Tansley review

The control of leaf development

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Contents

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Summary

The formation of a leaf is a basic aspect of plant development. This review provides an overview of our present understanding of the process from initiation to the final form of the leaf. Molecular genetic and cell biology approaches have yielded significant advances in this area, adding not only to our knowledge of leaf development but also to fundamental principles in plant biology. These principles will be highlighted, as well as areas where our understanding is still incomplete, in particular the problem of coordinating the multifaceted steps involved in the generation of the leaf structure.

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I. Introduction

Leaves constitute the basic organ of photosynthesis. They are central to the life strategy of the plant and are the eventual source of most food on the planet. An understanding of the processes underlying leaf development thus provides an insight into a basic process in biology, modulation of which may have far-reaching significance on strategies to improve crop performance.

This review will focus on our knowledge of the molecular processes controlling leaf development, highlight aspects where our understanding is limited and, in the spirit of the Tansley review series, attempt to integrate at least some of this knowledge into a working model of leaf development.

The article will follow the developmental progression of leaf formation, focussing on inception and the earliest stages of development, because it is during this window of opportunity that the developmental potential of the leaf is set.

II. The shoot apical meristem: the creation of a field of cells from which leaves can be formed

The formation of a leaf requires a field of cells from which a leaf can form. This original, naïve tissue is generated in a specialized organ, the shoot apical meristem (SAM). The SAM is characterized by a population of cells which maintain a relatively high rate of cell proliferation (Veit, 2004). Some of the daughter cells generated remain within the SAM to produce yet more cells (i.e. function as stem cells or initials), whereas some of the daughter cells on the flank and base of the SAM become incorporated into leaf and stem tissue, respectively. This balance of cell production in the SAM and the rate of cell loss via incorporation of cells into leaves and stem dictates the size of the SAM. Any image of the SAM must therefore be viewed as a snapshot in developmental time, reflecting a reality in which cells generally progress from the core of the SAM towards the edge, dividing as they go to generate a field of cells in which morphogenesis and differentiation can occur. Some recent research has focussed on this dynamic aspect of the SAM using novel methods of imaging and data analysis, the aim being to generate three-dimensional models of cell proliferation and displacement in the SAM against which real-time activity of proteins involved in regulating meristem function can be visualized (Grandjean *et al.*, 2004; Reddy *et al.*, 2004). The data generated by these studies demonstrate the dynamic properties of the SAM and the technical challenges involved. Further progress in this area can be expected and is required if we are to gain a full insight into how the SAM generates and maintains a population of cells from which the rest of the aerial portion of the plant is produced. Nevertheless, our essentially 'static' understanding of SAM function has progressed immensely over the last few years and had led to the following paradigm.

Essentially, the growth of the SAM is controlled by the opposing functions of two gene pathways: a positively acting pathway which promotes meristem growth (based on the homeodomain transcription factor WUSCHEL (WUS)) and a negatively acting pathway which suppresses meristem growth (based on a series of CLAVATA gene products) (Clark *et al.*, 1997; Brand *et al.*, 2000; Schoof *et al.*, 2000). The CLAVATA genes encode a small secreted ligand (CLV3) and two receptor-like proteins (CLV1 and CLV2). The two pathways interact so that the WUS-based pathway promotes activity of the negatively acting pathway, and the negatively acting CLV pathway suppresses activity of the positively acting WUS pathway (Fig. 1). Thus, any tendency for increased SAM growth via increased WUS activity leads automatically to suppression of WUS activity via the CLV loop. Moreover,

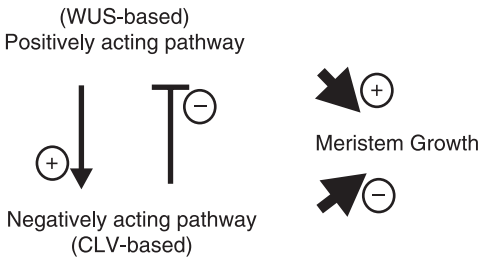

Fig. 1 The interaction of positively and negatively acting pathways controls meristem growth. The WUS-based pathway promotes meristem growth and, at the same time, promotes the activity of a negatively acting CLV-based pathway. The CLV-based pathway acts to suppress meristem growth via inhibition of the positively acting WUS-based pathway and, possibly, via a direct influence on meristem growth.

analysis of gene expression patterns shows that (at least in *Arabidopsis*) the WUS and CLV pathways occupy overlapping yet spatially separated regions of the SAM, indicating that local signalling must occur to coordinate the two pathways. The nature of this signalling is still to be fully elucidated.

The WUS/CLV paradigm provides a powerful insight into the mechanism by which the SAM generates a field of cells. However, a number of questions remain as to how the system actually works. Some of these address the mechanism of interaction between WUS and CLV. For example, does the WUS protein actually move within the SAM? (Indeed, based on recent observations suggesting that movement of proteins within the symplasm of the SAM is the default process (Wu *et al.*, 2003), one can pose the question differently: if WUS does not move within the SAM, why not?) What are the intermediaries by which WUS effects CLV3 gene expression and, similarly, what are the steps by which CLV3 feeds back on to WUS gene expression? Other open questions relate to the extent and polarity of signal movement. For example, does WUS signalling act only on cells distal to the region of WUS expressing cells? If so, why? What restricts the distance and polarity of WUS and CLV signalling? How is the system set up initially in the developing embryo? Finally, although demonstrated in *Arabidopsis*, is the WUS/CLV paradigm true for other plants, such as monocots? Answers to these questions are keenly awaited and can be expected to be forthcoming in the near future.

III. Patterning: the selection of leaf initiation site

As stated above, initiation of a leaf requires that a field of cells exists from which a leaf can be formed. In that respect, the mechanism by which the SAM is maintained is essential to leaf formation. However, there is little evidence that the process of cell proliferation in the SAM in any way determines the fate of those cells, although there has been some discussion on this point (Fleming, 2002; Klar, 2002). The SAM can thus be viewed as a cell-generating machine which produces a field of tissue in which the mechanism(s) of morphogenesis and

differentiation can act. However, not all cells in the SAM are equally competent for leaf initiation. In particular, although leaves can be generated at essentially any position around the circumference of the SAM, organogenesis does not occur on the tip of the SAM. This implies a mechanism actively precluding leaf formation at this position. Hypotheses to account for this include the flow of an inhibitory chemical from the apex of the SAM which prevents leaf initiation within its boundary, as well as more biophysical interpretations of morphogenesis which imply that the physical stresses converging at the apex of a growing dome or disc would prevent outgrowth occurring (Green, 1992).

Irrespective of the mechanism, the SAM produces a band of cells in which leaf initiation can occur. How are the sites of initiation selected? Classical studies led to the observation that the site of new leaf initiation was intimately linked with the positions at which previous leaves had been formed on the meristem (Snow & Snow, 1962). Coupled with data from experiments in which meristems and leaf primordia were physically manipulated and the outcome on leaf formation observed, these experiments led to the concept that new leaf primordia arose where there was 'available space' on the meristem, this 'available space' probably being dictated by the flux of inhibitory morphogens from recently formed leaves (reviewed in Steeves & Sussex, 1989). Thus, it was predicted that a new leaf arose at the site of a minimum of inhibitory morphogen which would, automatically, be the site most distant from the most recently formed leaves. Although the nature of the morphogen was unknown, the growth regulator auxin was implicated (Snow & Snow, 1937; Meicenheimer, 1981). Recent data indicate that, indeed, auxin plays a key role in the specification of site of leaf initiation and, moreover, these experiments have revealed the mechanism by which auxin flux in the meristem is controlled.

The key observations of this work were that, firstly, localized ectopic application of auxin on the surface of the meristem was sufficient to trigger the formation of leaf primordia (Reinhardt *et al.*, 2000). Secondly, localization of a family of proteins implicated in auxin transport (PIN proteins) revealed that they were expressed in the meristem in a pattern consistent with auxin flux being directed to the site of incipient leaf formation (Reinhardt *et al.*, 2003). Indeed, the pattern of PIN protein distribution suggests that auxin flux is primary restricted to the outer cell layers of the meristem and surrounding tissue, with the flux being directed from the surrounding tissue towards the meristem (Fig. 2a). In addition, newly formed leaves display a pattern of PIN protein expression indicating that such leaves act as a sink for auxin, i.e. auxin reaching the primordia along the epidermal layers is directed inwards at the presumptive leaf tip (Benkova *et al.*, 2004) (Fig. 2b,c). Thus, the presence of a leaf primordium depletes the auxin from the neighbouring region of the SAM. The integration of auxin sinks (new leaf primordia) around the circumference of the SAM leads by default to a maximum remaining auxin flux at the site furthest away from recently formed primordia (Fig. 2d). A site of maximum auxin flux initiates leaf formation which then acts as a new auxin sink, leading to a new pattern of auxin depletion.

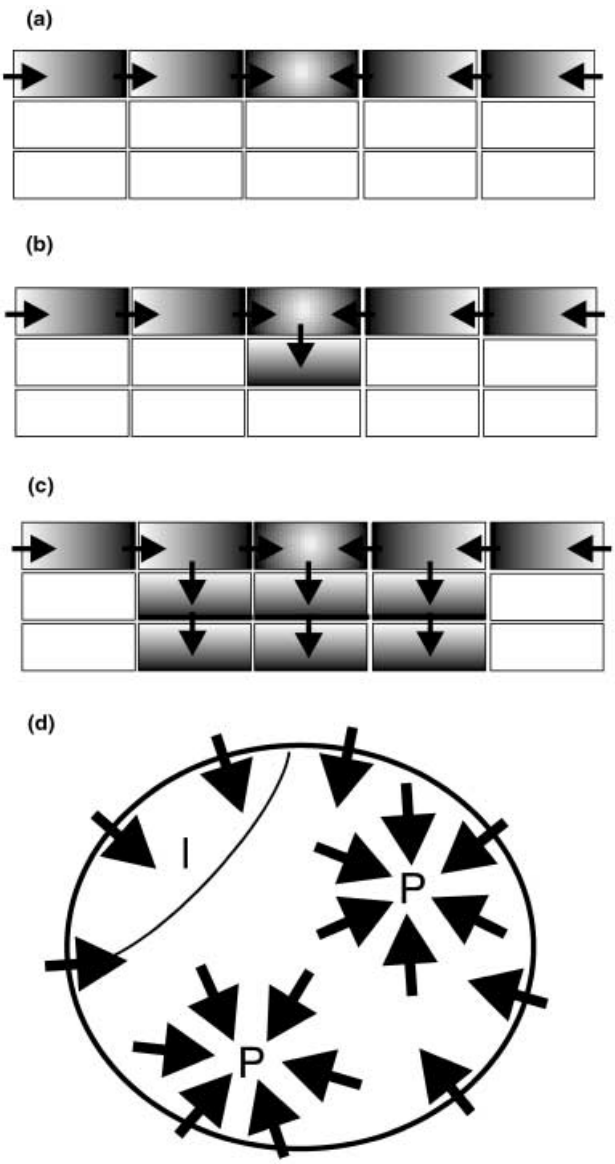

Fig. 2 Auxin flux dictates the site of leaf initiation. (a) Auxin flux in the SAM is restricted to the outer cell layer (grey cells). The direction of flux (arrows) is controlled by the asymmetric localization of a PIN protein (black band). (b) The PIN protein localization directs auxin flux towards the site of leaf initiation which is characterized by an inward flux of auxin. (c) The inward flux of auxin at this site becomes stabilized and amplified, leading to a local maximun of auxin at this position. (d) Sites of primordia formation (P) on the surface of a SAM are characterized by inward flux of auxin (arrows). The depletion pattern of auxin across the surface of the SAM, coupled with continued flux of auxin into the SAM from subtending tissue, leads to a position of auxin maximum (I) at a site most distant from the previous sites of leaf formation. This site will itself become a site of inward auxin flux, thus depleting auxin from that area of the SAM, thereby initiating a new pattern of auxin depletion.

This model neatly incorporates the observed data and provides convincing evidence that auxin is the key endogenous mobile signal involved in determining the site of leaf initiation. A number of questions, however, still need to be investigated. For example, if PIN protein distribution directs auxin flux to determine leaf initiation, and if leaf initiation sets up a new pattern of auxin flux, how does leaf initiation direct PIN pattern within the SAM? PIN protein distribution has been shown to be highly dynamic, so redistribution of PIN protein within a cell does not seem to be an issue (Geldner *et al.*, 2001). Rather, what is the relationship of PIN protein distribution to auxin? In other words, does auxin itself direct PIN protein localization and, if so, how? A further complication is provided by the possibility that PIN proteins may not actually transport auxin, rather that they are auxin transport-associated factors (Friml *et al.*, 2003). This implies that PIN proteins may act as part of a complex. What is the nature of the other component(s) of this complex?

One question arising from any model of leaf patterning is how the model accounts for or incorporates the observation that different plants may show different patterns of leaf initiation. The model described above was derived from experiments on plans showing spiral phyllotaxis (the most common leaf pattern). Recent data have provided some insight into the mechanism by which these patterns can be altered.

Maize plants show an alternate pattern of leaf initiation, that is the SAM generates a single leaf at each node and successive leaves are diametrically opposed. In the ABPHYLL mutant, plants generate two leaves at each node, with the leaves again being diametrically opposed (distichous pattern; Jackson & Hake, 1999). The ABPHYL1 gene has recently been identified and shown to encode a protein implicated in cytokinin signalling (Giulini *et al.*, 2004). At the same time, previous work has shown that the SAM of the ABPHYLL mutant plants is significantly larger than wild-type (Jackson & Hake, 1999). These data can be interpreted by a model in which the size of the SAM is influenced by the growth regulator cytokinin and in which the size of the SAM determines the dynamics of auxin flux, and hence determines the sites of auxin minimum generated by the positioning of leaf primordia. Indeed, simple modelling of leaf initiation suggests that any factor which affects SAM size, relative primordia size (auxin sink strength) and flux of auxin through the SAM will potentially influence the pattern of leaf initiation (Fig. 2d). However, as the field of competent meristem cells progressively increases in size, one might expect a switch from an alternate to a spiral pattern of leaf formation, yet a direct switch to a distichous phyllotaxis is observed in maize. This pinpoints the question: what is the mechanism by which particular patterns are favoured in particular plants? Is there a restriction on PIN patterning within the meristem, or a restriction of the area in which leaf initiation can occur (due to, for example, specific elements of auxin metabolism or signalling)? Finally, how is the system initiated? Does it depend on amplification of initial random fluctuations within the embryonic SAM in, e.g. auxin flux? If so, how does such amplification occur?

IV. Morphogenesis: the initial stages of leaf formation

The patterning mechanism discussed above leads to the determination of particular groups of cells in the SAM to form a leaf. What is the mechanism by which this pattern is transduced into actual change of form, i.e. the outgrowth of tissue to form a primordium? Molecular genetic analysis has revealed that leaf formation is accompanied by a panoply of changes in expression pattern of a number of transcriptional regulators (Veit, 2004). However, how these changes in pattern of transcriptional regulator are linked to the auxin patterning mechanism described above is unclear. Moreover, although it is clear that appropriate patterning of transcriptional regulators is required for appropriate leaf form and differentiation, it is as yet unproven that these regulators are absolutely required for leaf initiation.

A paradigm of leaf initiation is that the expression of homeodomain transcriptional regulators of the *Knotted1* class (*KNOX* genes) is down-regulated before and during leaf initiation (Jackson *et al.*, 1994). Moreover, misexpression of *KNOX* genes is associated with altered leaf morphogenesis, ranging from change in leaf shape to increased leaf complexity and even the formation of ectopic SAMs (Sinha *et al.*, 1993; Chuck *et al.*, 1996). However, the evidence that appropriate *KNOX* gene expression is absolutely required for leaf initiation is not incontrovertible. For example, in the above examples ectopic expression of *KNOX* genes leads to altered leaf morphogenesis but does not apparently affect the initiation process. Abrogation of *KNOX* gene expression can lead to various phenotypes (depending on specific gene and genetic background), but the initial phenotypes are not immediately linked to leaf initiation (Long *et al.*, 1996; Vollbrecht *et al.*, 2000). A complication in the interpretation of some of these data is that *KNOX* gene expression may be subject to a level of post-transcriptional regulation. Thus, although overexpression of a *KNOX* gene in *Arabidopsis* using the 35S promoter clearly led to ectopic expression in leaf tissue, the pattern of transcripts in the SAM appeared unchanged (Chuck *et al.*, 1996). On the other hand, indirect manipulation of *KNOX* gene expression in the SAM (resulting from modification of cell division pattern in the SAM) did not result in any overt alteration in leaf initiation (Wyrzykowska & Fleming, 2003), suggesting that the specific pattern of *KNOX* gene expression normally observed in the SAM is not required for leaf initiation.

A yet further complication to deciphering the importance of *KNOX* gene products in leaf initiation is the observation that KNOX proteins and RNA have the capacity to move within the SAM (Kim *et al.*, 2002) and even over long distances within the plant (Kim *et al.*, 2001). Because most of the analyses described above have focussed on *KNOX* RNA accumulation, it is difficult to precisely determine where the

protein accumulates. Even then, the question arises of whether the transcription factor is in the nucleus or cytoplasm, i.e. whether it is in the appropriate compartment for transcriptional activity. Efforts have been made to follow the movement of KNOX proteins within the SAM (Kim *et al.*, 2002), but the technical limitations of following a dynamic process in a dynamic organ in real time are formidable.

If the overall conclusion is that *KNOX* gene products are not causally involved in leaf initiation, this raises the question of the function of this developmentally important family of proteins. A strong argument can be made that they are involved in determining the fate of cells incorporated into leaves. This will be discussed later in this article.

As described above, auxin is intimately involved in leaf initiation. What, then, are the targets of auxin signalling in the SAM which lead to the initial outgrowth of a leaf promordium? An intuitive expectation is that auxin would lead to a local promotion of cell proliferation in the SAM and that this burst of cell division would be causally involved in the formation of a new leaf primordium. However, several lines of data indicate that this is not the case. For example, experiments in which cell proliferation has been promoted or repressed throughout the plant have led to various phenotypes, but leaf initiation per se does not seem to be affected (e.g. Cockcroft *et al.*, 2000; De Veylder *et al.*, 2002). It is possible to argue that in these experiments endogenous gradients of cell proliferation were maintained within the SAM and that these were sufficient to maintain a normal process of leaf initiation. However, a more direct approach in which cell proliferation was specifically promoted in a portion of the SAM failed to interfere with leaf initiation, indicating again that cell division rate within the SAM is not directly linked with leaf formation (Wyrzykowska *et al.*, 2002). Furthermore, experiments in which the orientation of cell division within the SAM was disrupted also did not disrupt leaf formation, indicating that the observed conserved pattern of cell division within the SAM is not required for organogenesis (Wyrzykowska & Fleming, 2003). These data argue against cell division being a primary target for auxin during leaf initiation. This then leads on to the question, if cell division is not the initial causal agent in leaf initiation, what is? A body of data supports the idea that the cell wall is the key to the problem.

Firstly, theoretical and experimental data indicate that the biophysical balance between internal pressure (acting to increase cell volume) and tensile forces within the cell wall (acting to counteract such pressure) are the key element in plant cell growth (Cosgrove, 2000). Provided that sufficient metabolic energy and raw materials are available to generate new cytosol, membrane and cell wall, and provided that sufficient turgor pressure is available to drive expansion, then the only factor restraining growth is the cell wall. The evidence suggests that cells in the meristem have abundant metabolic resources and turgor pressure. Therefore, it seems reasonable to suggest that the walls of SAM cells (and, in particular, of cells along the outer layers (tunica)) are under tensile stress, which is the only factor restraining growth. If this tension is decreased, then increased growth will occur until a new biophysical equilibrium is established. Moreover, because of the biophysical parameters of the SAM restricting tangential expansion, this new growth will have a vector essentially perpendicular to the plane of the surface of the SAM, and change in growth vector is central to the process of leaf initiation (Green, 1992). Are there any data to support this concept?

Experiments in which a cell wall protein, termed expansin, was locally applied to the surface of the SAM provided an initial insight (Fleming *et al.*, 1997). Expansins were identified by biochemical analysis of cucumber hypocotyls as proteins which could induce extension of plant tissue in an *in vitro* assay system (McQueen-Mason *et al.*, 1992) and, although the mechanism of action of expansins remains obscure, they can be used as a tool to loosen the cell wall. Thus, in the experiments described above, local application of expansin on the SAM led to morphogenesis, presumably via transiently altering this endogenous biophysical equilibrium. Other experiments confirmed this (Pien *et al.*, 2001) and showed that specific expansin genes are expressed within the SAM at the site of presumptive leaf initiation and that at least some expansin genes are responsive to auxin at the transcriptional level (Cho & Kende, 1998; Reinhardt *et al.*, 1998). Thus, expansins have the appropriate activity and are present at the right time and place to play a causal role in leaf initiation as a downstream target of auxin (Fig. 3). However, definitive data showing that down-regulation of expansin gene expression blocks leaf initiation are lacking.

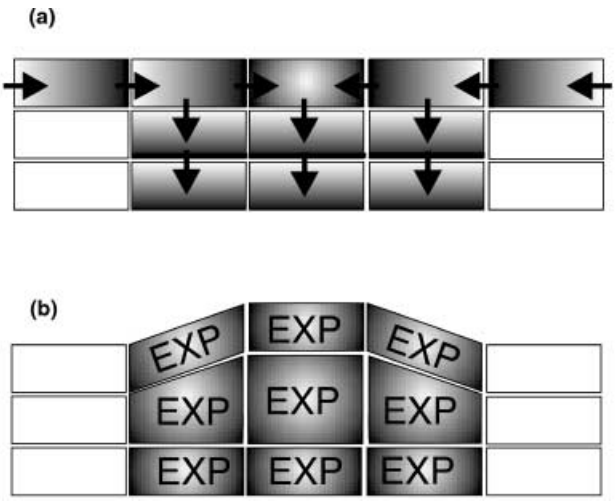

Fig. 3 Site of auxin maximum initiates morphogenesis. (a) The auxin patterning process leads to a local area of auxin accumulation. (b) Auxin can induce expansin (EXP) gene expression which promotes cell growth. Due to the biophysical pattern of stress and tension in the SAM, local increased growth is liable to occur in a radial direction, leading to tissue bulging out of the SAM.

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As will be discussed further in the next section, localized loss of *KNOX* gene transcript accumulation in the SAM leads to a number of downstream affects. One of these is the expression of an enzyme involved in gibberellin (GA) biosynthesis (Sakamoto *et al.*, 2001). Although ectopic application of GA to SAMs does not interfere with leaf initiation (Reinhardt *et al.*, 2000), it is clear that GA can influence leaf morphogenesis (Hay *et al.*, 2002). One possibility is that an increase in GA levels early in leaf initiation could serve to fix or amplify the switch in growth vector initiated via local change in cell wall extensibility, allowing the rapid polarized expansion of the leaf primordium.

A final question relating to *KNOX* gene expression in the SAM is whether auxin flux (which is directed towards the site of presumptive leaf initiation) is causally involved in the observed decrease in *KNOX* transcript accumulation at this site. No data have yet been published, but it would seem a natural linkage.

V. Differentiation and determination: transcriptional networks controlling determinancy

In plants with simple leaves (e.g. *Arabidopsis*, maize), *PHANTASTICA*-like genes (also termed AS1/Rough Sheath2/Phantastica (ARP) family of MYB transcription factors) are expressed at very early stages of leaf formation, mirroring the observed decrease in *KNOX* gene transcripts (Hay *et al.*, 2004). Indeed, PHANTASTICA-like proteins act to repress the expression of some *KNOX* genes and if the expression of the *PHANTASTICA*-like genes is abrogated, *KNOX* gene expression is apparent in the formed leaf (Ori *et al.*, 2000). As outlined above, ectopic *KNOX* gene expression in the leaf has a significant outcome for organ development. At the same time, other *KNOX* genes (*STM*-like) act to repress *PHANTASTICA*-like gene expression in the SAM, indicating a hierarchy of transcriptional switching in the SAM (Byrne *et al.*, 2001). This functions to, firstly, maintain cells within the SAM in an indeterminate state but, secondly, permits the controlled switching of groups of cells to a determinate, non-meristem fate (Fig. 4). It should be noted that this non-meristem state does not imply meristematic proliferation, i.e. although cells in the SAM proliferate, cells outside the SAM also continue to divide for some time. Moreover, although this review is focussed on leaf formation, most cells derived from the SAM become incorporated into the subtending stem, and this switching from SAM to stem is also associated with altered expression patterns of transcription factors such as those encoded by *KNOX* genes. The patterns of differentiation associated with stem tissue are intrinsically not too dissimilar to those observed in leaves, so care must be taken in assigning leaf specificity to changes of gene expression which are really associated with the switch from meristem to non-meristem state. Again, the defining element of the leaf is the change in growth vector associated with its morphogenesis rather than any acquisition of specific cell type.

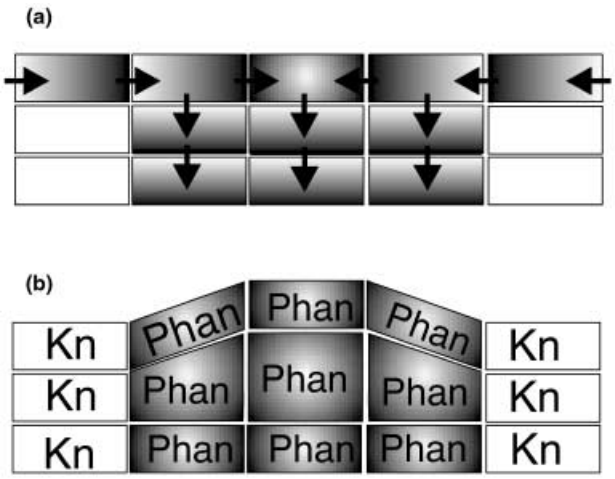

Fig. 4 KNOX (Kn) and PHANTASTICA (Phan) gene expression patterns presage the switching from indeterminate to determinate cell fate. (a) The auxin patterning process leads to a local area of auxin accumulation. (b) The local site of auxin accumulation corresponds to the area in which *KNOX* gene expression is lost. This leads to derepression of *PHANTASTICA*-like gene expression which is required for repression of *KNOX* gene expression in the young leaf primordium. Loss of *KNOX* gene expression and gain of *PHANTASTICA*-like gene expression leads to determinancy of cell fate.

It should also be noted that the transition from *KNOX* expressing to *KNOX* nonexpressing tissue at the SAM base is not as sharp as the transition shown by other molecular markers. Most notably, markers for photosynthesis (such as chlorophyll and RBCS) are totally absent from the SAM but are apparent within a few cell diameters in the subtending tissue (Fleming *et al.*, 1996). Because exposure of the SAM to light does not lead to the SAM cells expressing these markers, there is a developmental control excluding or preventing SAM cells undergoing this pathway of differentiation. The nature of this control is essentially unknown, yet it represents one of the most basic questions in cell biology, i.e. what controls the switch of a meristem cell to a particular differentiated pathway? Transcriptional regulators, such as the *KNOX* gene products, clearly play a role in setting the window within which such events can occur, but the actual molecular mechanism by which the switch occurs remains unknown.

VI. The elaboration of leaf form

In a typical angiosperm, a newly formed primordium undergoes lateral growth (i.e. becomes flatter) and growth along the proximal–distal axis (i.e. becomes longer). It has become apparent that the phase of lateral growth is dependent on the generation of a gradient within the primordium which differentiates adaxial and abaxial tissue. In essence, after primordium initiation, the future adaxial tissue becomes defined by a set of specific transcription factor activities, as does the abaxial tissue, and it is the juxtaposition of these two

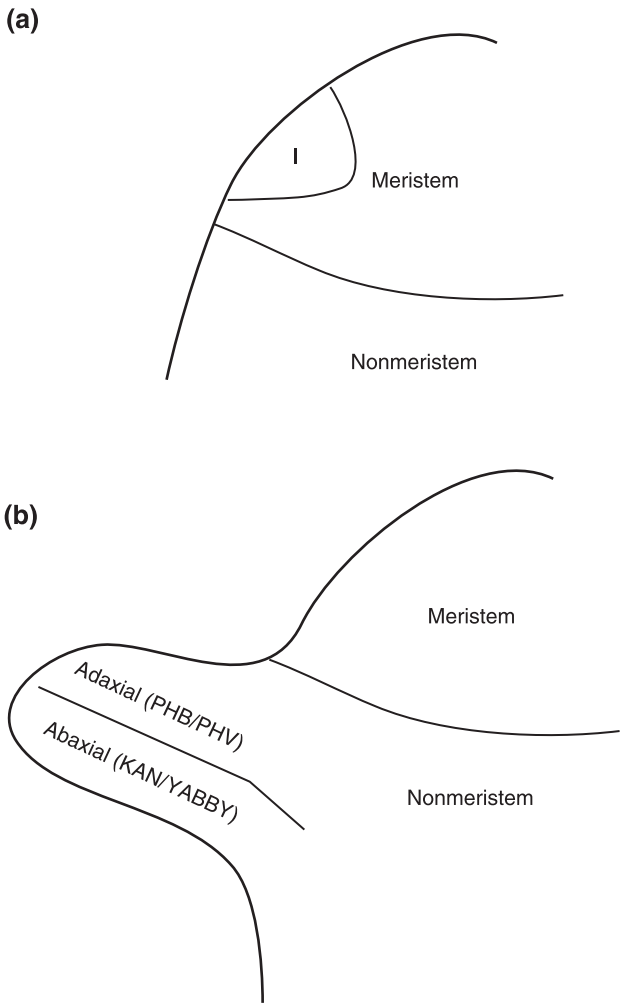

Fig. 5 Acquisition of polarity in the leaf. (a) The concerted action of patterning, effector and transcription factor activities in the incipient leaf (I) leads to the formation of a new organ. (b) This organ has nonmeristem identity and possesses a specific form. The acquisition of adaxial and abaxial fate in adjacent tissues occurs via the spatially controlled expression of specific transcription factors (PHB/PHV, KAN, YABBY), and this juxtaposition of adaxial and abaxial tissue allows the lateral growth of the leaf to form a flattened lamina.

different tissue identities which triggers lateral growth of the organ to generate the classical flattened lamina of the leaf (Waites & Hudson, 1995) (Fig. 5). The molecular mechanism underlying the spatially separated acquisition of adaxial and abaxial fate is still being explored, but an exquisite (if complicated) process of intercellular communication coupled to transcription factor patterning is being elucidated.

Three classes of putative transcription factors have been shown to be involved in this process: the *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*) genes (which encode class III homeodomain/leucine zipper (HD-ZIP) proteins) (McConnell *et al.*, 2001), members of the *KANADI* gene family (encoding Golden2/*Arabidopsis* response-regulator/Psr1 (GARP) transcription factors) (Eshed *et al.*, 2001; Kerstetter *et al.*, 2001) and *YABBY* genes (which also encode putative transcription factors) (Siegfried *et al.*, 1999). Before leaf initiation, the transcripts encoding these proteins seem to be uniformly expressed throughout the incipient leaf. Following leaf initiation, some of these transcripts become restricted to the adaxial domain of the leaf (*PHB/PHV*), whereas others (*KANADI*, *YABBY*) presage differentiation of the abaxial domain. The expression of the adaxial identity genes seems to preclude that of the abaxial identity genes and, conversely, ectopic expression of abaxial identity genes leads to suppression of adaxial identity. It was initially proposed that the activity of the PHB/PHV proteins in the initiating leaf primordium might be dependent on interaction with a (theoretical) small diffusible sterol-based factor emanating in a gradient from meristem, the idea being that this gradient would limit PHB/PHV function to the adaxial part of the leaf closest to the meristem source of this signal (McConnell *et al.*, 2001). This hypothesis has been complicated by the finding that plants express miRNAs which can interact with PHB/PHV and that these miRNAs accumulate in the abaxial domain of the primordium (Kidner & Martienssen, 2004). miRNAs have the capacity to direct turnover of their substrate mRNAs (Tang *et al.*, 2003), thus these new findings suggest that the loss of PHB/PHV transcripts from the presumptive abaxial domain is a result of miRNA-directed breakdown. Because it is also possible that miRNAs are mobile, the PHB/PHV miRNAs could act as an intercellular signal within the shoot apex (Juarez *et al.*, 2004). Intriguingly, the target sequence of the PHB/PHV miRNAs encompasses the mRNA sequence encoding the amino acid sequence predicted to be involved in sterol binding, i.e. the target of the putative adaxializing signal. Unravelling the signal mechanism involved in the acquisition of adaxial/abaxial identity in the leaf promises to shed new and important light on basic processes of plant development.

The observation that juxtaposition of adaxial and abaxial tissue is required for lateral growth in the meristem raises the question of how this functions. The answer is that we do not know. As demonstrated by Nath *et al.* (2003), the generation of a flat leaf lamina requires temporal and spatial coordination of differential growth throughout the leaf and disruption of this pattern of growth leads to abnormal leaf morphogenesis. Interestingly, miRNA-regulated expression of TCP-like transcription factors has been demonstrated to be involved in this process (Palatnik *et al.*, 2003). Elucidating the target processes for these transcription factors, and the mechanism underlying the control of miRNA expression and processing, will provide significant steps in our understanding of this important topic, i.e. the coordination of leaf growth over space and time.

VII. Early steps in leaf histogenesis

Shortly after a leaf primordium is formed, the first distinct changes in histology occur which presage the formation of different cell types (although, as mentioned above, by this

stage molecular and cytological changes associated with photosynthesis may have already occurred). The formation of prevascular tissue is one of these early steps as cells in this region undergo a pattern of oriented cell division. Auxin has been strongly implicated in this patterning process. In particular, localization of PIN proteins has indicated that at the earliest stages of leaf formation there is an inward flux of auxin at the tip of the forming primordium which streams down through the centre of the primordium (Benkova *et al.*, 2004). These data, along with observations linking auxin and leaf vascular patterning (Sachs, 1981), suggest that this initial polar flux of auxin predicts the fate of cells destined to form the main vascular bundle. As to the target genes involved in the initial differentiation process, recent data have implicated cytokinin signalling and cell wall proteins in the cellular events required for the establishment of vascular bundles (Bonke *et al.*, 2003; Motose *et al.*, 2004). Thus, the Altered Phloem Development (APL) MYB transcription factor is required for the acquisition of phloem or xylem tissue identity and an Arabino galactan protein is involved in xylem differentiation. However, these proteins are likely to be involved in relatively late events of vascular differentiation and are probably someway downstream from the initial patterning process. Thus, despite these key advances, we are still some way from having a clear understanding of the molecular mechanism underlying vascular differentiation. This is patently a key aspect of leaf development, not only because a vascular system is required for the import and export of photosynthate and water, but also because there is an intimate relationship between leaf form and vascular patterning (Mattsson *et al.*, 2003).

Subsequent to the initial stages of vascular differentiation, various other leaf tissues become established. For example, cells in the adaxial domain (defined by *PHB*/*PHV*) expression differentiate to form the palisade mesophyll and cells in the abaxial domain (*KANADI*/*YABBY*-defined) differentiate to form the spongy mesophyll. The mechanism underlying this differentiation process is unknown. However, an unexpected insight into the potential role of *PHANTASTICA*-like transcription factors in this process was recently reported (McHale & Koning, 2004). In *Nicotiana sylvetris*, *NSPHAN* is initially expressed throughout the leaf primordium but transcript accumulation gradually becomes restricted to the adaxial region and then to the middle mesophyll layers. When *NSPHAN* expression is repressed, the adaxial mesophyll cells appear to be less differentiated and this is associated with the ectopic expression of *KNOX* genes. These data fit with a model in which *KNOX* genes act to maintain cells in an indeterminate/immature state and PHANTASTICA acts to allow or promote palisade differentiation by repressing *KNOX* gene expression. Interestingly, one aspect of the phenotype displayed by *Nicotiana* plants in which *NSPHAN* expression is repressed is that ectopic laminas are formed on the adaxial face of the leaf. The interpretation of these data requires a slight modification of the paradigm that lamina formation requires juxtaposition of adaxial and abaxial tissue. Rather, it seems that what is required is a gradient of tissue determination; in other words, adaxial/immature and immature/abaxial gradients can function to promote lateral growth as well as adaxial/abaxial juxtapositions.

VIII. Later steps in leaf differentiation: epidermal cell fate

A final element of leaf differentiation to be discussed here is the acquisition of cell fate in the epidermis, most notably stomata and trichomes/hairs. Significant progress has been made in this area.

With respect to trichome formation in *Arabidopsis*, a number of positive-acting transcription factors have been identified (e.g. GL1, TTG and GL3) (Larkin *et al.*, 1994; Walker *et al.*, 1999; Zhang *et al.*, 2003). These factors promote trichome formation and are predicted to activate their own expression. Factors which repress trichome formation have also been characterized, including TRIPTYCHON (TRY) and CAPRICE (CPC) (Schnittger *et al.*, 1999; Schellmann *et al.*, 2002). Surprisingly, these negative regulators are expressed at a relatively high level in the cells which form trichomes. One interpretation of this observation is that the factors involved in promoting and repressing trichome formation act as part of a reaction–diffusion model (Meinhardt, 1982). If this model is appropriate for trichome patterning and differentiation, it predicts that the inhibitor factors would indeed accumulate at the site of trichome formation but that they would diffuse more rapidly than the positive effectors to the neighbouring cells, leading to the inhibition of trichome formation in these neighbouring cells. Although data to support this hypothesis are incomplete, it has been shown that at least one of the negative regulators (CPC) can move from cell to cell (presumably via plasmodesmata) (Wada *et al.*, 2002).

With respect to stomatal patterning, progress has not been so rapid yet a number of insights have been made. For example, a MAPKKK (YODA) has been identified as a key intermediary in stomata formation, with repression of YODA leading to ectopic stomata formation and overexpression of YODA resulting in the generation of leaves lacking stomata (Bergmann *et al.*, 2004). With respect to patterning of stomata across the epidermal surface, TMM (mutation of which leads to the formation of groups of stomata) has been shown to encode an LRR-kinase, suggesting that the protein acts as a receptor for some signal (not yet identified) involved in stomatal patterning (Nadeau & Sack, 2002). That this elusive signal may be peptide-based is suggested by the finding that another gene involved in stomatal pattern, *SDD*, encodes a subtilisin protease (Berger & Altmann, 2000). Such a protease may be involved in signal processing. However, cutin or fatty acid derived signals have also been implicated in stomatal patterning by the finding that the *HIC* gene (mutation of which disrupts environmental influences on stomatal density) encodes an enzyme potentially involved in cuticle formation (Gray *et al.*, 2002).

These findings suggest that final patterning of cell differentiation in the leaf epidermis depends on a series of intercellular signalling events which dictate the expression pattern of specific combinations of transcription factors. Significant progress has been made in the identification of these transcription factor networks, but clear evidence as to the identity and nature of the signalling elements remains elusive.

IX. Compound and simple leaves: variation on a theme?

A number of plants produce leaves in which outgrowths occur along the primordium proximal–distal axis while intervening tissue undergoes hardly any lateral growth. Moreover, these localized outgrowths can themselves undergo extensive lateral growth to generate structures which strongly resemble simple leaves. These individual segments are termed leaflets and the entire collection of leaflets (attached to what is now termed a rachis) is defined as a 'compound' leaf. (There has been some debate as to the use of the term 'compound' to distinguish such ornate leaf structures from their 'simple' cousins. Thus, the terms 'highly dissected' (Kaplan, 2001) and 'complex' (Sinha, 1999) have come into play. The term 'compound' is used here simply to relate particular leaf growth forms to the classical literature). A long-standing debate has focussed on whether the compound leaf structure represents a reiteration of the mechanism by which simple leaves are formed or whether compound leaves are modified forms of an initially simple leaf. Recent molecular data have provided an insight into the mechanism by which compound leaf structures form but have not yet fully resolved this issue.

The main players in this scheme appear (again) to be the *KNOX* and *PHANTASTICA*-like transcription factors. Plants that generate simple leaves are characterized by the repression of *KNOX* gene expression in the young developing primordia, whereas leaves that form compound leaves express *KNOX* genes shortly after leaf initiation (Bharathan *et al.*, 2002). This early phase of *KNOX* gene expression seems to be involved in compound leaf formation; for example, overexpression of *KNOX* genes in tomato leads to the generation of leaves that can be described as supercompound (highly dissected) (Hareven *et al.*, 1996). However, overexpression of *KNOX* genes in plants with simple leaves is not sufficient to switch the plant to making compound leaves. On the other hand, as we have seen in *Nicotiana*, suppression of *PHANTASTICA*-like gene expression in a simple leaf plant leads to the formation of ectopic lamina growths (McHale & Koning, 2004), whereas a similar down-regulation of a *PHANTASTICA*-like gene expression in tomato (a compound leaf plant) leads to the formation of palmate as opposed to pinnate leaves, indicating a reduction in compoundness (i.e. leaves are less dissected) (Kim *et al.*, 2003a). As described above, work on simple leaf plants indicates that *PHANTASTICA*-like proteins act to suppress *KNOX* gene expression, thus suppression of *PHANTASTICA* leads to ectopic *KNOX* gene expression, which promotes indeterminate/immature status, allowing the cells to undergo novel morphogenic processes. In plants with compound leaves, the relationship between *PHANTASTICA* and *KNOX* gene expression seems to have altered so that PHANTASTICA no longer suppresses *KNOX* gene expression in the context of the young primordium (Kim *et al.*, 2003b). This maintenance of a pool of indeterminate/immature cells in the primordia of compound leaves (which is also associated with a lack of abaxial differentiation) is presumably the basis of their ability to undergo the subsequent growth processes characteristic of these leaves. However, why in some plants (compound-type) this growth process should result in ornate leaflet structures whereas in others (simple-type) any ectopic structure is generally restricted in growth potential remains a mystery.

X. Changes in leaf form: developmental and environmental influences

The type of leaf formed by a plant at any one time is under both developmental and environmental regulation. Some of these changes may be very dramatic, such as the switch in leaf form displayed by some aquatic plants as the stem enters a gaseous environment. Other significant changes in leaf form and differentiation may also occur in response to different light regimes leading, for example, to the formation of shade leaves. At the molecular level, the greatest advances in our understanding have been gained from the developmentally controlled change in leaf form during the lifetime of a plant, so-called phase change.

In *Arabidopsis*, the juvenile and adult phases of vegetative development are characterized by differences in leaf size and shape and by the distribution of trichomes on the leaf blade. As a result of screening for mutations in genes affecting this phase change, Poethig and colleagues identified a number of gene products involved in this process. Interestingly, one of these is an ARGONAUTE-like protein implicated in the processing of miRNAs (Hunter *et al.*, 2003b). As described above, miRNAs have now been implicated in the acquisition of leaf adaxial/abaxial polarity and leaf shape and this provides another example suggesting that they play a fundamental role in many aspects of leaf development. Other genes implicated in leaf phase have also been identified and these share the characteristic of being implicated in the regulation of nuclear transport (Bollman *et al.*, 2003; Hunter *et al.*, 2003a). The precise significance of this is still to be elucidated, but the data suggest that the control of movement of (as yet uncharacterized) factors between the nucleus and the cytoplasm are involved in phase change.

XI. A model of leaf formation

The information in this review provides an indication of the complexity of the process of leaf formation. Many different

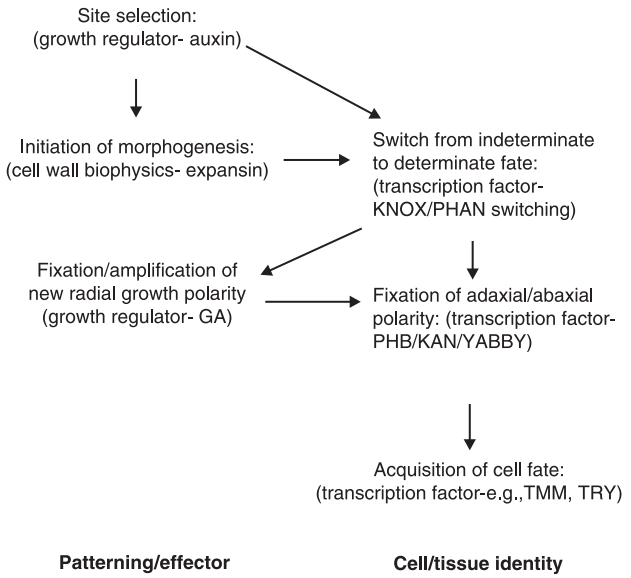

Fig. 6 Scheme of leaf formation. Leaf formation involves an interplay between patterning/effector molecules and transcription factor activities which delineate cell and tissue fate. However, at least some of the transcription factor proteins and their encoding RNAs may be mobile within the tissue, thus can act both as patterning and cell identity determinants. Arrows do not necessarily imply direct causal relationships, rather a developmental flow chart of key events in leaf formation.

steps are involved, yet these steps must be coordinated in time and space. An overview of the entire process is provided in Fig. 6 in the form of a tentative flowchart of events.

The first phase in leaf formation involves patterning of a band of cells around the periphery of the SAM. Strong evidence indicates that the growth regulator auxin is intimately involved in this patterning process. Although the target genes/processes of this flux of auxin are not verified, a change in cell wall extensibility to allow for the switch in growth vector required for leaf primordium outgrowth seems necessary. A number of lines of evidence suggests that the cell wall protein expansin is likely to be involved as the mediator of this process. Before leaf initiation occurs, *KNOX* gene expression is down-regulated. This may also be a direct consequence of auxin flux at the site of presumptive leaf formation. One consequence of decreased *KNOX* gene expression is to promote GA biosynthesis which may fix and/or amplify the altered growth vector characteristic of the new leaf primordium. At the same time, *PHANTASTICA*-like gene expression is up-regulated in the primordium. Gain of PHANTASTICA activity and loss of KNOX activity determines the fate of the cells incorporated into the leaf. At the same time, signalling processes emanating from the SAM lead to the fixation of adaxial tissue identity (via the expression of PHB/PHV genes in this tissue) and, conversely, loss of PHB/PHV activity in the abaxial tissue leads to gain of abaxial identity (via expression of *KANADI* and *YABBY* genes). The juxtaposition of these adjacent tissue identities allows lateral growth to occur to form the lamina. As the leaf grows from an initial bulge on the SAM, intercellular patterning signals are created within the leaf which lead internally to the specification of different tissues. In the case of the vascular tissue, auxin is intimately involved. Similarly, in the leaf epidermis, intercellular signalling pathways delineate the cell-specific expression patterns of transcription factors which define the fate of those cells.

This model provides a general outline of the process of leaf formation. Some aspects have been characterized in some detail, but many parts involve speculation, in particular how the different aspects are coordinated. It is clear that appropriate timing and spatial coordination of transcription factor activity is key to the formation of a functioning leaf and the characterization of these factors continues apace (e.g. Eshed *et al.*, 2004). At the same time, it is clear that intricate and possibly novel forms of communication occur within and between the SAM and the young leaf. Elucidation and manipulation of these signalling pathways will be essential for a full understanding of the control of leaf development.

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