Chapter 14
Auxin and Self-Organisation

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Abstract This essay develops a conceptual framework to understand the role of auxin for the genesis of plant organisms. This framework has to consider the specificities of the plant lifestyle and underscores the fact that plant organisation is highly modular. The assembly of these modules is controlled through robust self-organisation driven by autocatalytic loops linked to lateral inhibition which can be formally described as reaction–diffusion system in sensu Turing. Instead of actual inhibitory molecules as in the original Turing model, they achieve lateral inhibition by mutual competition for an activator (auxin). This can be demonstrated for phyllotaxis, but also for vascular differentiation. We study self-organisation in cell strains from tobacco and find that individual cell divisions within a file are synchronised through weak coupling based on a directional flow of auxin. We use this system as a simple minimal organism we have identified an oscillatory circuit as central element of self-organisation. This self-referring circuit connects auxin-dependent remodelling of the actin cytoskeleton with actin-dependent remodelling of auxin flux. The essay concludes with the working hypothesis that the contiguity of plant organisms is manifest in time ("rhythm") rather than in space ("body") and describes an experimental model where the induction of cell axis and polarity as base for self-organisation can be studied de novo in regenerating protoplasts.

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1 Why Plant Organisms Are Really Different

1.1 It All Starts with Biophysics

All life has to balance supply with consumption. Supply has to occur through the surface, whereas consumption is a function of volume. A growing cell will increase surface by the second power of the radius, but volume by the third power. As a consequence, supply (surface, r^2) and consumption (volume, r^3) will diverge progressively. To bridge this gap, metabolic efficiency has to be elevated—however, this is possible only due to the innate constraints set for instance by protein structure. When this tunable component of metabolic efficiency is enriched, the surface has to be increased by invaginations or protrusions, a phenomenon already observed already in unicellular organisms. Such surface increases confer a selective advantage, because a larger organism acquires buffering against environmental fluctuations and, most important, is less readily devoured by competitors.

As a consequence of their photosynthetic lifestyle, plants have to augment their surface by centrifugal extension, generating a considerable degree of mechanical load (Fig. 14.1a). As long as plants remained aquatic, this load was at least partially relieved by buoyancy, allowing considerable sizes even for fairly simple architectures. However, when plants began to conquer terrestrial habitats, they had to develop flexible, yet robust, mechanical supports. The invention of vasculature-based modules, so-called telomes (Zimmermann 1965), became a decisive factor for the evolutionary success of land plants (Fig. 14.1b). Mechanical load shaped plant architecture down to the cellular level: Plant cells are endowed with a rigid cell wall with specific and fundamental consequences for cell division and cell expansion. These cellular specificities are of tremendous agronomical impact. For instance, the reduction of lodging in cereals is considered as pivotal factor for the success of the so-called Green Revolution (for the cellular details, refer to Nick 2012).

The central point of these considerations is that plants were channelled towards a sessile lifestyle due to biophysical constraints.

1.2 A Consequence: Cells Versus Organisms—Why the Plant Approach Is Different

In addition to plant architecture, the sessile lifestyle has shaped the mode of morphogenesis. In animals, the *Bauplan* is laid down early in development. In some cases, even maternal factors have been found to complement the DNA of the embryo providing a kind of "morphogenetic inheritance". For instance, the anterior–posterior polarity in the *Drosophila* embryo is determined by a gradient of maternal, untranslated mRNA encoding transcription factors such as BICOID or

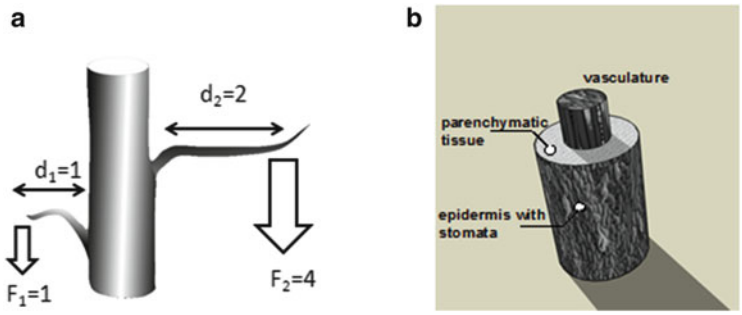

Fig. 14.1 Plant architecture is shaped by mechanical load. (**a**) The lever force produced when a branch doubles its length (from $d_1 = 1$ to $d_2 = 2$) grows fourfold. (**b**) The development of telomes as load-bearing architectural module in the early Devonian was decisive for the evolutionary success of land plants

NANOS (Nüsslein-Volhard 1995). Even in classical models for epigenetic morphogenesis, such as the amphibian embryo (Spemann 1936), the dorsiventral polarity of frog eggs is established by autocatalytic feedback of polarising signals (gravity, sperm entrance) upon inherited patterns. These inherent patterns include not only preformed morphogenetic movements, but also transport and translation of maternal mRNA coding for cytoskeletal proteins and polar determinants (Elinson and Rowning 1988). In these models, the *Bauplan* is laid down during early development, often prior to cellularisation. Differentiation proceeds from the level of the entire organism down to the level of individual cells.

Again, plants are different: The genetic determination of plant shape is not as stringent as for animal development, but depends strongly on the environment. As central feature of this open morphogenesis, growth is not confined to early development, but continues throughout the entire life cycle. The ability to adjust growth in response to environmental stimuli is central for the adaptation of the individual plant to the challenges of its habitat. As a consequence of the rigid cell walls, cellular movements, a central mechanism in animal development, are not relevant for plant morphogenesis. The basic morphogenetic unit in plant development is the individual cell. Differentiation initiates from the level of individual cells and subsequently proceeds up to the level of the entire organism. This fact is highlighted by the ability to regenerate entire plants from almost any plant cell. In animals, such totipotency is confined to the fertilised egg cell and, sometimes, to its immediate descendants (Spemann 1936).

Thus, the principal difference (although there are definitely transitions that are now ignored for the sake of being clear) between plant and animal morphogenesis can be condensed into the following statement: In animals, the organism produces cells, whereas in plants, cells produce an organism. This means that the potency to form an organism must be enshrined in the individual plant cell.

2 Why Plants Need Coordinative Signalling?

2.1 Open Patterning

Plant cells are flexible in their developmental potency. Cell expansion is under control of phytohormones and environmental factors such as light (Lockhard 1960). The rapid expansion of cells is complemented by a slower addition of morphogenetic elements (cells or organ primordia), which does not occur randomly, but is ordered in space and time. This pattern formation (in sensu Bünning 1965) depends, on the one hand, on intrinsic signals that are obviously defined by genetic factors (otherwise there would be no base for classical plant taxonomy!). On the other hand, plant patterning can integrate signals from the environment. Environmental integration is evident, when a shoot meristem is committed for flowering controlled by day length and subsequently will form floral instead of vegetative organs. In animal patterning, the elements that are organised during pattern formation are generated prior to being differentiated. In a fruit fly embryo, for example, numerous nuclei are produced before they are patterned depending on gradients derived from maternal factors. Plants follow different developmental rules—here, the pattern is perpetuated in an iterative manner when new elements are continuously added *during* the patterning process.

This pattern iteration could be achieved, in principle, by assigning different developmental fates to the daughter cells during cell division. The pattern would then result from an ordered sequence of such formative divisions. Such a mechanism had been proposed for the root meristem of the model plant *Arabidopsis thaliana*, which is characterised by a highly stereotypic cell lineage (Scheres et al. 1994). However, elegant laser ablation experiments (Van den Berg et al. 1995) and functional analysis of mutants with aberrant tissue layers (Nakajima et al. 2001) revealed that even in this stereotypic system, cell fate was defined by signals (transcription factors) from adjacent cells and not by cellular genealogy.

Generally, the principal totipotency of plant cells is difficult to reconcile with a strong impact of cell lineage. Patterning in plants must result from coordinative signals between the already defined (older) regions of the pattern and the newly formed elements of the field that still have to acquire a specific identity.

2.2 Coordinative Signalling During Patterning Is Evolutionary Ancient

Plants acquired photosynthesis by sustainable symbiosis with autotrophic cyanobacteria. Functional multicellularity is already present in this class of prokaryotes. Filamentous cyanobacteria are capable of a simple cell differentiation yielding so-called heterocysts that can convert atmospheric nitrogen into

ammonium and thus overcome the limitations of nitrogen bioavailability. Since filamentous cyanobacteria combine open patterning with developmental flexibility, coordinative signalling would be expected already in these prokaryotic precursors of the plant lifestyle. The nitrogenase required for the fixation of nitrogen dates back to the earliest anoxic phases of life on this planet and is therefore highly sensitive to oxygen. To safeguard the functionality of nitrogenase, any photosynthetic activity (releasing oxygen) has to be excluded from heterocysts. Thus, the heterocysts must be supplied with assimilates from their photosynthetic neighbours. Nitrogen export and assimilate import have to be balanced even though the total number of cells grows continuously, which represents a classical problem of open patterning. This balance is regulated by iterative algorithms, whereby preexisting heterocysts suppress the differentiation of new heterocysts over a range of around ten cells. When, as a consequence of cell division, the distance between the heterocysts exceeds this threshold, a new heterocyst will differentiate between them. Using patterning mutants in *Anabaena*, the factor responsible for this lateral inhibition could be identified as the diffusible peptide patS (Yoon and Golden 1998). Differentiation (including the synthesis of patS) will begin in clusters of neighbouring cells. However, one of these cells will excel the others and then immediately start to suppress further differentiation in its neighbourhood (Yoon and Golden 2001).

Thus, already the photosynthetic pluricellular prokaryotes do not use a predetermined cell fate but "negotiate" differentiation by signalling between neighbouring cells.

3 The LEGO Principle of Plant Morphogenesis

Plants use a modular version of "organism" and "identity". Due to their centrifugal architecture, plant organisms lack contiguous borders, a hierarchy of the "body" over its parts (which is linked with a strong cell autonomy). Moreover, there is not any impact of cellular genealogy on the set-up of the *Bauplan*, nor a predefined developmental programme. Nevertheless, they are able to defend their identity against the fluctuations of their environment. Their buffering capacity even excels that of animals by orders of magnitude. Despite strong variations in the details of individual development (which is tuned with the respective environmental conditions), the characteristics of each plant species emerge as a specific way to develop, respond and propagate. Without this specificity, no classical plant taxonomy would be possible.

We encounter here a seemingly paradox combination of flexible and species characteristic development. This paradox can be resolved considering the pronounced modularity of plant development. To use a metaphor: plant development resembles a play of LEGO bricks. Each brick is simple in shape and robust enough to survive most if not all challenges posed by a young architect. The assembly of these bricks is extremely flexible, though, and allows for almost any conceivable

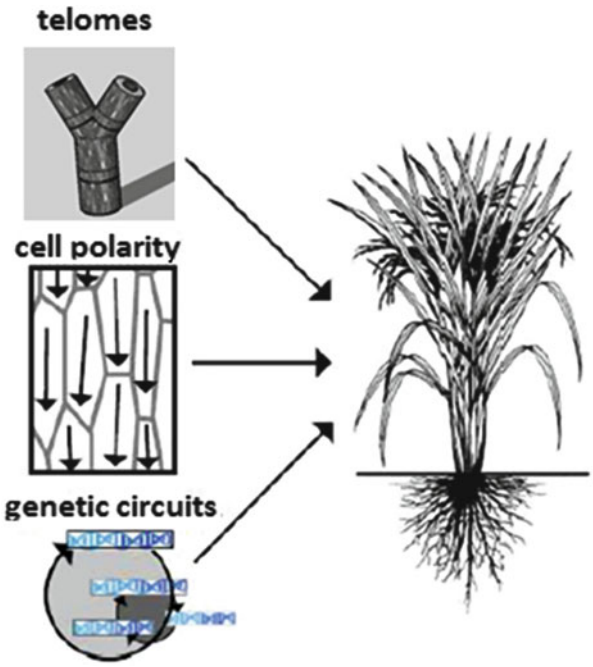

Fig. 14.2 Modular organisation of plant development. Plant architecture is based on morphological modules (telomes) that are combined in a flexible manner depending on environmental conditions. Individual plant cells are endowed with innate directionality (cell polarity) that is dynamically aligned by signal flow through the morphological modules. Self-referring robust genetic circuits guide the differentiation of the cellular and the morphological modules and are recombined in temporal patterns

variation of architecture. Where would be genetic information be placed in this metaphor? Probably less in the inspiration of the young architect, but rather in the way the bricks are produced in the factory.

What are these "LEGO bricks" of plant development? There are principally three types of bricks: architectural, cellular, and genetic (Fig. 14.2).

3.1 Architectural Modules

The architectural "LEGO bricks" are the telomic modules arranged in a flexible way integrating mechanical load with a panel of environmental signals (with light as central component). The arrangement of telomic modules is under control of auxin flux from the aerial organs towards the roots driving the differentiation of ground tissue into vasculature (as core element of the developing telome). This architectural principle is simple, robust, and flexible. Cellular details of recently discovered fossil finds of the progymnosperm *Archaeopteris* (Rothwell and Lev-Yadun 2005) suggest that already in the Upper Devonian, 375 million years before our time, the arrangement of telomes was controlled by auxin flow. The secret of the land plant success story seems to reside in this modular morphology.

3.2 Cellular Modules

The polarity of vascular cells is aligned with the shoot–root axis and represents the cellular correlate of the "LEGO bricks" forming the base for the telomic principle. The directional transport of auxin (see Chap. 4) is brought about by the combination of multidirectional, "exploratory" influx and directional efflux (due to the polar localisation of auxin efflux carriers). The polar localisation of auxin efflux carriers is a continuous process rather than a fixed structure: These carriers cycle continuously and rapidly (the lifetime of the carriers at the membrane are in the range of a few minutes!) between an endocytic compartment and the site of their activity at the plasma membrane. The intensity of cycling depends on the presence of auxin (Paciorek et al. 2005) and differs between the different poles of the cell establishing such a polar distribution (Dhonukshe et al. 2008) providing the positive amplification loop required for the auxin canalisation mechanism driving vascular patterning. Sensory input on the auxin distribution of surrounding cells continuously "questions" this loop either reinforcing the existing directionality of the cell or leading to a new polarity. Since morphology and cellular architecture are brought about by modular, self-organised processes, the genetic control might be relatively simple.

3.3 Genetic Modules

The genetic "LEGO bricks" underlying the *Bauplan* of land plants might be robust regulatory circuits that are launched under control of fairly permissive temporal patterns. By shifting these programmes in time (so called heterochrony), new architectures can be generated that at first sight are very spectacular. The power of heterochrony is illustrated by the case of the "Skye" ecotype of the model plant *Arabidopsis thaliana* (thale cress). Normal thale cress plants produce a leaf rosette but, upon bolting, switch to the formation of small, single leaves protruding from the elongating inflorescence. This switch is impaired in the "Skye" ecotype resulting in a fundamentally altered architecture with aerial rosettes formed from the axillary meristems of the bolting inflorescence (Grbić and Bleecker 1996). It could be shown that this spectacular change of the *Bauplan* was caused by mutations in two relatively inconspicuous genes that modulate, among numerous other factors, the timing of developmental processes. The mutations simply delay the inactivation of the vegetative programming during bolting. The ongoing vegetative development at simultaneous launch of a floral programme accounted for a fundamentally different morphology that at first sight seemed to result from "macroevolution". A comparative approach on plant development rapidly reveals that evolutionary adaptations of plant architecture can often be deduced from heterochronic shifts between fairly simply morphogenetic processes (for review, see Li and Johnston 2000).

3.4 The Secret of Plant Morphogenesis: Robust Modules, Flexible Assembly

In summary, plant organisms assemble morphological, cellular, and genetic modules to accommodate challenges from the environment with the innate necessities of physiology. These modules stem from fairly robust self-organisation providing a mechanism to maintain the specific quality of the respective plant. The assembly of these modules is rather flexible and can be tuned with the exogenous necessity of environment. Since the modules are relatively robust and autonomous, the signals that regulate modular assembly may be very simple. Complexity is provided by the receiving modular process, not by signal triggering this process. Indole-acetic acid, the natural auxin, is astonishingly small and simple. However, it combines three molecular properties (none of which is spectacular): Auxin is a small organic acid and therefore easily moves through the acidic environment of the apoplast. Auxin carries a lipophilic indole ring and therefore can permeate the cell membrane from any direction, which allows a cell to "explore" the auxin levels in its neighbourhood. Auxin is a weak acid and thus readily trapped in the neutral cytoplasm and has to be actively exported by carriers, which allows to create a directionality of auxin efflux. When the localisation of the efflux transporter is shifted under the control of auxin itself, this will generate a self-regulatory circuit that perfectly meets the criteria of a reaction–diffusion system in sensu Turing (1952).

4 Auxin and Coordinative Signalling

4.1 Plant Patterning and Coordinative Signalling: Phyllotaxis

Phyllotaxis allows to optimise the position of leaves to maximise photosynthetic efficiency. A prospective leaf primordium will form at maximal distance from the older primordia indicating inhibitory fields (Schoute 1913). In fact, when the youngest primordium is isolated from its environment by tangential incisions, this will be the subsequently formed primordia will shift their position (Snow and Snow 1931). This shift was originally interpreted in terms of the additional space created by the incision that would allow the incipient primordia to move to a position where they otherwise were excluded (first available space model). Later, inhibitory fields emanating from the older primordia have been proposed, but the nature of these inhibitory signals has been under debate for a long time. Basically, there were two standpoints in this debate: biophysics versus biochemistry.

4.2 Biophysical Model of Phyllotaxis

Buckling from the older primordial would, under conditions of the tissue tension present in a growing meristem, inhibit by mechanical stresses the formation of new primordial in the neighbourhood. In fact, the position of prospective primordial could be perfectly predicted by models of stress–strain patterns (for review, see Green 1980). As expected from a biophysical model, local release of tissue tension by beads coated with the cell wall loosening protein extensin could invert the phyllotactic pattern (Fleming et al. 1997). As early event of incipient primordial commitment, membrane-associated microtubules reorient sharply and subsequently align with the stress–strain pattern (Hardham et al. 1980). By means of GFP-labelled microtubules, this phenomenon could be followed in a non-invasive manner in living shoot apices of *Arabidopsis thaliana* (Hamant et al. 2008). A combination of live cell imaging with mathematical modelling of stress–strain pattern revealed that cortical microtubules align in the direction of maximal mechanical stress in a transcellular pattern. When the outer meristem layer was removed by laser ablation, microtubules reoriented in orientations predicted by the mathematical model, followed by a compensatory bulging of the apex. The impact of cortical microtubules is further corroborated by recent evidence for a role of the microtubule-severing protein katanin for meristem patterning (Uyttewaal et al. 2012).

4.3 Biochemical Model of Phyllotaxis

As alternative to the biophysical inhibition, chemical signals from the older primordia were proposed to inhibit the initiation of a new primordium in their proximity. This model was supported by studies in apices that had been freed from primordia by application of auxin transport inhibitors (Reinhardt et al. 2000), an experimental system that allows study of the *de novo* generation of a pattern in the absence of any prepattern. These studies showed that the coordinative signal depends on auxin. Unexpectedly, the preexisting primordia did not act as sources, but as sinks for auxin. This leads to mutual competition for free auxin within the apical belt that is competent for the initiation of leaf primordia. Since pre-existing primordia attract auxin fluxes from the meristem, they drain their neighbourhood from diffusible auxin, such that the initiation of new primordia is inhibited (Reinhardt et al. 2003). When the auxin efflux regulator PIN1 is mutated, this will, as a consequence, strongly perturb phyllotaxis, supporting the importance of directional auxin efflux for the pattern. However, a recent detailed analysis of the *pin1* mutant revealed that the phyllotactic pattern is disturbed but not eliminated, indicating that PIN1 is not the only factor capable of guiding the pattern (Guenot et al. 2012).

4.4 Synthesis: The Auxin–Microtubule–Tension Loop

As for most dichotomous debates in biology, reality seems to be a synthesis of the two seemingly exclusive standpoints: By measuring tissue rigidity with Atomic Force Microscopy in the vegetative apex of *Arabidopsis thaliana*, an auxin-dependent local softening of the cell wall could be demonstrated. It was further shown that this relaxation of wall tension was mediated by a specific modification of wall pectins homogalacturonan de-methyl-esterification (Braybrook and Peaucelle 2013). Interestingly, when this modification was administered in the absence of auxin transport, it was not effective. Thus, both functional auxin transport and local reduction of mechanical stress were necessary and sufficient for phyllotactic patterning. The resulting model is based on a regulatory feedback loop between auxin transport and tissue tension and requires that the direction of mechanic stress can be transduced into altered localisation of auxin efflux transporters. Microtubules that can guide the localisation of PIN1 (Heisler et al. 2010) and simultaneously perceive mechanic stress might be the mechanistic link. This auxin–microtubule–tension loop might thus provide the synthetic bridge reconciling the traditional antagonistic viewpoints on phyllotaxis.

4.5 Plant Patterning and Coordinative Signalling: Vasculature

All land plants (except the archaic mosses) are made of load-bearing modular elements, the telomes. These telomic modules consist of conductive woody vasculature embedded in a cylinder of parenchymatic tissue with the potency for vascular development, enclosed by an epidermal layer. The flexible arrangement of telomes represents the core process of plant architecture. The vasculature can be adjusted by differentiation of parenchymatic cells to tune mechanic load and transport with the perturbations, for instance, from wounds (Sachs 2000) or from growth as in the venation of developing leaves (Mattsson et al. 1999). All cells of the parenchymatic tissue are competent to differentiate into vasculature. This differentiation depends on the flow of auxin through these cells.

Although auxin can enter the cells of higher plants through specific import channels, for the pattern of vasculature, the non-directional influx through the membrane is important (discussed in more detail in Sect. 6). The major natural auxin, indolyl-3-acetic acid, is a weak acid and relatively small. In the acidic environment at the outer face of a plant cells, it will be uncharged and therefore can permeate through the membrane even without the help of influx carriers. In the more or less neutral cytoplasm, auxin is deprotonated and thus acquires a negative charge that will prevent its spontaneous exit. Due to this ion-trap mechanism, auxin will accumulate in the cell. However, it can exit by means of specific export pumps that are localised asymmetrically, guided by cell polarity. The combination of

non-directional influx and directional influx produces a mutual competition of individual cells for free auxin and a directional flow in the direction of cell polarity. A cell with more active or more localised auxin exporters will transport more auxin than its neighbours and therefore cause a drainage of auxin. This mechanism for lateral inhibition of individual elements is now combined by autocatalytic feedback: The differentiation from the ground state into a vascular cell fate is induced by the flux of auxin passing through the respective cell. Conversely, this differentiation promotes cell polarity resulting in a stronger gradient of auxin exporters what, in turn, will further stimulate auxin drainage of the neighbourhood.

This positive feedback, in combination with a lateral inhibition (caused by mutual competition for auxin), drives the pattern of conductive tissue and thus the arrangement of telomes. This "auxin canalisation" model has been extensively studied and modelled mathematically and is capable, for instance, to predict venation patterns in leaves (for review, see Berleth and Sachs 2001).

4.6 Plant Patterning: Order Without a "Great Chairman"

Biological patterns are shapes that become manifest on the level of a population of cells or organs. They are holistic in quality and represent classical system properties that emerge when the system is considered as an entity. At first glance, this would call for a strong hierarchy controlling the behaviour of the individual elements. To use a metaphor from human societies: collectivism is usually bound to strong (and often autocratic) leader personalities. This approach will not work for plant development, though. As pointed out above, plant cells maintain a high level of autonomy and are not easily subdued to the rule of a "Great Chairman". In addition, plant cells behave in a highly stochastic manner, a property that ultimately can be attributed to the diffuse organisation of environmental sensing (details are given in Nick 2006): Plants lack specialised sensory organs. In shorthand, each individual cell is able to sense most environmental signals in a monadic way and therefore has to employ extreme amplification of the sensory input resulting in all-or-none type outputs on the level of individual cells. If all cells of a tissue would respond in a homogenous manner, plant responses would be saturated already at very low input. In case of light, the input from the new moon would produce the same output as full sunlight at noon time. Due to the strong variation of sensory thresholds, plants can differentiate between weak and strong stimuli by the frequency of individual cells where sensing is activated. By integrating over the population of activated cells via cell–cell communication, plants can extend their dynamic range of sensing combining high sensitivity with differential responses to different signal input.

What can we generalise from phyllotaxis and vascular patterning? Both patterns resist stochastic fluctuations of the initial situation, they are based on lateral inhibition between the elements of the pattern, and they contain qualitative decisions generated via autocatalytic feedback loops. Both patterns can be described by the mathematics of reaction–diffusion systems that were adapted to biology by

Turing (1952) and have been quite successfully used to model various biological patterns such as foot-head patterning in *Hydra* (Gierer et al. 1972) and segmentation in *Drosophila* (Meinhard 1986), but also leaf venation (Meinhard 1976). In reaction–diffusion systems, a locally constrained, self-amplifying feedback loop of an activator is linked to a far-ranging mutual inhibition (Gierer and Meinhard 1972). Auxin-dependent patterning differs in one aspect from the original model, where the inhibitor is usually described as a positive entity (such as the patS peptide acting in cyanobacterial patterning). In auxin-dependent patterning, lateral inhibition is brought about by mutual competition for the activator.

Self-activation combined to mutual competition also provides *proportional harmony*, a system property of many organisms meaning that a new holistic organisation can emerge independently of size, when the original system is either divided or fused. In plants, this astounding ability becomes manifest as the lack of physical body individuality: the plant body can be subdivided and the parts will readily organise a new independent plantlet that in shape and architecture resembles its progenitor organism.

5 Organismic Modules Are Built by "Auxin Resonance"

5.1 "Leaves in the Test Tube": Experimental Reduction of Plant Self-Organisation

Plants add, during their entire lifetime, new cells to the tip of roots and shoots. As shown for the root meristem by elegant laser ablation experiments (van den Berg et al. 1995), cell differentiation in the mitotically active meristems is controlled by signals from the neighbouring, already differentiated, cells. However, when the meristem becomes accessible to cell biological inspection, differentiation is already channelled. At this stage, it is very difficult, if not impossible, to manipulate the pattern in a fundamental manner. Thus, meristems are beautiful systems to study how patterns are perpetuated, but for the analysis of pattern induction, simpler systems are needed, where determination has not progressed that far. Several years ago, we have introduced cell lines derived from the ground tissue of tobacco shoots as experimental system to study the primordial stages of division patterning (Campanoni et al. 2003). These cell lines can be readily cultivated in suspensions maintained under continuous rotation. Plant suspension cell lines are generally considered as dedifferentiated and have even been termed "HeLa cells of plant biology" (Nagata et al. 1992). However, they often preserve certain features from their source tissue, such as the ability to generate the structured cell wall thickenings characteristic for vascular cells (Nick et al. 2000), the ability to generate, through a series of axial cell divisions, cell files with a clear axis and polarity, and

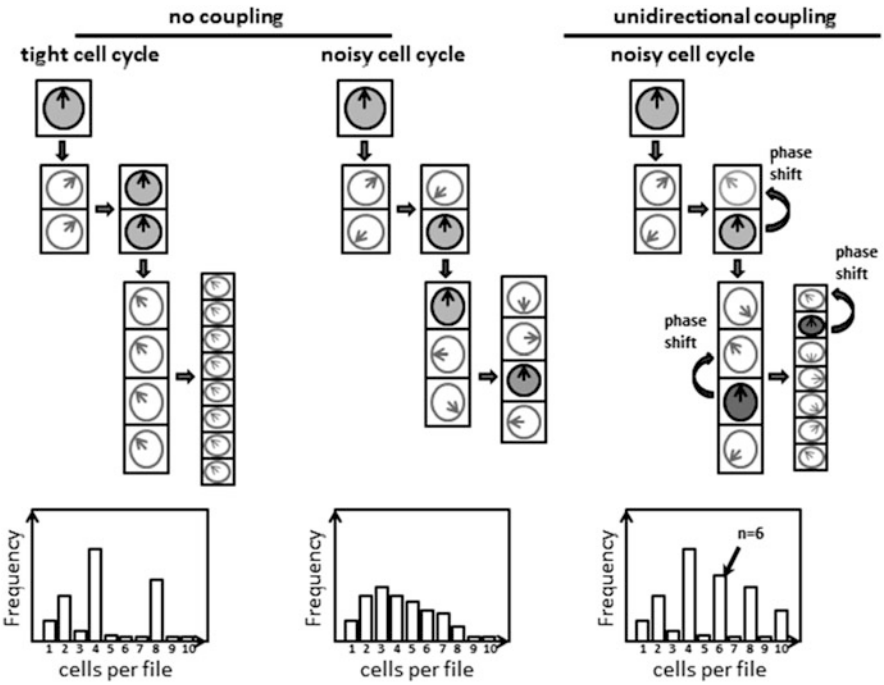

Fig. 14.3 Models for the synchrony of division patterns in cell lines derived from tobacco parenchyma. There are two concurrent possibilities—either individual cells act autonomously (no coupling of their cell cycles, *left*) or they show temporary, unidirectional coupling of their cell cycles (*right*). The schematic clocks represent the position of the cell cycle for the respective cell. A position at "high noon" stands for the onset of mitosis. The predicted frequency distributions of cell number per file are shown in the *lower panel*. Three principle cases are shown: (1) In the absence of coupling, but under tight control of cell cycle duration would result in a sequence, where frequency peaks are predicted for 2, 4, 8,. . ., 2^n cells per file (*left-hand column*). (2) In the absence of coupling and for a noisy cell cycle, there should be no clear frequency peaks, but odd- and even-numbered cell files should occur at the same frequency (*central column*). (3) In case of unidirectional coupling, a cell entering mitosis generates a signal that is conveyed to its down-stream neighbour. This signal causes a phase shift by accelerating the cell cycle of the receiver cell. For this unidirectional coupling, even for noisy cell cycles, a partial synchrony is predicted with frequency peaks at 2, 4, 6,. . ., 2*n* cells per file (*right-hand column*). This model is the only that predicts a frequency peak for six cells per file. This frequency peak (*arrow*) is diagnostic for unidirectional coupling (bidirectional coupling would lead to a pattern as observed for uncoupled cells under tight control of the cell cycle; see *left-hand panel*)

they have preserved responsiveness to the controlling signal, auxin. Since these files derive from singular cells, they cannot rely on positional information inherited from the mother tissue. Patterns of competence within a cell file must originate de novo during the culture cycle.

5.2 Weak Coupling of Autonomous Oscillators

During the work with these tobacco cell files, we observed that files consisting of even numbers of cells were dominating over files with uneven cell numbers (Campanoni et al. 2003; Maisch and Nick 2007). At first sight, frequency peaks of even-numbered files might occur, when the cell cycle proceeds with a precise timing (Fig. 14.3). This should generate files in a sequence of

$$f(n) = 1, 2, 4, 8, \ldots 2n$$

individual cells (with n representing the number of cell cycles). However, the length of individual cell cycles varies over a broad range, and there is, in addition to the expected peaks at $2n$, a curious frequency peak for files composed of six cells (in some cases accompanied by a smaller peak of ten cells). This observed feature could be simulated using a mathematical model derived from non-linear dynamics, where elementary oscillators (cycling cells) with a high level of noise (variation in the length of individual cell cycles) were weakly coupled, and where the number of these oscillators was not constant, but grows with time (Campanoni et al. 2003). In contrast to concurrent models, this weak coupling algorithm was able to predict the observed frequency peak of hexacellular files. Moreover, this model predicted several non-intuitive properties of the experimental system. A striking feature of the model was the prediction that coupling must be unidirectional, i.e. that the coordinating signal is transported in a polar fashion. The coupling is seen as a phase shift in the cell cycle, i.e. a dividing cell will cause its downstream neighbour to accelerate its cell cycle such that it will also initiate mitosis. Unidirectional signalling is a diagnostic feature of auxin transport. In fact, the predominance of even-numbered cell files could be eliminated by low concentrations of 1-*N*-naphthylphthalamic acid, a specific inhibitor of auxin exporters (and thus of directional auxin transport). Although the noise in this system was considerable, with high variation in the cycling period over the cell population, the division of adjacent cells was synchronised to such a degree that files with uneven cell numbers were rare compared to files with even numbers. Frequency distributions over the cell number per file thus exhibited oscillatory behaviour with characteristic peaks at even cell numbers (Fig. 14.3).

5.3 Sensitive Muscles: The Actin–Auxin Oscillator

Auxin efflux carriers are not static, but undergo dynamic cycling between intracellular compartments and the plasma membrane. Treatment with the fungal toxin Brefeldin A (BFA) traps the carriers in the intracellular compartments (Geldner et al. 2001). This trapping is suppressed by cytochalasin D, an inhibitor of actin assembly suggesting that actin is involved in the cycling of auxin efflux carriers. On

the other hand, the cargo of these carriers, auxin, controls the conformation of actin, whereby the massive bundles prevalent in the absence of auxin are rapidly detached into finer filaments after addition of auxin (for review, see Nick 2010). Auxin can stimulate its own transport by improving the polar localisation of the auxin efflux carriers at the cell poles (Paciorek et al. 2005), suggesting that these transporters are more efficiently moved along the finer actin filaments in response to auxin. This model was tested in rice seedlings expressing different levels of the actin-binding domain of mouse talin in fusion with the yellow fluorescent protein (Fig. 14.4). By feeding radioactively labelled auxin to the tip of the seedling, the amount of radioactivity recovered in an agar block at the seedling base (quantified by a scintillation counter) could be used as measure for the efficiency of auxin transport. Based on this experimental system, the debundling of actin filaments by exogenous auxin could be shown to precede the concomitant stimulation of transport efficiency (Nick et al. 2009). Upon overexpression of the talin marker, actin filaments were constitutively bundled accompanied by a reduced capacity to transport auxin. However, when exogenous auxin was added, these bundles relaxed into numerous fine strands of actin filaments followed by a promotion of auxin transport. These findings demonstrate that

1. Actin reorganisation into fine strands precedes the stimulation of auxin transport.
2. Fine strands of actin are necessary for efficient auxin transport.
3. Actin reorganisation into fine strands is sufficient to promote auxin transport.

Thus, manipulation of actin can be used as tool to manipulate auxin transport—at least in experimental systems, where polar auxin flux is elevated to an extent that the steady-state level of active transporters at the membrane becomes limiting. We therefore transferred this strategy to further dissect the role of auxin transport for division synchrony in the tobacco cell model. If actin is part of an auxin-driven feedback loop, it should be possible to manipulate auxin-dependent patterning through manipulation of actin. To test this prediction, we had to create a situation, where actin is excessively bundled. For this purpose we employed a transgenic approach, where we expressed the actin-binding domain of mouse talin in fusion with the yellow fluorescent protein. Mouse talin competes with endogenous actin depolymerisation factors for binding sites on actin such that the actin filaments are progressively trapped in a bundled configuration (Ketelaar et al. 2004). In fact, overexpression of the construct in tobacco cells produced constitutively bundled fluorescent actin filaments. As predicted, the synchrony of cell division was impaired in this line, but could be restored by addition of auxins along with a normal organisation of actin. A screen for actin-binding proteins mediating the effect of auxin upon actin organisation identified tobacco actin depolymerisation factor 2 (NtADF2) as central player. A cell line overexpressing this factor is impaired in division synchrony in a highly specific way—in this line, the frequency peak at $n = 6$ diagnostic of unidirectional weak coupling is absent, but can be rescued by addition of PIP2, a phospholipid specifically sequestering ADFs (Durst et al. 2013).

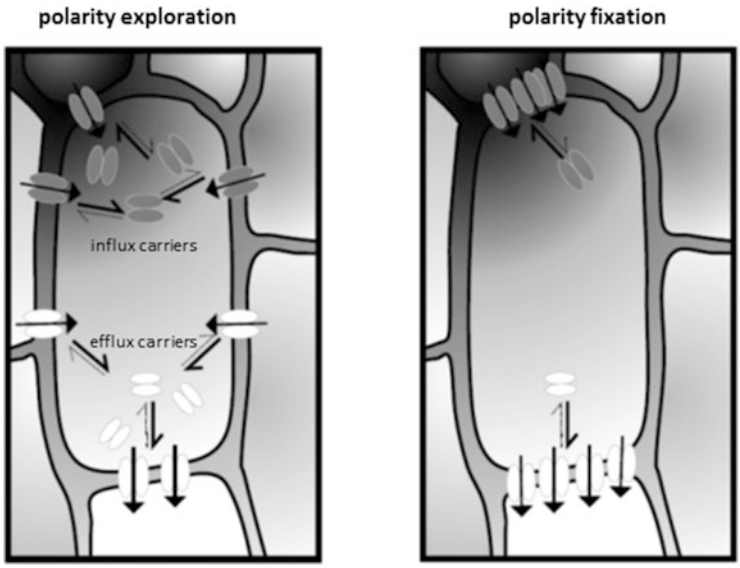

Fig. 14.4 Two-phase model for the exploration of space by auxin in plant self-organisation. A self-amplifying feedback between non-directional auxin influx through ion-trapping and gradient-dependent cycling of auxin efflux and influx carriers allows to integrate auxin concentration over the environment of a given cell and to generate a polar flux driving self-organisation

We therefore arrive at a model of a self-referring regulatory circuit between polar auxin transport and actin organisation, where auxin promotes its own transport by shaping actin filaments. This circuit seems to contribute to the self-amplification of auxin transport, a central element in current models of auxin-dependent patterning. The implications of this model are to be explored, but already at this stage it can be used to derive characteristic properties of basipetal auxin transport. For instance, the model predicts that the transport of IAA should oscillate. Auxin will induce fine actin strands that will partition auxin efflux carriers more efficiently to the plasma membrane, such that the intracellular auxin concentration will decrease. This decrease will cause bundling of actin filaments and, as a consequence, efflux carriers will be sequestered in intracellular compartments, culminating in a reduced efflux such that auxin received from the adjacent cells will accumulate and trigger a new cycle. The frequency of these oscillations should depend on the dynamics of actin reorganisation (around 20 min) and the speed of carrier cycling which is in the range of 5–10 min (as inferred from the comparison of auxin uptake in control versus BFA-treated cells; Paciorek et al. 2005). From these parameters, auxin transport is predicted to oscillate with a period of about 25–30 min. In fact, such oscillations with a period of 25 min had been observed during classical experiments on basipetal auxin transport in maize coleoptiles (Hertel and Flory 1968).

5.4 New Approaches to Morphogenesis: Chemical Engineering

The polarity of ground tissue cells provides the cellular base for the alignment of telomes as architectural modules. A self-referring, oscillatory circuit involving actin remodelling, rapid cycling of auxin efflux carriers, non-directional auxin influx, and directional auxin efflux has been identified as core element of this cell polarity. To dissect this circuit, it is not sufficient to identify molecular players such as ADF2 (Durst et al. 2013), but it is necessary to generate and manipulate the spatial patterns of molecules at subcellular resolution. Although genetic engineering allows to target transgenes to specific compartments using localisation motives, the spatial resolution of this strategy is too coarse-grained. New strategies are warranted to increase spatial resolution. To achieve this goal we used chemical engineering based on caged auxin that can be released by localised irradiation in single cells or even parts of a cell (Kusaka et al. 2009). Caged compounds conventionally use 2-nitrobenzyl-esters as caging group. However, the ester bond was found to be enzymatically hydrolysed in plant cells such that auxin was released prior to photolysis producing high unspecific background activities. By molecular modelling of the active centres of these enzymes, an esterase-resistant caging group, (2,5-dimethoxyphenyl)(2-nitrobenzyl) ester, could be designed and employed successfully. We administered this tool to the actin–auxin oscillator to demonstrate in a proof-of-principle experiment that a biological response can be controlled by light at cellular resolution. By using an auxin-inducible promoter (DR5) driving a GFP reporter, we were able to confirm that auxin was released only in the irradiated cell. Subsequently, we used the cell line overexpressing talin in fusion with the yellow fluorescent protein. In this cell line, actin is constitutively bundled, but can be rescued by addition of exogenous auxin (Maisch and Nick 2007). By feeding caged auxin to this cell line and irradiating individual cells of a file, we could trigger a specific reorganisation of actin filaments that was confined to the irradiated cell (Kusaka et al. 2009). Thus, chemical engineering using light-switchable triggers can now be exploited to steer auxin gradients during self-organisation of the tobacco cell model. At present, we are completing a study, where auxin is released in different cells of a file during specific stages of the culture cycle accompanied by specific changes in division patterns. Recent experiments using protoplasts from fluorescently tagged actin marker lines could demonstrate that even intracellular auxin gradients can be produced that will then be transduced into intracellular gradients of actin organisation (Liu et al. 2013).

6 The Influx Issue: Auxin as Exploratory Molecule

The generation of spatial patterns by coordinative signals requires that space can be explored in different directions. This seems to contrast with the pronounced polarity of auxin transport. This directionality has been classically explained by polar efflux of auxin (Rubery and Sheldrake 1974). The exploratory part of coordination would be the non-directional influx of indolyl-acetic acid through the plasma membrane maintained by a chemical gradient, where IAA is stripped from its proton in the more or less neutral cytoplasm. The molecular identification of auxin influx carriers (Bennett et al. 1996) that are localised in a polar fashion opposite to the PIN efflux carriers (Swarup et al. 2001) has shifted focus a bit. These findings led to a model, where not only efflux but also directional influx contributes to the polarity of auxin flow.

A molecule that is easily transported through the acidic environment of the apoplast, but that is readily trapped in the cytoplasm and then has to be actively exported is ideally suited to convey lateral inhibition between neighbouring cells. When the localisation of the efflux transporter is placed under the control of auxin itself (Paciorek et al. 2005), this will establish a perfect reaction–diffusion system in sensu Turing (1952). This system is able to establish a clear cell polarity from even minute and noisy directional cues. However, when auxin *influx were exclusively* directional, due to the polar localisation of the influx carrier AUX1, exploration of space as prerequisite of coordinative signalling for pattern formation, would not work.

This apparent dilemma might be less dramatic as it seems at first sight. The impact of carrier-based auxin influx depends strongly on apoplastic pH: since the pK_s value for indole-acetic acid is 4.75, the proportion of the anionic form that definitely requires a carrier to enter the cell is relatively high for pH 5 (74 % IAA^-); for a pH of 5.5 even 95 % of auxin are present in the anionic form (Swarup and Péret 2012). However, is this the relevant pH of the cell wall? To determine the pH of plant cell walls is far from trivial, due to ion exchange at the carbon hydrate matrix. Most measurements of cell wall pH systematically underestimate the acidity of the chemical environment for the apoplastic auxin. Reliable measurements can only be achieved by using a pH-stat approach, because here the metric component is buffered. Using this strategy, the physiological pH of the cell wall has been found to range between 4.0 and 4.5 (Lüthen et al. 1990), i.e. in a range, where the uncharged form of auxin predominates. Thus, "exploratory" ion trapping is a substantial component of auxin influx.

Alternatively, exploration of space might be achieved by the cycling of the efflux carrier as well (see Chap. 8). When the auxin effect on the cycling of PIN proteins (Paciorek et al. 2005) is not homogenous over the auxin-stimulated cell, but depends on the local auxin concentration at the respective flank of the cell, this would provide a mechanism, by which a cell can "explore" gradients of auxin across a tissue. This mechanism has been proposed for phyllotaxis (Jönsson et al. 2006) and has been integrated into models for auxin channelling that are

congruent with predictions from the classical auxin canalisation model (Roeland et al. 2007).

A polar transport of auxin can be detected already in several lines of multicellular algae (see Chap. 13; Dibb-Fuller and Morris 1992; Cooke et al. 2002) including *Chara* as close relative of the land plant ancestor (Boot et al. 2012; for review see Raven 2013). In phaeophycean algae, polar auxin transport has been recruited for the establishment of polarity (Basu et al. 2002). Transcellular auxin gradients are necessary for polarity, because when these gradients are overrun by exogenous IAA symmetry break is suppressed in *Fucus* (Whitaker 1942). This indicates that the central role of auxin in cell communication developed from evolutionarily quite ancient preadaptations already present prior to the transition to a terrestrial lifestyle. However, in order to integrate plant architecture, the directional output must be integrated with input that is non-directional. The cell must explore its neighbourhood in different directions, which is possible through the ion-trap mechanism of auxin influx. This does not exclude that the resulting cellular polarity will subsequently reinforce the main route of influx by partitioning auxin influx carriers of the AUX1/LAX family to the sites, where ion trapping was most active. In fact, both mechanisms of auxin influx might act in concert (Fig. 14.4): the ion-trap mechanism would be used in a phase of polarity exploration, whereas repartitioning of influx carriers (along with repartitioning of efflux carriers) would provide a fixation of the initial, still flexible, polarity.

Patterning of a tissue is a complex phenomenon, and at the time that tissues become amenable to experimental manipulation, cell polarity is already laid down. This means that in tissues it is possible to investigate pattern perpetuation. How a pattern is laid down requires experimental systems, where cell polarity is still on the move.

7 It Is All Geometry: The *Tabula Rasa* Approach

Polarity induction de novo has been classically studied in the brown alga *Fucus* (Goodner and Quatrano 1993; Hable and Hart 2010). The spherical zygote undergoes asymmetric division yielding progenitor cells for thallus and rhizoid. The orientation of this division can be aligned by unilateral blue light inducing a calcium influx at the shaded flank, where later the rhizoid will emerge (Jaffe 1966). A cap of dynamic actin filaments is formed at this site and attracts vesicles transporting cell wall material driving the outgrowth of a rhizoid. The polarity seen in response to blue light is produced by reorientation of a preformed polarity, but truly generated de novo, demonstrated by induction with strong plane-polarised blue light producing a high fraction of birhizoidal twins. This beautiful system has enabled a wealth of phenomenological, physiological, and cell biological insights into polarity induction, but it suffers from limited molecular accessibility.

Comparable systems, where spherical cells undergo formative divisions, are rare in higher plants. The closest version, developing microspores, are quite different, in

that they harbour a distinct preformed polarity that becomes manifest as nuclear movements as well as asymmetric cell fate of the daughter cells: the generative daughter will inherit immortality, whereas the vegetative cell is doomed to death at fertilisation, giving a neat illustration of Weismann's germ line/soma concept. By colchicine or other antimicrotubular drugs, this developmental asymmetry can be eliminated (Twell et al. 1998).

To obtain symmetric, apolar cells in higher plants is possible, however, when the cell is stripped off its wall by cellulase. These protoplasts correspond to a tabula rasa situation and lack any axis and polarity, but retain the ability to regenerate complete plants as shown in spectacular experiments on tobacco (Nagata and Takebe 1970). Thus, protoplasts resemble the zygotes of *Fucus* with respect to de novo generation of polarity. The observation that regenerating protoplasts of the moss *Physcomitrella patens* show a redistribution of calcium channels (visualised by a fluorescent channel antagonist (Bhatla et al. 2002) indicates that the underlying mechanisms might be similar.

We therefore used protoplasts of tobacco BY-2 cells to study how polarity and axis are induced de novo (Zaban et al. 2013). The presence of fluorescently tagged transgenic marker lines allowed us to follow the behaviour and role of the cytoskeleton during this phenomenon. The system could be standardised to such a degree that the temporal pattern of regenerative stages could be investigated on the quantitative level such that functional analysis became possible. Using anti-cytoskeletal compounds and inducible expression of actin-bundling proteins it could be shown that a dynamic population of actin was necessary for polarity. When actin dynamics were suppressed, curious tripolar cells ensued in analogy to the twinned embryos observed in *Fucus* for induction by strong polarised light.

In the meantime, we succeeded to integrate this tabula rasa system into a microfluidics platform, which allows us to provide gradients of auxin through controlling the flux through the system and preformed geometries through rectangular microvessels (Sun et al. 2009). Using this system we currently investigate how the regenerating protoplast induces polarity after having explored the geometry of its environment.

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