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Dynamic Integration of Auxin Transport and Signalling

Review

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Recent years have seen rapid progress in our understanding of the mechanism of action of the plant hormone auxin. A major emerging theme is the central importance of the interplay between auxin signalling and the active transport of auxin through the plant to create dynamic patterns of auxin accumulation. Even in tissues where auxin distribution patterns appear stable, they are the product of standing waves, with auxin flowing through the tissue, maintaining local pockets of high and low concentration. The auxin distribution patterns result in changes in gene expression to trigger diverse, context-dependent growth and differentiation responses. Multi-level feedback loops between the signal transduction network and the auxin transport network provide self-stabilising patterns that remain sensitive to the external environment and to the developmental progression of the plant. The full biological implications of the behaviour of this system are only just beginning to be understood through a combination of experimental manipulation and mathematical modelling.

Introduction

The most common naturally occurring type of auxin is indole-3-acetic acid (IAA). It is a simple molecule, related to tryptophan, but it is remarkable stuff. It is involved in both local and long distance communication within plants and in communication between plants and their environment. Throughout the plant, an intricate network of transmembrane proteins distributes auxin within and between tissues to create both highly stable and highly dynamic patterns of auxin distribution and flow. These patterns are driven by multiple interacting feedback loops [1], probably supplemented by localised sites of increased auxin synthesis and degradation [2]. Through the action of this distribution system, auxin accumulates in patterns, which are read out to direct plant growth and development [3]. The read-outs are many and various. They include patterning of the main organ systems of the plant, regulation of the amount and direction of growth, and the initiation and activation of new axes of growth, in the form of lateral branches in both the roots and shoots.

The system can be likened to a particularly complex and dynamic road network through which traffic flows. Auxin, like traffic, can leave or enter the network, and can flow around the network with, for example, a one-way system channelling it along specific routes. The distribution of traffic in the system can influence the rules for flow. This can involve relatively minor adjustments, such as opening a new lane to increase capacity along a particular route, or more radical changes such as laying entirely new roads or reversing the direction of a one-way street. Rapid progress in recent years has led to the identification of many of the key components of the auxin road network and the auxin signal transduction machinery. Now, the two central challenges for auxin biologists are to decipher the auxin 'highway code', and to determine how the behaviour of auxin in this system is read-out to drive diverse patterning and growth processes. At the heart of both of these questions is auxin signal transduction. Read-outs from the auxin distribution network are dependent on local sensing of auxin. Furthermore, the network is so heavily regulated by feedback that auxin signalling is central to its operation too.

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Auxin Signal Transduction

Transcriptional Responses to Auxin

The last two decades have seen the elucidation of a complete transduction pathway from auxin to gene expression. Array experiments demonstrate that hundreds of genes change their expression in response to auxin [4]. The best understood auxin-responsive genes belong to three gene families, the GH3s, the SAURs (small auxin upregulated RNAs) and the Aux/IAAs (Auxin/Indole-3-acetic acid inducible genes) [5]. Members of each of these families are up-regulated rapidly in response to auxin addition, independent of protein synthesis, thus defining them as primary auxin response genes. Indeed, treatment with the protein synthesis inhibitor cycloheximide alone induces the transcription of these genes, suggesting that they are under the control of a rapidly turned over repressor protein. Typical of the many levels of feedback regulation in auxin biology, GH3s encode auxin conjugating enzymes, which act to reduce free auxin levels [6], and Aux/IAAs encode the rapidly turned over transcriptional repressors of auxin-inducible genes, predicted by the cycloheximide experiments [7].

Analysis of the promoter regions of these gene families has allowed the identification of auxin response elements (AREs) [5]. In natural promoters, these elements are usually composite, but the consensus TGTCTC element when concatamerised is sufficient to mediate strong auxin-inducible expression of reporter constructs [8]. Synthetic promoters containing this element have, therefore, become a powerful tool in the analysis of auxin-regulated gene expression. Yeast one hybrid analysis identified the Auxin Response Factor (ARF) protein family, members of which bind to the TGTCTC–ARE [9]. There are 22 ARF genes in the *Arabidopsis thaliana* genome [10]. They are characterised by an amino-terminal DNA binding domain of the plant-specific B3 type, and carboxy-terminal dimerisation domains, known as domains III and IV [11]. The ARF family can be classified into sub-groups depending on the composition of the middle region

between the DNA binding and dimerisation domains. The five ARFs with glutamine-rich (Q-rich) middle regions act as transcriptional activators, whilst the remaining 17 have serine-rich (S-rich), serine-glycine-rich (SG-rich), serine-proline-rich (SP-rich) or serine-proline-leucine-rich (SPL-rich) middle regions and representatives of each type have been shown to act as repressors of transcription. The three sections of the ARF proteins act essentially independently. The dimerisation domains are shared by the Aux/IAA protein family, which has 24 canonical members in *Arabidopsis* [10], and the ARFs and Aux/IAAs have been shown to form homo- and heterodimers both within and between the families [12]. Domains III and IV are located at the carboxyl terminus in the Aux/IAAs, whereas at the amino terminus are two additional highly conserved domains. Domain I is a potent transcriptional repression domain, whilst domain II is responsible for the characteristic instability of the Aux/IAAs and holds the key to auxin-regulated transcription.

Aux/IAAs and ARFs were discovered through a molecular biological route, but genetic analyses have also made a significant contribution to our understanding of their function. A role for Aux/IAAs in mediating downstream auxin responses was established through the analysis of the phenotypes of a range of dominant or semi-dominant auxin response mutants showing diverse auxin-related phenotypes [10]. These mutations were all found to be in domain II of Aux/IAA family members and, where tested, were shown to stabilise the proteins [13–16].

Targeted Protein Degradation in Auxin Signalling

The importance of regulated protein stability in auxin signalling was further supported by the molecular analysis of three auxin-resistant mutants of *Arabidopsis* — *axr1, axr6* and *tir1. TIR1* encodes an F-box protein [17]. F-box proteins are subunits of SCF-type ubiquitin-protein ligases, which poly-ubiquitinylate specific substrate proteins, thus targeting them for degradation [18]. The SCFs are named after three of their subunits; Skp1, Cullin, and the F-box protein. A forth subunit, Rbx1, forms a dimer with Cullin, which has ubiquitinylating activity. The Skp1 protein links the Rbx/Cullin dimer to the F-box protein. F-box proteins are characterised by an amino-terminal F-box motif, which interacts with Skp1. At the carboxyl terminus they have one of a range of protein–protein interaction domains, which are responsible for recruiting the target protein to the SCF for ubiquitinylation. In the case of TIR1, these are carboxy-terminal leucine rich repeats.

There are nearly 700 F-box proteins in the *Arabidopsis* genome, compared to 24 in *Drosophila*, 14 in yeast and 337 in *Caenorhabditis elegans*, suggesting that targeted protein degradation is a commonly used regulatory mechanism in plants [18]. TIR1 is part of a small group of five genes, at least four of which have been shown to be involved in auxin signalling [19] and hence have been named the 'AFBs', for Auxin-related F-Box proteins. The importance of SCFs in auxin signalling was further supported by the demonstration that the *axr6* auxin resistant mutant phenotype was caused by mutations in Cullin1 [20], and that *axr1* auxin resistant mutants have mutations in a subunit of the RUB1 activating enzyme [21]. RUB1, known as Nedd8 in animals, is a small protein related to ubiquitin, which is conjugated to Cullin and affects the activity of SCFs [22]. Conjugated RUB1 is removed by the COP9 signalosome, a multi-protein complex [23]. This cycle of RUB addition and removal is apparently involved in SCF assembly and disassembly [24]. It is unclear why the phenotypes of loss of AXR1 function are virtually all attributable to defects in auxin response, as in theory defects in the RUB cycle could affect all 700 SCFs. Certainly there have been suggestions that specific *axr1* phenotypes are related to other signalling pathways such as light signalling [25], but the evidence is rather circumstantial and auxin is involved in so many processes none of the phenotypes described can be excluded from being auxin-dependent. There is a close homologue to AXR1 in *Arabidopsis*, so it is possible that AXR1 is particularly important in RUB addition to SCF^{AFBs} [26]. However, given the minimal impact of loss of function of this second gene [26], it is more likely that the activity of SCF^{AFBs} is particularly sensitive to RUB cycle defects.

A targeted protein degradation mechanism on the one hand, and a family of transcriptional repressors, whose instability is required for normal auxin response, on the other, suggest an obvious mechanism for auxin-induced gene expression which is now well supported by experiment. Auxin regulates transcription by promoting the interaction between the Aux/IAAs and TIR1 and the closely related AFBs, thus targeting them for degradation by the 26S proteasome [14,19,27,28]. Domain II is both necessary and sufficient for most of this effect, because 14 amino acids from domain II can confer these properties on heterologous reporter proteins [14,27,29], a 16 amino acid domain II peptide interacts with TIR1 in an auxin-stimulated fashion [14], and domain II mutations that stabilise the Aux/IAAs reduce or abolish this interaction [14].

Auxin-induced transcription shows parallels to many regulated degradation systems across eukaryotes, where a signal destablises a regulatory protein by promoting its interaction with a protein-ubiquitin ligase. In most cases, this requires the signal-induced modification of the target protein. However, uniquely so far, auxin signalling was shown to act by modification of the SCF [30]. The final link in the chain came from the demonstration that this modification involves the direct binding of auxin to TIR1, defining TIR1 as an auxin receptor [31,32].

The Effects of Aux/IAA Degradation

So, what is the effect of Aux/IAA degradation? A basic-level answer is easy to come by: Aux/IAAs dimerise with Q-rich ARFs, bringing them to the promoters of auxin-inducible, ARE-regulated genes, preventing transcription of these genes. Auxin promotes the degradation of the Aux/IAAs, freeing the Q-rich ARFs to promote transcription (Figure 1). There are examples where this seems to be sufficient to explain some auxin regulated outputs. For instance, in the early embryo of *Arabidopsis*, the interaction between the Q-rich ARF5/MONOPTEROS (MP), and the Aux/IAA IAA12/BODENLOS (BDL) is required to specify the embryo root pole [33].

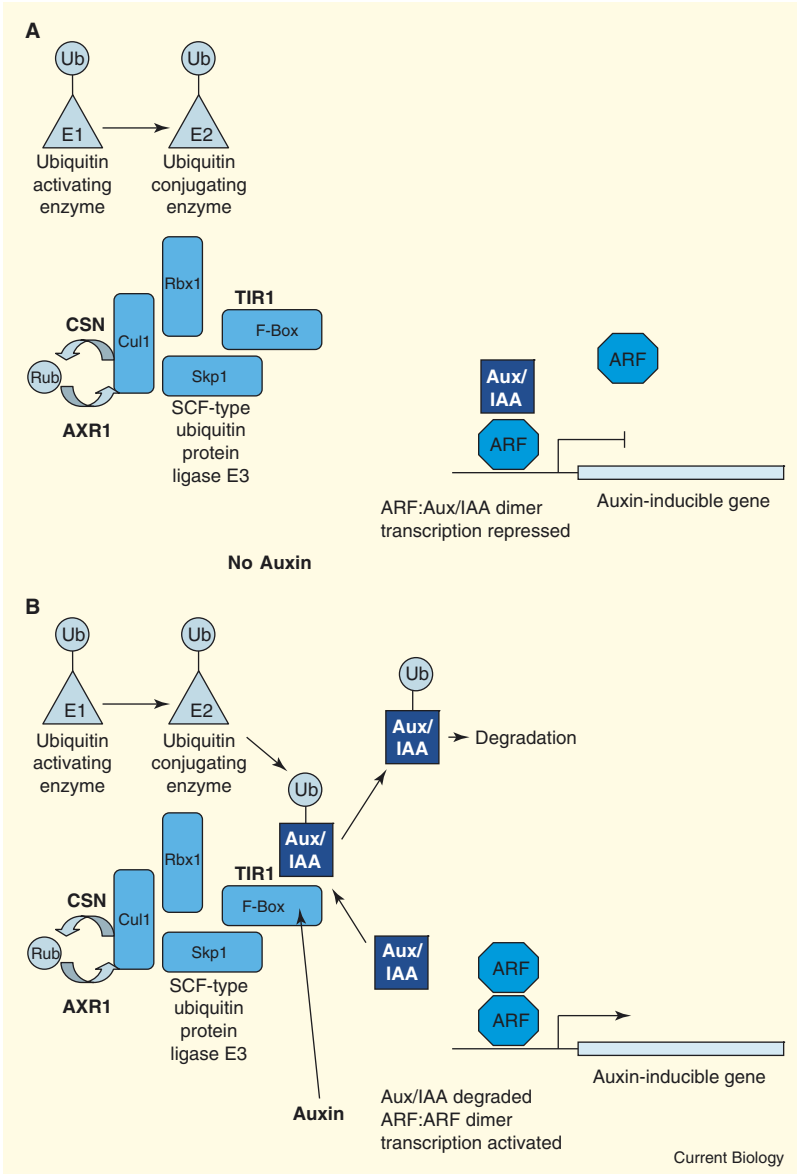

Figure 1. Regulation of gene expression by auxin.

Auxin-responsive genes are activated by members of the ARF family with Q-rich transcriptional activation domains. (A) Activation is blocked by dimerisation with members of the Aux/IAA family, which have a powerful transcriptional repression domain. The Aux/IAAs are unstable, and their degradation is mediated by the ubiquitin-proteasome pathway. Ubiquitin is activated by ubiquitin activating enzyme, E1, transferred to ubiquitin conjugating enzyme E2, and then via a ubiquitin-protein ligase E3 to the Aux/IAA targets. Once tagged with a poly-ubiquitin chain, the Aux/IAAs are degraded. The E3 in question is of the SCF-type, characterised by a Cullin–Rbx dimer that transfers the ubiquitin to the Aux/IAA and an F-box protein, TIR1 or one of its close relatives, which recruits the Aux/IAAs to the complex. These parts are connected by the SKP1 subunit which acts as a scaffold. For efficient activity, cycles of addition and removal of the ubiquitin-like protein, Rub1, to and from the Cullin subunit are required. Addition requires the Rub1 activating enzyme of which AXR1 is a subunit, and removal requires the Cop9 signalosome (CSN). (B) Auxin promotes degradation of the Aux/IAAs by binding directly to the F-box protein (TIR1 or its close relatives), promoting interaction between the F-box protein and the Aux/IAAs, releasing ARFs to form homodimers and promote transcription.

Polarity in the embryo is established with the first cell division of the zygote, which is asymmetric. This basic axis is elaborated, culminating in the establishment of the root apical meristem at the root pole, and the shoot apical meristem, positioned between the two seed leaves or cotyledons, at the shoot pole. Post-embryonically these meristems act as pools of stem cells, which divide to maintain the meristems, while giving rise to the entire root and shoot systems, respectively. Auxin plays a central role in patterning the apical–basal embryonic axis. At the root pole, auxin levels are monitored through the accumulation of the BDL protein. In mutants carrying domain II mutations in BDL, the protein has both increased basal stability and it is resistant to auxin-mediated degradation. The high levels of BDL that accumulate in these mutants block differentiation of the root pole in the adjacent more basal cells, resulting in a root-less embryo [33,34]. The root-suppressing effects of *bdl* result from its interaction with MP, as evidenced by the fact that loss of MP function results in a phenocopy of *bdl* phenotypes [33,35]. Specificity in the system is apparently conferred by a combination of the specific patterns of expression of the Aux/IAA and ARF family members involved, and variation in their dimerisation preferences [36,37].

However, this basic model (Figure 1), while representing the hub of the system, is clearly over-simplistic. Major unresolved issues include the following: What is the function of the repressing ARFs? What is the function of their interaction with Aux/IAAs, as they are already repressors? What is the function of Aux/IAA–Aux/IAA dimers? There is evidence that some repressing ARFs do not interact well with Aux/IAAs and that they do not affect auxin-regulated transcription [38], so they may have little to do at all with auxin signalling. Nevertheless, the role of these ARFs in auxin signalling cannot be conveniently ignored. For example, one *Arabidopsis* ARF, ARF3/ETTIN, does not have dimerisation domains, yet it binds AREs and its loss of function confers auxin-related

phenotypes in the flower [39]. So how does this work? One possibility is that repressing ARFs and Aux/IAA–Aux/IAA dimers may affect the Q-rich ARF-Aux/IAA relationship through competition. Different ARFs may compete for AREs, and ARFs and Aux/IAAs can compete for dimerisation partners. Thus, while the basic model described above is likely to be the heart of the matter, the balance of the system may be affected by the presence of non-Q-rich ARFs competing with Q-rich ARFs for the AREs, and Aux/IAAs and ARFs competing for dimerisation partners. For example, some Aux/IAAs might function primarily to sequester other Aux/IAAs and prevent them from interacting with ARFs. Acting in this capacity they would effectively be transcriptional activators. This is an attractive idea, because there are several examples where stabilising domain II mutations in closely related Aux/IAA family members confer completely opposite phenotypes. For example, mutations in IAA3/SHY2 result in longer root hairs than wild-type, due to precocious root hair initiation, whereas mutations in IAA17/AXR3 inhibit root hair initiation, resulting in bald roots. These phenotypes can be recapitulated by expression of the two genes from the same heat-shock inducible promoter, suggesting that the effect is at least partly mediated by the proteins, rather than their expression patterns or dynamics [37].

Other key properties of these proteins are their basal stability and the effect of auxin on stability. There is certainly considerable diversity in these properties. For example, the basal half lives of Aux/IAA family members can vary by an order of magnitude [13,14], and recently highly stable and non-auxin responsive family members have been described [40]. The fact the AFBs also are a small gene family, each member of which is likely to show variation in expression level between tissues and variation in Aux/IAA affinities, provides yet another layer at which diversity in response can be encoded in the transcriptional regulatory network [19].

Specificity of the Auxin Readout

As described above, the AFB–Aux/IAA–ARF protein network is central to the measurement of auxin levels across the plant. The fact that the downstream consequences of changes in auxin levels are so different in different tissues may be partly encoded in the tissue specific and/or environmentally-induced configuration of the network, establishing unique ARF–Aux/IAA–AFB balances. This is consistent with the specific properties of different members of these gene families, coupled with their specific expression patterns [41–45].

A striking example of tissue-specific auxin responses is the shoot apical meristem. In the meristem, leaf formation is triggered by patterns of auxin accumulation in the peripheral zone of the meristem. Ectopic addition of auxin in the peripheral zone can trigger ectopic leaf initiation, but the apex of the dome, which immediately abuts the peripheral zone, is completely resistant to this effect [46]. A likely explanation for this could be that the dome apex has a tissue-specific configuration of the auxin signalling system that severely attenuates auxin response, or more interestingly, specifically attenuates leaf differentiation outputs. This latter model could involve, for example, dome-specific expression of particular inhibitory ARFs, which compete with activating ARFs for the promoters of auxin-regulated leaf specification genes. The evidence for this question is currently rather unclear with conflicting reports suggesting either normal or attenuated auxin response in the dome apex, as assessed by expression driven by a synthetic auxin responsive promoter [47,48]. It is clear that the multiple multi-gene families together form an auxin signalling network that regulates transcription. The complexity that is possible within the network through competitive interactions of the different members provides an explanation as to how auxin might have such profoundly different effects in different tissues.

Auxin Distribution: The Road Network

The sections above have described how auxin levels can be transduced to specific changes in gene expression. These functions are essential to interpret the pattern of auxin distribution across the plant; however, they are also essential for establishing that pattern.

Auxin is so far unique among plant hormones in being actively moved around the plant by a series of transmembrane pumps or pump components [1]. The chemiosmotic hypothesis is a long-standing and widely accepted model for the basic operation of this system [49]. Auxin is a weak acid, and at the extra-cellular (apoplastic) pH a significant fraction is protonated and hence apolar. As such is can freely diffuse into the cell, where the pH is higher, resulting in ionisation. The auxin ions are then trapped in the cell and can only leave through active transport, energised by the electrochemical gradient across the plasma membrane. The auxin efflux activity can be localised to a specific part of the cell surface. Thus, in a file of cells that are all polarised in the same direction, auxin movement will be unidirectional. A widely discussed example of this is the so-called ‘polar transport stream’, in which auxin synthesised in the young expanding leaves at the shoot apex is pumped in cell files associated with the plant vascular system down the stem, into the roots, and to the root tip.

This shoot-derived auxin has many roles. Principally, it can be considered to be a reporter for the health and vigour of the primary shoot apex. If leaves are being produced and are expanding at the primary shoot apex, auxin production will be high and this will be reflected in the amount of auxin flowing in the polar transport stream. This auxin inhibits the activity of lateral shoots and promotes the production of lateral roots, ensuring appropriate root–shoot balance. For example, the initiation of root branching in young *Arabidopsis* seedlings is triggered by a pulse of shoot-derived auxin produced as the first leaves start to expand [50]. Later, if the primary apex is damaged (or more likely eaten by a herbivore), the growth of lateral shoots is activated to replace it [51].

The polar transport stream is a highly stable route for auxin movement down the plant, supplemented by phloem transport, which can be considered to be an expressway that delivers auxin in bulk to the root tip [52]. The polar transport stream has impressive

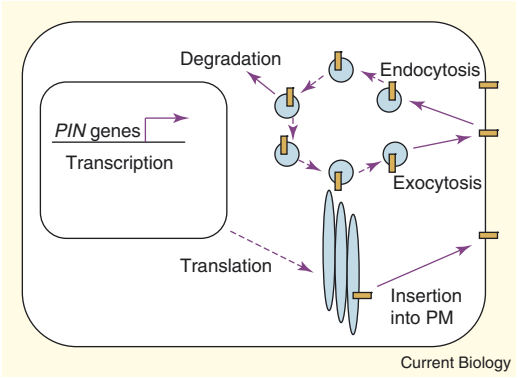

Figure 2. Auxin regulates many aspects of PIN function.

PIN auxin efflux carriers dynamically cycle between the membrane and intracellular compartments. Many aspects of PIN (orange rectangles) accumulation and localisation are affected, directly or indirectly, by auxin (full purple lines) including transcription, polarity of plasma membrane insertion, endocytotic removal from the plasma membrane and degradation.

self-repair abilities. If it is interrupted by wounding, auxin accumulates at the upper edge of the cut, triggering cell division to bridge the gap, and then re-establishing polar auxin transport, re-connecting the broken stream [53]. This is an example of a more general canalisation phenomenon, which has been a central feature of models for auxin transport for many years [53]. The canalisation hypothesis proposes that routes for auxin transport are established by positive feedback loops in which the transport of auxin through a cell results in increased and more polarised transport capacity in the cell. Through this mechanism, stochastic variation in transport between localised auxin sources and sinks is amplified to become a narrow channel for auxin flow. Repair of the polar transport stream is one example of this. Another well studied example is the specification of vascular patterning in leaves, where a localised auxin source at the leaf margin and localised sink out of the petiole into the stem become connected by canalised paths of auxin transport, which trigger vascular differentiation, and hence the pattern of veins on the leaf [53,54].

Many of these ideas were developed based on physiological data, sometimes coupled with mathematical modelling. They have been given added impetus by the recent molecular characterisation of several components involved in auxin efflux [1]. There are three classes of transmembrane protein that have been implicated: the PINs (a plant-specific transporter family), the PGP/MDRs (Multi-Drug Resistant-like transporters) and the KUPs (Potassium transporter-like [55]). The relationship between these classes is unclear; however, mutants in many of the genes encoding these proteins result in auxin transport defects. Both the PINs and the MDRs appear to be able to transport auxin directly and at least partially independently of each other [56,57]. The PINs are the best characterised *in planta*, owing to good antibodies and GFP fusion proteins allowing the collection of a large amount of localisation data both in fixed tissue, and more recently using spectacular real time imaging [58]. The results show an excellent correlation between PIN localisation to a particular cell face, and the direction of auxin transport [1,59]. PIN targeting appears to be a highly dynamic process with continuous cycling of the PINs between the cell surface and an intracellular compartment (Figure 2), a process dependent on ARF-GEF proteins such as GNOM, and sensitive to vesicle trafficking inhibitors such as brefeldin A [60,61].

The cycling of efflux carrier components via these compartments is likely to be important for their redistribution between different cell faces. There are now many examples of rapid changes in the polarity of PIN localisation. These include a startling flip in the direction of auxin flow in the early *Arabidopsis* embryo, which correlates with a flip in the localisation of the PIN7 protein [62]. In the two-cell embryo, PIN7 is localised to the apical face of the basal cell, and auxin accumulates in the embryo apex, where it is required for efficient establishment of the shoot pole. At the 32-cell stage, the polarity of PIN7 localisation flips completely, correlating with auxin accumulation at the root pole, the specification of which is apparently equally dependent on local auxin accumulation.

The environment can also influence PIN polarity, correlating with the redistribution of both auxin and growth. In the root tip, the auxin distribution network acts to cycle auxin around the tip (Figure 3). As described above, auxin is transported to the root tip in the central vascular cylinder of the roots, via both the phloem and the polar transport stream. At the tip, the auxin reaches the root apical meristem, which is responsible for the production of the growing root. In the centre of the meristem is a group of mitotically relatively inactive cells, the quiescent centre, surrounded by rapidly dividing initials which act as stem cells, replacing themselves whilst producing concentric rings of root cell types. Behind the quiescent centre, these are the epidermis, cortex, endodermis, pericycle and stele, containing the vascular tissue. In front of the quiescent centre, root cap initials give rise to the columella root cap, and to the sides, the lateral root cap, which protect the growing root as it pushes through the soil. Auxin, arriving from the shoot, accumulates at an auxin maximum centred on the quiescent centre and columella root cap initials. Auxin leaves this maximum down through the columella, and is redistributed back up the root through the lateral root cap and into the epidermis. Here it contributes to the regulation of cell elongation, in the root elongation zone, and behind that, the initiation of root hair differentiation in the differentiation zone. The auxin is then recycled back into the central tissue of the root and returns to the auxin pool at the quiescent centre [63].

The input of the environment into this system is best understood in the case of gravity. Upon reorientation of the root, so that the tip is perpendicular to the gravity vector, PIN3 in the columella root cap becomes polarised to the lower surface of the cells, directing auxin toward the lower side of the root, where it is transported back through the lateral root cap to the elongation zone [64]. The differential distribution of auxin to this lower side inhibits growth relative to the upper side, thus driving reorientation of the root tip and, therefore, root growth toward the gravity vector. The differential accumulation of auxin on the lower side is amplified by

increased proteasome-dependent degradation of PIN2 on the upper side in the lateral root cap and epidermis [65]. This degradation is dependent on vesicular trafficking, emphasising the importance of vesicle cycling in the regulation of PIN function [65]. As well as PINs, the gravitropic response requires the AUX1 protein, which has recently been shown to act directly as an auxin influx carrier [66]. Although auxin can enter cells by diffusion, influx can be speeded up by the actions of an energised carrier, and this is thought to be required to prevent dissipation of the differential accumulation of auxin on opposite sides of the root by diffusion round the root in the apoplast [67]

The pattern of auxin distribution in the root tip is interesting, because — despite the fact that it can be skewed by gravitational cues — the basic pattern, with a maximum centred over the quiescent centre and columella initials, is remarkably robust, and is maintained even if large amounts of auxin are added to the outside of the root [68]. In contrast, the pattern is rapidly disrupted by inhibitors of polar auxin transport [68]. Consistent with this observation, the pattern of auxin distribution correlates with the pattern of PIN localisation, with specific PIN family members expressed along parts of the auxin route, with different polarities depending on whether they are on the outer route away from the tip, or the inner route toward the tip. Strikingly, if individual PINs are eliminated by mutation, there is little phenotypic effect because in these mutants, different PIN family members change their expression pattern, and even sometimes the polarity of their cellular localisation, to compensate for the missing PINs [63,69]. Multiple PIN mutations are necessary before the pattern breaks down. This suggests feedback regulation, whereby deviations in the pattern of auxin distribution trigger alterations in PIN gene expression, and correction of the pattern. In the root tip, this feedback is mediated by two closely related members of the PLETHORA family of transcription factors [70] and such feedback is widespread and can occur at multiple levels, as predicted by the canalisation phenomena commonly observed in the establishment of auxin transport conduits throughout plant development. For example, more direct regulation of PIN gene transcription by the AFB-Aux/IAA-ARF network has also been observed in various contexts such as the shoot apical meristem and the embryo [58,69] and there is good evidence that auxin itself can regulate PIN protein stability [65,71].

This self-correcting, self-organising and self-stabilising system is presumably needed during normal root growth to maintain the auxin distribution pattern in the root tip as the root grows, with the added benefit of providing environmental responsiveness and damage repair systems [72]. Although the pattern of auxin distribution looks stable, it must be remembered that it exists in a growing organ so that the cells participating in the root tip auxin cycle are constantly changing as cells exit into the mature root, and new cells are added at the level of the initials. Therefore, this auxin distribution pattern is really highly dynamic.

A more blatant example of dynamic patterns of PIN and auxin distribution is the shoot meristem [58]. The shoot meristem is responsible for the production of

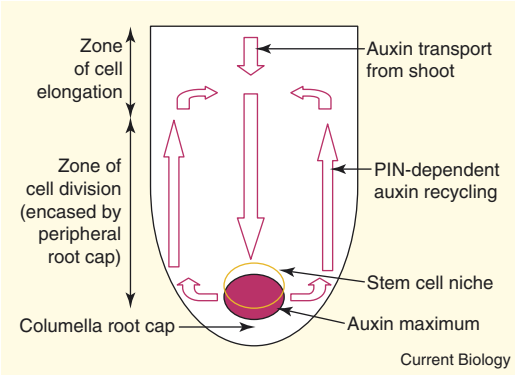

Figure 3. Dynamic patterns of auxin flow in the root tip result in stable patterns of auxin accumulation.

A high concentration of auxin accumulates in a localised region at the root tip, and this is required for correct cell type specification in the growing root. Auxin flows into this region from the centre of the root and is recycled away in the epidermal layer where it is required for cell division and elongation in turn. It then returns to the central root and to the tip.

the shoot and consists of a dome of cells, with a central zone of relatively slowly dividing initials, which feeds cells into a surrounding ring of rapidly dividing cells that make up the peripheral zone. Stem tissue is laid down under the meristem, and in the peripheral zone leaves are initiated in a fixed pattern, or phyllotaxy. Leaf specification in the peripheral zone appears to be triggered by a local accumulation of auxin at the site of leaf specification, driven by the auxin efflux machinery including the PINs [46,58]. Blocking auxin efflux with auxin transport inhibitors, or by mutation in PIN1, prevents organ initiation at the apex, without preventing meristem activity and stem production [46]. It was the resulting pin-like shoot structure, produced as a result of loss of PIN1 function, that gave the PIN family its name. When auxin is applied locally to the peripheral zone of these pins, initiation is triggered at the site of auxin application. Immunolocalisation studies demonstrated that PINs are localised in the meristem in such a way as to direct auxin toward incipient and young organ primordia [46]. More recently, PIN1–GFP fusion proteins have been used to generate spectacular time-lapse movies showing polarisation of PIN toward the sites of incipient organ initiation, followed by repolarisation toward the next primordium [58]. This also showed that auxin induces the transcription of PIN1, so that GFP accumulation in the meristem is also likely to correlate with auxin levels and/or response. The movies clearly show an increase in GFP accumulation at the sites of organ development, consistent with efflux polarisation driving local sites of auxin accumulation, which in turn specifies organ fate. This entire system operates in the meristem epidermis, which is why real time imaging is possible. As in the root tip, auxin is thought to be kept in the epidermal layer by expression of the AUX1 influx carrier in this layer [46]. This restriction of auxin movement to the epidermis is eventually broken by the establishment of an auxin stream down into the internal tissues from the centre of the developing organ. This stream initiates the patterning of the organ vascular system [46,58].

In an attempt to understand the auxin transport system better, mathematical models have been constructed that approximate the observed patterns of leaf initiation [48,73]. These two models are based on similar assumptions about the patterning mechanism involved, derived from the experimental results described above. The models demonstrate that these assumptions can produce a reasonable, although not very stable, phyllotactic pattern. Interestingly, the models require the inclusion of one, non-intuitive assumption to produce the observed self-organising pattern. It is necessary to propose that PIN polarity in a cell is regulated by auxin in neighbouring cells, such that PIN distribution polarises toward the cells with the most auxin. This idea is contrary to classical models in which PINs polarise toward cells with low auxin concentration, but high auxin flux, for example the canalisation models mentioned above [53]. It seems unlikely that there will be a single rule for PIN polarisation and certainly there are examples where PINs are directed away from high auxin levels, such as in the collumella root cap [70]. Nonetheless, this is a good example of where the construction of a formal mathematical model has presented a clear testable hypothesis, and thrown the spot light on a central mystery in auxin biology — the regulation of auxin flow, the auxin highway code [74].

The Highway Code

The examples of the auxin distribution network in action that we have discussed so far clearly demonstrate the importance of regulated and dynamic targeting of auxin efflux activity. Understanding the mechanisms that control this process is, therefore, central to understanding the auxin 'highway code'. Encouragingly, remarkable parallels in PIN behaviour have been observed in apparently disparate auxin-driven patterning processes such as lateral root formation, leaf initiation, and leaf vascular patterning, suggesting that common regulatory modules are involved [75,76]. However, detail about the nature of these mechanisms is currently tantalisingly patchy.

There are clearly both tissue specific and PIN protein specific aspects to the targeting system. For example, in the root tip auxin circuit described above, PIN1 is expressed in the cortex and positioned on the cell face at the root tip end of cells, pumping toward the quiescent centre. PIN2 protein is similarly expressed and localised in the cortex, but in the epidermis PIN2 is localised on the opposite cell face, pumping auxin back up the root toward the elongation zone. In the *pin2* mutant, the PIN1 expression domain expands to include the epidermis, where it localises on the shoot-facing cell surface, like epidermal PIN2 [63,69]. These data suggest a tissue specific determinant of PIN localisation. Consistent with this idea, reestablishment of correct PIN polarities in the root tip following damage depends on tissue respecification [72]. However, there is also good evidence for PIN-specific elements in the system, because when PIN1 is misexpressed in the epidermis from the PIN2 promoter, it adopts its cortical type of orientation [59], and differently tagged PIN variants have been shown to localise differently [59].

Thus, the basic properties of the PIN protein family members, and cell-type specific targeting machinery combine to target PINs. However, these mechanisms are of limited value in explaining the dynamic nature of PIN polarisation. Certainly many examples of PIN retargeting are associated with cell specification events, but by no means all of them are, and in no case is any detail known about the mechanism of polarisation. As mentioned above, it seems likely that cycling of components of the efflux machinery between the plasma membrane and intracellular vesicles is important [60]. Several known pharmacological inhibitors of polar auxin transport apparently act by blocking this vesicular trafficking, suggesting that the trafficking itself may be necessary for efflux activity [77]. More recently, however, it has been shown that short-term treatment with auxin appears to trap PINs on the plasma membrane and this correlates with an increase in auxin efflux, arguing against a requirement for PIN cycling for efflux [78]. In addition, this observation raises the interesting possibility that targeting of auxin efflux to a single cell face may be achieved by differential removal of efflux components from the other faces. In this scenario, auxin efflux components would be synthesised and inserted randomly into the plasma membrane, but they would be re-internalised into intracellular vesicles from all but one cell face. Over time, this mechanism would lead to the accumulation of auxin efflux components on a specific cell face. This model provides a possible mechanism to achieve the suggested polarisation of auxin efflux toward high auxin, described above [48,73]. If extracellular auxin can inhibit PIN internalisation, then the cell face experiencing the highest levels of auxin will accumulate PINs, and pump more auxin out. This positive feedback loop will be broken only if uptake of auxin by the neighbouring cell is so rapid as to deplete the intercellular space of auxin. This would require an extracellular auxin receptor, for which there is good evidence [79,80]. Although this is an attractive hypothesis, it must be noted that longer auxin treatments can increase internalisation and PIN degradation, so the significance of the short-term plasma-membrane trapping of PINs is unclear [65]. A modification of the auxin-induced trapping idea is that it is the activity of the carrier itself that prevents its internalisation, so that active carriers remain on the membrane. This mechanism could only polarise the pumps if auxin was more likely to be pumped from one face; for example, if the pumps were activated by a signal derived from a neighbouring cell.

The alternative to these 'targeted removal' models is the more intuitive 'targeted insertion' model, in which auxin efflux components are trafficked from intracellular compartments to a specific cell face. This mechanism would achieve polarity more quickly than targeted removal, but at present there is little to go on with respect to the mechanism by which it might occur. The main clues so far come from the analysis of the *PINOID* gene (*PID*). PID is a serine/threonine protein kinase and its loss of function results in phenotypes similar to loss of the PIN1 protein, with the shoot apical meristem loosing the ability to initiate organs [81,82]. However, this phenotype is not caused by loss of PIN1, but rather by its mis-targeting to basal, rather

than apical membranes in the shoot apical meristem [83]. Furthermore, overexpression of PID can also trigger a flip in PIN1 polar localisation [83], and *PID* transcription is regulated by auxin, providing yet another layer to the feedback system [82] (Figure 2).

Conclusions

Auxin action is driven by the interplay between context-sensitive transcriptional readouts of auxin concentration and active positioning of auxin within tissues by localised and oriented transporters, with likely additional contributions from localised synthesis and catabolism. These systems are interconnected at many levels, allowing the generation of self-organising repeating patterns, or self-stabilising standing waves. There are obvious advantages to such a system in maintaining the environmental responsiveness that is a touchstone of plant development, as well as in providing triggers for genetically regulated developmental progression.

Simultaneously, the system provides a mechanism for long-range communication between plant tissues [84]. This can take on at least four forms. The first involves changes in the amount of auxin produced in one tissue and transported to another, with an unchanging transport network. For example, the initiation of leaves at the shoot apex results in an auxin pulse, which is transmitted to the roots in the polar transport stream and triggers root branching [50]. A second mechanism involves adjusting the transport network to redirect auxin from one tissue to another, as in the case of gravitropism where auxin in the root cap is non-uniformly redistributed to one side of the root [64]. Here the gravity-sensing machinery in the root cap communicates to the cells of the root tip elongation zone by modulation of the transport of auxin away from the tip. A third system for long range communication could occur if sink tissues compete for a limited auxin supply in a process that is conceptually similar to lateral inhibition. Such a mechanism has been proposed to account for the even spacing of initiating lateral roots, and is central to phyllotactic patterning of leaf initiation [46,75,84]. The fourth mechanism, in which auxin sources compete for an available sink, is essentially the opposite. This can have a regulatory effect in a situation where auxin removal is essential for some process to proceed, such as in the export of auxin through the centre of a newly initiated leaf primordium. In this situation, auxin movement out of a tissue could be blocked if auxin backs up through the transport network due to a bottleneck elsewhere in the system. There is growing evidence that shoot branching is regulated partly in this way [85].

The possibilities for communication in this system are thus extraordinary in their complexity and diversity. There are effects driven by auxin concentration, with the strong possibility that intracellular and extracellular auxin are measured independently. In addition, there are effects based on competition between tissues for auxin sources or auxin sinks, which can be driven by the capacity for auxin flow. On top of this, there is the as yet unsupported possibility that auxin flux through transporters into and/or out of cells could also be directly monitored. All these systems are interdependent and regulate one another. There is no doubt that a serious effort in mathematical modelling will be required, closely linked to experiment, to have any chance of understanding auxin biology.

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