in the development of the vascular network. By performing local auxin applications on pea hypocotyls, he showed that auxin was able to induce the formation of tracheary elements from the site of auxin application to the central vein already present. The differentiation of such narrow vascular strands could not be explained by diffusion, so he proposed that an active mechanism—which he called canalization—was acting to connect auxin sources (i.e., the local applications in his experiments) to auxin sinks (the central vein). He proposed that auxin fluxes across cells are able to enhance auxin transport and that this feedback of auxin flux on transport drives the selection of strands of cells that will then differentiate into vascular tissues.

Mitchison further tested the ability of auxin flux-feedback model to generate patterns (Mitchison 1980, 1981). He proposed two variants whereby the canalization process could be realized through either facilitated diffusion or polarized auxin transport. In the first case, auxin transport is mediated via passive channels which are bidirectional, increasing the ability of auxin to move in both directions. In the second variant, the number of transporters can vary on each side of the interface. The direction of flux is then an important component of the model: auxin transport is reinforced only when there is a net efflux of auxin across the interface. Mitchison showed that both variations of the model were able to generate discrete channels of auxin flow within a uniform field of cells, similar to those observed during vascular development in leaves. One constraint for the model to work is that the relationship between the increase in transport rate and auxin flux must be nonlinear, thus resulting in amplification of the flux.

The actual discovery of the PIN transporters and their localization permitted a more precise examination of the canalization hypothesis. To further validate Mitchison's model, Rolland-Lagan and Prusinkiewicz performed simulations on the polar transport version of Mitchison's model and compared it with PIN1 expression data (Rolland-Lagan and Prusinkiewicz 2005). During midvein initiation in leaves, PIN proteins polarize away from auxin maxima at the leaf tip towards the vasculature below (Bayer et al. 2009; Reinhardt et al. 2003a; Scarpella et al.

2006). Rolland-Lagan and coworkers showed that the model was able to reproduce this behavior of PIN1. In addition, they demonstrated that canalization models were able to reproduce a large range of venation patterns that occur *in planta*, including connected veins and discontinuous strands in mutants (Bayer et al. 2009; Reinhardt et al. 2003a; Sauer et al. 2006; Scarpella et al. 2006).

However, some predictions from these classical models remain in (at least apparent) contradiction with experimental data. Sachs supposed that preexisting veins were acting like auxin sinks, with low auxin concentrations compared to surrounding tissues, thereby attracting developing veins towards them. However, as preexisting strands exhibit a high DR5 expression (Scarpella et al. 2006), it is likely that they contain high auxin concentrations. To assess these shortcomings, different hypotheses were made. A model proposed that the number of PIN proteins within a cell is fixed and that different regions of the plasma membrane compete for intracellular PINs (Feugier et al. 2005). In that case, fully developed veins exhibited high auxin concentration. In another model, the AUX/LAX influx carriers were proposed to facilitate accumulation of auxin in vascular cells (Kramer 2004).

The discovery of PIN proteins also revealed that auxin transport is not always occurring along narrow channels. This is typically the case at the shoot apical meristem, where PIN polarities adopt highly complex patterns throughout the tissue. This might seem in contradiction with the canalization hypothesis, but recent simulations showed that this is not necessarily the case. Stoma et al. (2008) demonstrated that this phenomenon could be due to a low amplification response to flux of the epidermal cells. Indeed as long as this flux-based amplification remains linear, no canalization occurs, but only diffuse patterns are obtained.

Another observation that is in apparent contradiction with canalization is that PIN1 proteins at the meristem surface tend to polarize towards high auxin concentrations that correspond to sites of organogenesis. The same is true for the root, where an auxin maximum is maintained at the root tip, with auxin fluxes going in and out. However, flux-based models do not exclude such a situation. Analogous to water flowing in and out of a basin, it is perfectly possible for canalized auxin fluxes to go against or downstream of preexisting gradients. Indeed, Stoma et al. showed that canalization could explain not only the venation patterns but also the patterns of auxin maxima observed in root and shoot. At the shoot, meristems produce leaves and flowers in highly ordered patterns. These organs can be produced in opposite, spiraled, or whorled arrangements, also called phyllotaxis. Stoma et al. tested if canalization could reproduce these patterns using a small field of virtual cells. This was indeed the case, and importantly, a flux-based model was also able to reproduce realistic patterns of PIN distribution in virtual tissues representing root and shoot meristems.

Transport Against the Gradient

The complex patterns of PIN distribution at the shoot apical meristem surface suggest fluxes of auxin towards local auxin maxima. This leads to propose an alternative scenario for auxin transport, where auxin would flow against a gradient.

In these so-called up-the-gradient models, each cell senses auxin concentrations in neighboring cells and subsequently polarizes its PINs towards neighbors with higher concentration. It assumes the existence of a short-range signaling pathway that gathers information about auxin content in surrounding tissues.

Computational models based on this concept were able to reproduce a large variety of phyllotactic patterns observed *in planta*, including spiral, distichous, decussate, and tricussate ones. Even switches between patterns could be reproduced by modifying model parameters (Jonsson et al. 2006; Smith et al. 2006). In principle, such a mechanism could also account for the formation of veins in leaves. Merks et al. (2007) showed that an up-the-gradient mechanism could in principle also lead to a moving maximum. This *traveling wave* mechanism could in principle account for the formation of veins. However, a limitation of the up-the-gradient model is that it does not easily explain, in contrast to canalization, the orientation of PIN transporters away from an auxin maximum as observed in the root and during vein development in the shoot meristem, for example.

Combined Model

As the up-the-gradient model does not provide a straightforward explanation for PIN transporters pointing away from auxin maxima, Bayer et al. (2009) proposed a hybrid model, where both canalization and up-the-gradient would coexist. In this model, cells with low auxin concentrations follow the *up-the-gradient* mode, whereas above a certain threshold cells would switch to a canalization mechanism. In this scenario, relative low auxin levels at the meristem surface would induce an *up-the-gradient* behavior, which would lead to the local accumulation of the hormone. These local maxima would then cause a switch to canalization towards the inner tissues. This combined model was able to reproduce observed PIN polarization patterns in both phyllotaxis and vein formation. Several predictions made by this model were validated. First, the hybrid model predicts high auxin concentrations in the forming vein as observed in reality. It therefore does not require extra hypotheses to explain this phenomenon, in contrast to the canalization hypothesis, which requires the presence of an auxin importer to keep auxin at sufficiently high levels (see above). Second, the model also predicted a temporary orientation of PIN transporters in inner tissues towards the auxin maximum at the surface, an event that was again observed in reality and not easily explained by canalization. However, whereas canalization and up-the-gradient only need a single mechanism, the hybrid model is not a minimal hypothesis and requires the coexistence of two different mechanisms.

2.2.3 Towards a Mechanistic View of Patterned Auxin Transport

To compare and further test the theoretical frameworks we have just discussed, more details are required on the actual cellular and molecular processes involved.

These models indeed ask the question of how incoming and outcoming fluxes or concentrations are sensed. Although our understanding remains limited, recent findings provide a number of interesting leads.

Cell–Cell Communication via the ROP Pathway

As we have seen above, both PIN localization and microtubule organization are strongly correlated in several tissues. In leaves, this coordination likely involves several ROP proteins that mutually inhibit each other (Xu et al. 2010). More specifically, ROP2 would address PIN to the plasma membrane via an actin-driven process, while ROP6 would cause the attachment and organization of microtubules. Since both ROP proteins mutually inhibit each other, it is easy to see how this would lead, through stochastic variations, to alternate microtubule-rich and actin-/PIN-rich domains (Fig. 1). Xu et al. (2010) also found that auxin itself promotes ROP activity via the auxin receptor AUXIN BINDING PROTEIN1 (ABP1), a process that does not involve transcriptional regulation. Importantly, the two proteins show a differential sensitivity to auxin as the activation of ROP2 is saturated at lower hormone concentrations than that of ROP6. Computer simulations taking into account all these properties have shown that, in a multicellular context, such a system would lead to complementary patterns of ROP2/PIN and ROP6/microtubule distributions along neighboring membranes. Although this remains to be proven, such a mechanism could explain how directed fluxes could arise in all tissues throughout the plant. It also remains to be seen whether this system would rather correspond to a canalization process, transport up the gradient, or something completely different.

A Role for Biomechanics?

Whereas the mechanism mentioned above can potentially explain how very local chemical interactions between cells generate patterns, a second line of evidence points at the importance of mechanical feedbacks. There is strong evidence that microtubules themselves are at least in part reacting to mechanical tension within tissues (Hamant et al. 2008). Such tensions can arise, for example, when cells in neighboring regions grow at different rates. In both roots and shoots, microtubules align along the predicted main force directions. Since they are also controlling the orientation of the cellulose microfibrils in the wall in the same direction, they might reinforce the cell wall to resist the forces, causing organ outgrowth and tissue folding. If microtubules respond to stresses and PIN localization is correlated with microtubule directions, it is tempting to propose that auxin transport itself is also influenced by mechanics. This hypothesis was further corroborated by experiments where mechanical tensions were perturbed. Cell ablations as well as drug treatments affecting the cell wall caused concerted changes in microtubule organization and PIN localization, and together the data suggested an interesting

explanation for the *up-the-gradient* model. In this context, Heisler et al. (2010) tested if mechanical signals would be sufficient to explain the observed patterns of PIN1 using a computer model. They investigated the hypothesis that PIN1 in each cell localizes towards the wall that is most mechanically stressed. An important postulate was the existence of stress-induced signals from the cell wall that act only locally to promote accumulation of PIN1 at the nearest membrane. The model also assumed that auxin-induced cell wall loosening in response to auxin concentrations inside a cell is limited to its own wall. In other words, the cell would sense via biomechanical signals transmitted through the cell wall, which of its neighbor has the highest concentration of auxin, and polarize its PIN proteins towards this cell.

Mechanistic Insights: Conclusions

A better understanding of the cellular mechanisms leading to polar PIN localization is currently absolutely required. The results regarding the role of ROPs and the cytoskeleton are very promising and have provided a first basis for more mechanistic models for auxin transport. In parallel, the link with the membrane traffic machinery should also be further explored. A step in this direction was taken by Wabnik et al., for example, who combined the feedback of auxin on both transcription and endocytosis in a single model (Wabnik et al. 2010). Combined with extracellular perception, this model was able to reproduce venation patterns, suggesting that it could behave like a canalization model.

3 From Auxin Distribution to Downstream Gene Expression Patterns

Once auxin gradients are established, the cells need to perceive local hormone concentrations and respond accordingly. If auxin acts as a morphogen, one should expect a dose-dependent response. The reality, however, appears more complex than that. First, there seems to be a tissue-specific difference in competence to react to auxin.

For instance, auxin causes the upregulation of a DR5 reporter at the periphery of the meristem, while it is not able to do so in the meristem center (Barbier de Reuille et al. 2006; Vernoux et al. 2011) (Fig. 2). Second, there is a tissue-specific modulation of auxin response: auxin triggers organogenesis at the periphery of the shoot apical meristem, leads to vascular strands development in young leaves, and also induces lateral root formation (Reinhardt et al. 2000; Scarpella et al. 2006). Therefore, it is not possible to understand auxin-based pattern formation without considering local differences in competence and tissue-specific responses. This, in turn, suggests that we have to consider the downstream signal transduction pathway as a plausible key regulation level of auxin responses.

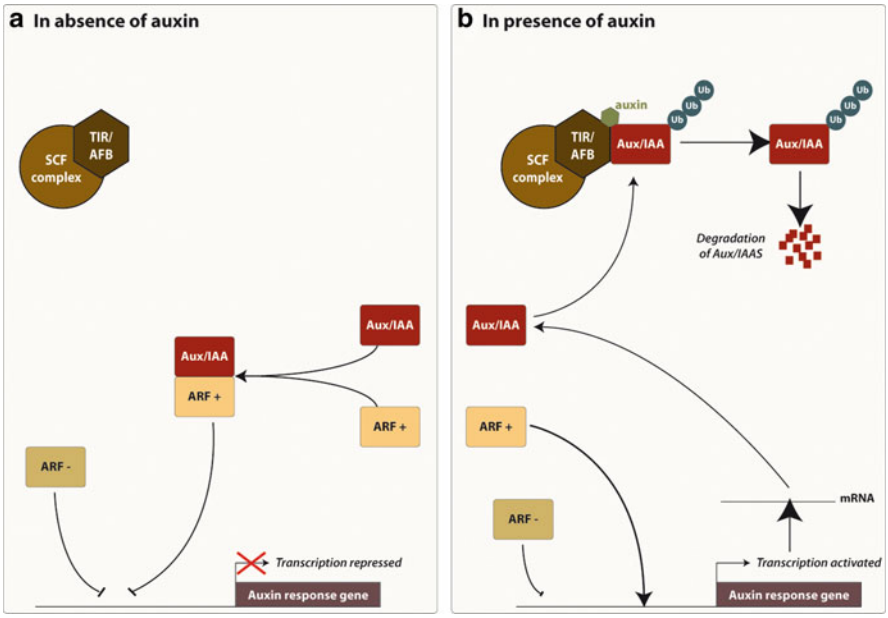

Fig. 3 Regulation of gene expression by auxin. The auxin signal transduction requires two families of transcriptional regulators: Aux/IAAs and auxin response factor (ARFs). At low auxin levels, Aux/IAAs bind to the ARFs preventing them from regulating target genes. When auxin levels rise, the F-box protein (TIR1 or its close AFB relatives) interacts with Aux/IAA repressors in an auxin-dependent manner, leading to their degradation by the 26S proteasome. Aux/IAA degradation likely dissociates corepressors from ARF proteins residing on promoters of auxin response genes, allowing them to promote transcription. This includes activation of most of the *Aux/IAA* genes themselves, thus establishing a negative feedback loop. Note that the ARF family is composed of both transcriptional repressors (ARF−) and activators (ARF+) and both of them may regulate the same target genes. The Aux/IAAs mainly interact with ARF+

The auxin signal transduction requires two families of transcriptional regulators: Aux/IAAs and auxin response factor (ARFs) which are composed, respectively, of 29 and 23 different elements. Aux/IAAs and ARFs have been shown to form homo- and heterodimers both within and between families (Chapman and Estelle 2009). In absence of auxin, Aux/IAAs bind to the ARFs and, by recruiting corepressors of the TOPLESS family, prevent them from regulating auxin target genes. In the presence of auxin, Aux/IAA proteins bind to the TIR1 F-box of SCF complex (or its AFB homologues) and act as auxin coreceptors. This interaction leads to the ubiquitination and subsequent degradation of Aux/IAAs thereby allowing the ARFs to regulate transcription (Fig. 3). This includes activation of the *Aux/IAA* genes, thus establishing a negative feedback loop. Importantly, sequence analysis and transient assays suggest that most ARFs act as transcriptional repressors, while only 5 ARFs are activators (Guilfoyle and Hagen 2007).

To understand how the auxin transduction pathway might modulate local auxin responses, not only the expression patterns of the effectors of the pathway but also

information on protein interactions and identification of ARF targets is required. Relevant information has been recently obtained in different auxin-dependent developmental contexts. During lateral root initiation, it was shown that two different sets of ARFs/IAAs (a crucial early IAA14–ARF7–ARF19-dependent auxin response module, followed by a second IAA12–ARF5 module) were expressed successively and that both were required from proper development (De Smet et al. 2010). It suggests that auxin could coordinate distinct consecutive developmental steps through the expression of different response modules over time. In *Arabidopsis* embryo, a key role for specific ARF-Aux/IAA pairs has been shown (Weijers et al. 2005, 2006) and an expression map of all 23 ARFs during embryogenesis indicates that the transcriptional regulation of ARFs generates combinatorial patterns that might create a prepattern essential in the regulation of cell fate (Rademacher et al. 2011).

In a recent study, a full ARF-Aux/IAA interactome was obtained. This work revealed three clusters corresponding to the Aux/IAAs, ARF activators, and ARF repressors, respectively, and showing stereotypical interaction capacities (Vernoux et al. 2011). Aux/IAAs interact with themselves and with the ARF activators. The ARF activators mainly interact with AUX/IAAs and poorly with themselves. Finally the ARF repressors show low or no interactions with the rest of the network or with themselves. The expression of these genes was tested at the shoot apical meristem and 25 of them were detected (among which some Aux/IAAs, ARF activators and repressors). Importantly, here the situation is different from what was observed in the embryo since most of the Aux/IAA and ARFs are co-expressed, albeit at different levels: weakly in the center of the meristem, where the stem cells are located, and much more strongly at the cell periphery where organs are initiated. Mathematical modeling of the network predicted that the differences in expression levels between the center and the periphery of the meristem result in different auxin sensitivities (high sensitivity at the periphery, low at the center). The demonstration of high level of auxin both in lateral organs and at the center of the meristem using DII-VENUS (Vernoux, Brunoud), while DR5 is activated specifically in lateral organs, confirmed this prediction (Fig. 2). In addition, this work suggests a surprisingly simple scenario for the shoot apical meristem, where auxin-activated gene expression would be controlled by a *double brake* system (Vernoux et al. 2011). In this scenario activating ARFs would be both inhibited by interactions with the AUX/IAAs and by competition with the ARF repressors for binding sites in the promoters of target genes. Increasing auxin concentrations would cause the degradation of AUX/IAAs but not influence the competition with the ARF repressors. This high level of inhibitory regulation can buffer local variations in auxin levels (Vernoux et al. 2011), thus likely contributing to the robustness of patterning in the shoot meristem.

This illustrates clearly that auxin responses do not only depend on auxin concentrations but also on the competence to react to hormone levels with different mode of regulation observed in different tissues.

Importantly, a recent report has shown yet another level of complexity in auxin signaling. Aux/IAAs and TIR/AFBs have been demonstrated to be both necessary and sufficient for auxin binding thus defining pairs of coreceptors (Calderon

Villalobos et al. 2012). As there are, as mentioned, 6 different TIR/AFBs and 29 different Aux/IAA proteins, an important combination of coreceptors can be formed. Interestingly, each of them has a different affinity to auxin, suggesting that the combination of coreceptors expressed within a cell might determine the range of auxin concentration to which the cell can react. Further work should reveal a key role for this mechanism in patterning in response to auxin.

4 Conclusions and Perspectives

Auxin is a versatile signaling molecule in plants that is involved in many developmental processes. The polar transport of auxin has been shown to play a crucial role in controlling auxin-regulated development since it is able to redistribute auxin in specific patterns, in different tissues. Most of the attention has been paid to understand how the PIN network can self-organize to generate such specific patterns. It seems now established that both chemical (auxin itself) and mechanical signals influence PIN polarization in cells, but the precise molecular mechanisms by which these different signals are integrated to precisely control PIN orientation still remain a major issue.

Once distributed in specific patterns, auxin is locally interpreted by its perception pathway. Given the abundance and structure of the downstream network, cellular responses to auxin depend not only on hormone concentrations but also on the interaction of all the effectors of the signaling pathway. Besides, auxin is able to induce a wide range of responses in different tissues by activation of specific pathways and it is yet not fully clear where the specificity lies.

Although genetic studies have made clear that polar transport is essential in controlling auxin-dependent patterning, biosynthesis might also be an important component in this process. Indeed, it has been shown in different developmental contexts that local biosynthesis of auxin is required for proper patterning (Cheng et al. 2006, 2007a, b, 2008; Stepanova et al. 2008), suggesting that it might be an important mechanism to stabilize PIN-dependent patterns. In this case, the control of patterning by auxin would not be so different from animal morphogens: both would be locally produced and distributed in a directional fashion. It will thus be really crucial to integrate biosynthesis data into current models of auxin patterning.

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