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Signalling: The Green Light to Leaf Development

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ABSTRACT

A characteristic feature of plant development is the continual elaboration of lateral organs such as leaves, from the flanks of the shoot apical meristem (SAM). To maintain this pattern of growth, cells destined for organ formation are constantly generated by a small group of pluripotent stem cells located in the apex of the SAM. While this developmental strategy differs from that of animals, many of the underlying mechanisms regulating cell proliferation and cell fate are similar. For instance, positional cues play an important role in regulating cell identity in plants as they do in animal systems, suggesting the presence of extensive cell-cell signalling networks operating within plants. The last decade has seen considerable progress in identifying the molecular components of these signalling pathways. In some cases the signal operates over short distances, and typically involves the activation of transmembrane receptors by ligands. In other cases, signals are conveyed using RNA, transcription factors or hormones and may operate over greater distances both within developing organs and between organs of the plant. The goal of this review is to provide both a historic prospective as well as current insights into signalling pathways regulating leaf initiation and patterning.

Keywords: adaxial-abaxial polarity, auxin, cell-cell signalling, HD-ZIPIIIs, leaf development, miRNAs, stomata, ta-siRNAs, transcription factors

CONTENTS

INTRODUCTION

Plant lateral organs such as leaves, bracts and floral organs are determinate structures that arise from dome-like meristems in regular and predictable patterns. They come in a multitude of sizes, shapes and colours reflecting functions that range from being the primary photosynthetic organ to attracting insect pollinators. Despite these differences, the underlying mechanisms regulating organ initiation and subsequent growth are similar in all higher plants. The first step in organ formation occurs when one or more founder cells are specified in the flanks of the shoot apical meristem (SAM) or floral meristem (FM). These cells begin to proliferate rapidly leading to the formation of an outgrowth, termed a primordium (**Fig. 1A, 1B**). The newly emerged primordium continues to grow, first by cell division and then by cell expansion, until it attains an appropriate size and shape (Donnelly *et al.* 1999). In many species of plant, such as the dicot *Arabidopsis*, the process of differentiation begins soon after primordia emergence, leading to the formation of morphologically and often physiologically distinct cell types along the three spatial axes of the organ. The greatest range of cell types is often apparent along the adaxial-abaxial (also referred to as dorsal-ventral) axis of the leaf blade. For instance, in *Arabidopsis*, the adaxial epidermis is composed of large pavement cells and infrequent stomata (small pores involved in gaseous exchange), whereas the abaxial epidermis has smaller and more jigsaw-shaped pavement cells as well as a higher stomatal index (**Fig. 1C**). Internally, adaxial arising palisade mesophyll cells are both larger and possess more chloroplasts than the underlying abaxial spongy mesophyll cells. In contrast to palisade mesophyll cells, which tend to be closely packed, spongy mesophyll cells support airspaces that are essential for gaseous exchange (**Fig. 1C**). The resulting asymmetric distribution of cell types ensures that the leaf is well adapted for photosynthesis. How this pattern of cell types is established during leaf development is the main focus of this review.

Chimaeras reveal the importance of signalling during organ development

Early insight into the mechanisms coordinating growth and

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Invited Review

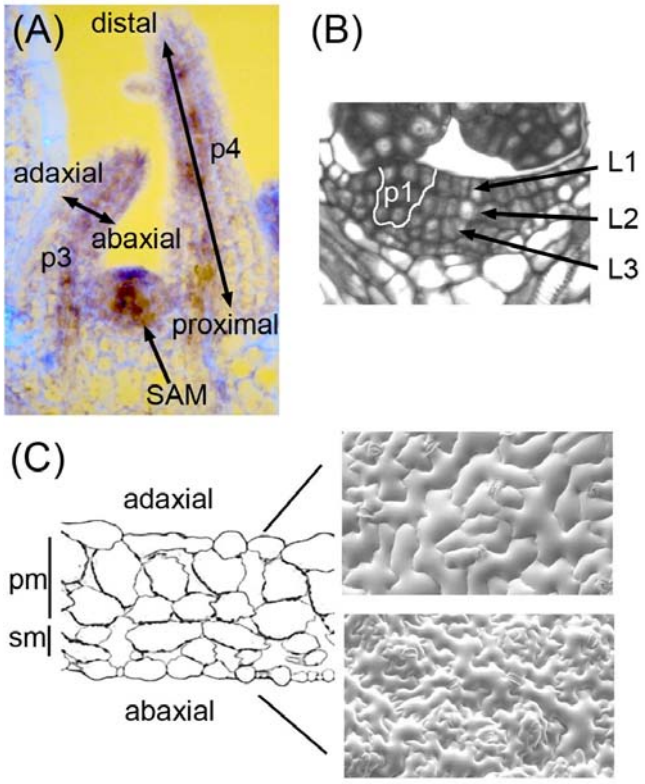

Fig. 1 Structure of the shoot apical meristem and leaves. (**A**) Longitudinal view of the *Arabidopsis* vegetative shoot apical meristem (SAM) stained with the fluorochrome Calcofluor to highlight cell walls. Due to the spiral arrangement of organs around the meristems, this section shows progressively older leaves (p3 and p4) but excludes younger primordia (p1-p2). The proximal-distal and adaxial-abaxial axes are shown relative to the SAM. The adaxial side is closest to the meristem and the abaxial side furthest away. Brown stain marks expression of the *HD-ZIPIII* gene *PHB* in the SAM and adaxial sides of the leaf. (**B**) Enlargement of the *Arabidopsis* SAM showing the three clonally distinct layers (L1-L3) and an initiating leaf primordium (p1). (**C**) As the *Arabidopsis* leaf matures, adaxial cells become morphologically and physiologically distinct from cells in the abaxial domain. For instance, adaxial epidermal cells are large and irregular in shape (top panel), whereas abaxial epidermal cells are much smaller and more jigsaw-shaped (bottom panel). Internally, adaxial palisade mesophyll (pm) cells are large and packed full of chloroplasts, whereas the abaxial spongy mesophyll (sm) is smaller, contain less chloroplasts and support airspaces that are essential for gaseous exchange.

cell fate during leaf development has come from the study of genetic mosaics. Typically these plants harbour small groups of cells (sectors) that are phenotypically distinct from the rest of the plant, allowing their growth and pattern of differentiation to be monitored throughout development. When mosaics arise from pluripotent stem cells located in the SAM, the resulting sector encompasses a much larger portion of the plant and spans some or all of the tissue derived from these cells (Marcotrigiano 2001). Examples of this type of mosaic, which are termed chimaeras, include variegated ornamentals favoured by horticulturists. Characterisation of chimaeras has shown that the SAM of higher plants is composed of stem cells located in clonally distinct layers and that cells from each layer are incorporated into developing organ primordia. The layered structure of the meristem is also apparent histologically with the outermost *tunica* being composed of one or two layers of cells that divide anticlinally (plane of cell division perpendicular to the meristem surface) and an inner *corpus* where anticlinal and periclinal (plane of cell division is parallel to the meristem surface) cell divisions occur more or less randomly. In many plants, including the model plant *Arabidopsis*, the layers of the tunica are referred to as L1 and L2, while the corpus is derived from the L3 layer (**Fig. 1B**).

The characterisation of periclinal chimaeras has also helped to define the different types of cells that are derived from each layer of the SAM following their incorporation in leaves or the stem of the plant. For instance in leaves of dicot plants, cells derived from the L1 layer form the epidermis, whereas subepidermal mesophyll cells are derived from the L2 layer and those of the L3 produce the ground tissue and vasculature (reviewed in Tilney-Basset 1986). It should be noted however, that patterns of cell fate vary between species, and even between lateral organs arising within the same species. In *Arabidopsis*, marginal subepidermal cells of the petal are derived from the L1 layer instead of the L2 layer (Jenik and Irish 2000). While this analysis shows that cells from any one layer are predestined to adopt a limited range of fates, observing rare interlayer cell displacements in periclinal chimaeras has shown that displaced cells almost always adopt fates expressed by cells in the receiving layer (Stewart and Derman 1975). This implies that cell fate is largely influenced by 'position' rather than 'lineage' (although there are some exceptions, see Kessler *et al.* 2002), and that there is an exchange of information between neighbouring cells. Similarly, chimaeras in which layers of the meristem are derived from plants or mutants with different growth potentials have shown that the underlying layers (L2, L3) influence growth of the L1 layer (reviewed in Tilney-Basset 1986; Szymkowiak and Sussex 1996; Marcotrigiano 2001). In other studies, the L1 layer seems to play a more important role in regulating organ and plant growth (Savaldi-Goldstein *et al.* 2007). Irrespective of the exact contribution made by each layer to leaf size control, it is clear that signals moving between the layers play an important role in coordinating growth during organ development.

The analysis of chimeras has therefore provided important insight into the extent of signalling during organ development. By analogy to signalling in animal systems, it is possible that cell-cell communication during organ development is regulated by short range signalling molecules such as receptor-ligand combinations. Given that there are over 600 receptor-like kinases in the *Arabidopsis* genome, this scenario does not seem unlikely, although to date few of these receptors have been implicated in development and in most cases the ligands are unknown (Shiu and Bleecker 2003). Alternatively, signalling may involve the localised movement of RNA and transcription factors between cells via small channels called plasmodesmata that connect the cytoplasm of adjacent cells. Finally, as in animal development, short and long distance signalling may involve hormones. In this review, we consider each of these different signalling mechanisms giving examples of how they regulate specific processes in leaf development. The aim is to highlight some of the many recent advances that implicate a role for signalling during leaf development and to indicate the direction that this field may take in the future. The types of signals involved in growth control will not be discussed here, as they have been the subject of several recent reviews (Ingram and Waites 2006; Savaldi-Goldstein and Chory 2008).

Leaf initiation PIN-ed on auxin

One of the more intriguing aspects of plant development is the mechanism that determines the pattern of lateral organs that arise along the stem or in a flower, referred to as phyllotaxy. The first insight into the mechanism regulating phyllotaxy came when microsurgical studies carried out over 50 years ago established that a signal generated from pre-existing organs influences future sites of organ formation (reviewed in Golz 2006). Recent studies have shown that auxin gradients within the SAM play a key role in determining sites of organ initiation and as such may be the principal signal involved in phyllotactic patterning.

Auxin gradients arise through a combination of auxin synthesis and transport. In the case of indole-3-acetic acid (IAA), the best studied of the auxins, the biosynthetic pathways involved in its production are relatively well under-

stood (Davies 2004). Given the importance of auxin in almost all aspects of plant growth and development, surprisingly little is known about the genes that encode IAA biosynthetic enzymes or indeed where they are expressed in plants. This situation has recently changed however, with the identification of several families of genes involved in auxin biosynthesis in *Arabidopsis* (Zhao *et al.* 2001, 2002; Cheng *et al.* 2006; Stepanova *et al.* 2008; Tao *et al.* 2008). Two of these, the *YUCCA* family of flavin monooxygenases and the trytophan aminotransferases *TAA1* and *TAR2*, are expressed in the developing embryo and post-embryonically in the apical regions of the shoot, incipient leaf primordia and the vasculature (Cheng *et al.* 2007). This implies that auxin is mainly synthesized in the young aerial tissue of the plant before moving to other regions of the plant. Initially movement of auxin from the site of synthesis through the apoplast and into cells involves passive diffusion along a concentration gradient. However once inside a cell auxin becomes charged, preventing any further diffusion. As a result, the bulk of auxin movement in a plant involves a system of active transport. Proteins involved in polar auxin transport in *Arabidopsis* include members of the *PIN-FORMED* (*PIN*) family of transmembrane efflux carriers, the *AUXIN PERMEASE1/LIKE AUX* (*AUX1*/*LAX*) family of influx carriers and members of the *MULTIDRUG RESISTANT*/*P-GLYCOPROTEIN* (*MDR*/*PGP*) ABC transporter family that either function as influx or efflux carriers (Bennett *et al.* 1996; Gälweiler *et al.* 1998; Blilou *et al.* 2005; Terasaka *et al.* 2005; Lewis *et al.* 2007; Bainbridge *et al.* 2008).

The importance of auxin gradients in regulating developmental processes becomes apparent when plants are grown in the presence of chemicals that block polar auxin transport. For instance, plants grown on media containing NPA produce stems and/or inflorescence lacking leaves or floral buds (Okada *et al.* 1991; Reinhardt *et al.* 2000; Scanlon 2003; Navarro *et al.* 2004). Similarly, *Arabidopsis* mutants defective in either polar auxin transport, such as *pin-formed1* (Okada *et al.* 1991) and *pinoid* (Bennett *et al.* 1995; Michniewicz *et al.* 2007) or auxin signalling such as *monopteros* mutants (Berleth and Jürgens 1993) also form naked pin-like inflorescences, providing further support for the view that auxin is required for organ formation.

Due to the small size of the meristem and the difficulties in detecting active auxin *in planta*, the relationship between auxin and organ initiation has been difficult to investigate. However, this situation has changed over the last few years with the development of molecular reporters that can be used to infer auxin distribution within plant tissue. For instance fusing putative auxin efflux transporter proteins to the fluorescent protein GFP has shown that these proteins specifically accumulate in the side of the cell exporting auxin in both shoots and roots of *Arabidopsis* (Gälweiler *et al.* 1998; Benkova *et al.* 2003; Blilou *et al.* 2005). The pattern of PIN proteins can therefore be used to determine the likely direction of auxin flow within a particular tissue and hence the likely distribution of auxin (Gälweiler *et al.* 1998; Benkova *et al.* 2003; Reinhardt *et al.* 2003; Blilou *et al.* 2005; Heisler *et al.* 2005). In addition to PIN protein localisation, analysis of the auxin responsive promoter *DR5* has also provided insight into the pattern of auxin accumulation within the plant. *DR5* is composed of multiple synthetic auxin response elements (AuxREs), which are recognised by a family of transcription factors called AUXIN RESPONSE FACTORs (ARFs) that function either as activators or repressors (Ulmasov *et al.* 1997, 1999). When the concentration of auxin in a cell is low, ARFs are kept in an inactive state by binding to AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) proteins. However when the intracellular concentration of auxin increases, ARFs become active due to an auxin-induced degradation of AUX/IAAs by the ubiquitination pathway (Guilfoyle and Hagen 2007). Thus, by examining *DR5* expression, the magnitude of auxin responses throughout plant development can be monitored (Sabatini *et al.* 1999; Mattsson *et al.* 2003).

Looking at the cellular distribution of PIN1 in the vegetative and inflorescence SAM of *Arabidopsis* plants using either immunolocalisation or PIN1:GFP fusions, has shown that this protein preferentially accumulates in L1 cells (Benkova *et al.* 2003; Reinhardt *et al.* 2003; Heisler *et al.* 2005). In the bulk of the meristem PIN1 is localised to the side of the cell facing the apex suggesting that auxin is likely to flow through the L1 layer towards the tip of the SAM. However in the peripheral zone of the SAM, PIN1 accumulates in membranes facing future sites of organ initiation. These sites are also marked by elevated *DR5* expression suggesting that auxin flows from surrounding tissue into this region, creating a zone of high auxin concentration (Benkova *et al.* 2003; Reinhardt *et al.* 2003; Heisler *et al.* 2005; de Reuille *et al.* 2006; Smith *et al.* 2006). As *PIN1*

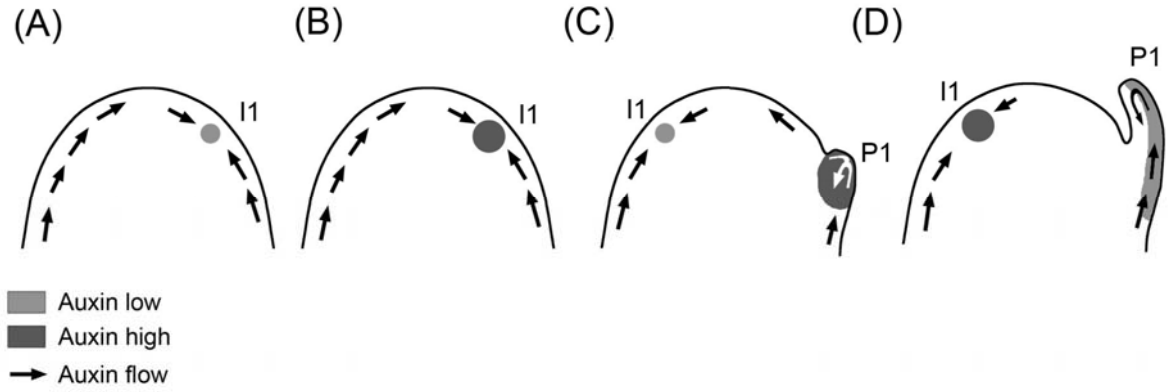

Fig. 2 Auxin signalling in phyllotaxy. A side view of the SAM showing likely directions of auxin flow (arrows) based on PIN1 protein localisation in SAM cells. Accumulation of auxin within the SAM and primordia is indicated with shading. (**A**) Based on the apical localisation of the PIN1 protein in cells of the SAM, it is thought that auxin flows predominantly through the L1 layer towards the summit of the apex. In the flanks of the SAM, PIN1 accumulates in cell membranes facing a future site of organ initiation (I1). As a consequence, auxin flows into this site from adjacent regions of the SAM leading to its accumulation. (**B**) Accumulation of auxin at I1 results in an elevation of *PIN1* expression and the increased accumulation of auxin at this site – creating so called auxin maxima. Once the concentration of auxin has passed a critical threshold, organ formation is initiated. (**C**) As the primordium develops, it becomes a sink for auxin flowing up from developmentally more advanced primordia. At this time there is also rapid reversal in the subcellular localisation of PIN1 protein in the SAM region adjacent to the primordium. This reversal is likely to redirect auxin away from this area and back towards the apex summit. As a result of the primordium becoming an auxin sink, a zone of auxin depletion surrounds the developing primordium preventing further organ initiation from occurring in this region of the SAM. Auxin will, however, accumulate at sites furthest from developing primordia, marking the next site of organ formation. (**D**) The continued development of the primordium leads to PIN1 protein preferentially accumulating in the abaxial domain as well as in the developing vasculature. This suggests that auxin accumulates abaxially in the developing organ primordia. Eventually the developing leaf becomes a source of auxin, presumably through the activity of the *YUCCA* genes. Figure redrawn from Reinhardt *et al.* (2003).

expression is positively regulated by auxin (Reinhardt *et al.* 2003; Heisler *et al.* 2005), the build up of auxin at this site is presumably enhanced by an increase in PIN protein accumulation. Following primordium initiation, the localisation of PIN1 in SAM cells adaxial to the initiating organ rapidly reverses, such that PIN1 now accumulates in membranes facing away from the primordium. This shift in PIN1 localisation presumably leads to auxin flowing back towards the summit of the apex (Heisler *et al.* 2005). Within the initiating organ PIN1 continues to direct auxin flow into the centre of the primordium, and then as the primordium emerges, PIN1 directs auxin along the abaxial side of the organ towards the tip. As the organ develops, PIN1 gradually accumulates in basal membranes of provascular elements, suggesting that auxin eventually flows out of the organ (Reinhardt *et al.* 2003; Heisler *et al.* 2005). Based on these and earlier observations, it has been proposed that the pattern of organ formation in the SAM is largely influenced by auxin distribution (Reinhardt *et al.* 2000, 2003; Heisler *et al.* 2005). According to this model, organ formation is confined to the periphery of the SAM where auxin accumulation is highest (**Fig. 2**). As primordium development is initiated, a zone of auxin depletion surrounds the organ, caused in part by the rapid PIN1 reversal in the SAM. As a consequence of this reversal, auxin begins to accumulate in regions of the SAM furthest from pre-existing organs, which subsequently triggers the formation of a new primordium and an associated auxin depletion zone. This model largely accounts for how pre-existing organs influence future sites of organ formation and when tested computationally, it successfully predicts stable phyllotactic patterns seen in many species of plant (de Reuille *et al.* 2006; Jonsson *et al.* 2006; Smith *et al.* 2006).

In addition to organ initiation, dynamic fluxes in auxin movement are involved in other aspects of plant growth and development. These fluxes are apparent throughout embryogenesis where they are instrumental in specifying apical-basal and central-peripheral patterning, as well as cotyledon formation. Auxin also regulates the patterning and growth of roots and plays an important role in mediating gravitropism. And as will become apparent in later sections of this review, auxin has roles in determining organ polarity and shape.

Signals involved in organ polarity – A *PHB*ulous story

Classic microsurgical experiments in several plant species have shown that separating leaf primordia from the meristem at an early stage of development leads to the formation of radial leaves lacking adaxial cell identity (Snow and Snow 1954a, 1954b; Sussex 1951, 1955; Hanawa 1961). More recent laser ablations studies not only confirm the importance of contact between the meristem and the developing primordium for organ polarity, but also demonstrate that this function involves the L1 layer (Reinhardt *et al.* 2005). One possible explanation for these observations is that during the early stages of leaf development, a polarising signal moves through the L1 layer of the meristem into the site of organ initiation, where it promotes adaxial identity (**Fig. 3**). Interestingly, this signal is only required transiently as organs separated from the meristem at later stages of development lack polarity and growth defects (Sussex 1951, 1955; Reinhardt *et al.* 2005). This implies that at a certain time after leaf emergence, the control of cell fate becomes independent of the meristem. Since its discovery the nature of this signal (hereafter referred to as the 'Sussex' signal) has intrigued many plant biologists, but perhaps due to the small size of the meristem and/or the restricted distance over which the signal operates, has thus far eluded identification.

In recent years there has been considerable progress in understanding the molecular mechanisms regulating polarity establishment in *Arabidopsis* and other plant species. This has led to the identification of regulatory genes that function in a complex web downstream of the polarising signal. The first polarity mutant to be identified and cloned was *PHANTASTICA* (*PHAN*), a *MYB*-like transcription factor from *Antirrhinum majus* that promotes adaxial cell identity (Waites and Hudson 1995; Waites *et al.* 1998). In addition to abaxialisation, leaves of severe *phan* mutants are radially symmetric suggesting that adaxial-abaxial patterning is necessary for blade growth. Support for such a model comes from the analysis of intermediate *phan* mutants where patches of abaxial tissue arising in the adaxial domain of mutant leaves triggers the formation of small ectopic blades (Waites and Hudson 1995). Collectively, these observations suggest that blade initiation occurs in the region of the leaf where adaxial and abaxial cells are juxtaposed. The link between organ polarity and blade growth may also account for the formation of radialised leaves following surgical separation from the SAM.

While the function of *PHAN* appears to be conserved in tomato and tobacco (Kim *et al.* 2003; McHale and Koning 2004), mutations in the *PHAN* orthologue of *Arabidopsis* (*asymmetric leaves1*, *as1*) and maize (*rough sheath2*, *rs2*) do not cause obvious organ polarity defects. Instead, these mutations are associated with the ectopic expression of the meristem specific *KNOTTED-LIKE HOMEOBOX* (*KNOX*) genes in their leaves (Timmermans *et al.* 1999; Tsiantis *et al.* 1999; Byrne *et al.* 2000; Ori *et al.* 2000). As a result, the normally ovate and mildly serrated *Arabidopsis* leaf becomes deeply lobed in *as1* mutants due to reduced growth between the serrations (Ori *et al.* 2000), whereas ectopic

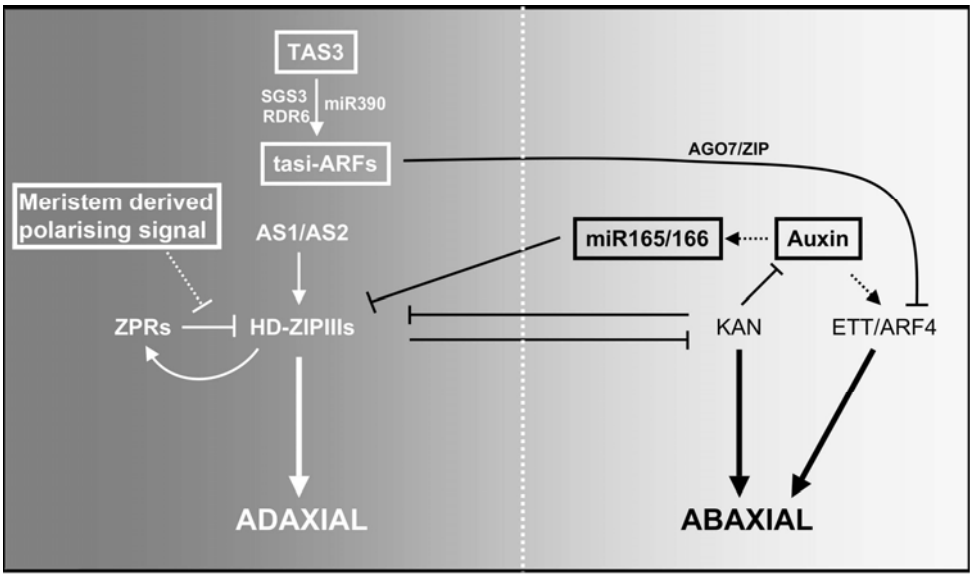

Fig. 3 Genetic pathways promoting adaxial-abaxial cell identity in *Arabidopsis* leaves. Genetic pathways promoting adaxial identity promoting pathways are shown on the left (dark shading) and abaxial one on the right (light shading). See text for details of each pathway. Arrows indicate positive regulation, whereas negative regulation is indicated with a T-shaped line. Boxes indicate components that have the potential to act as signals either directly by moving between cells, or activating pathways that produce a signal. Dashed lines indicate proposed functions that have not yet been demonstrated experimentally.

KNOX expression in *rs2* leaves causes proximal-distal patterning defects leading to 'roughening' in the basal part of the leaf (Schneeberger *et al.* 1998). The lack of obvious adaxial-abaxial polarity defects in either of these mutants may be explained if other genetic pathways function redundantly with *AS1*/*RS2* in promoting adaxial cell identity. Consistent with this scenario is the observation that mutations in *ASYMMETRIC LEAVES2 (AS2)*, a gene that functions with *AS1* to repress *KNOX* genes (Semiarti *et al.* 2001; Byrne *et al.* 2002; Iwakawa *et al.* 2002), displays polarity defects in certain genetic backgrounds (Xu *et al.* 2003; Xu *et al.* 2006; Ueno *et al.* 2007; Pinon *et al.* 2008; Yao *et al.* 2008). Further evidence that *AS1-AS2* pathway promotes adaxial cell identity comes from the observation that ectopic expression of *AS2* in the bottom-side of leaves results in a conspicuous loss of abaxial cell identity (Iwakawa *et al.* 2002; Lin *et al.* 2003; Xu *et al.* 2003).

Besides the *AS1-AS2* pathway, members of the Class III *HOMEODOMAIN-LEUCINE ZIPPER* (*HD-ZIPIII*) transcription factor family, *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*), function redundantly to establish adaxial cell identity in developing organs (McConnell and Barton 1998; McConnell *et al.* 2001; Emery *et al.* 2003; Prigge *et al.* 2005). Consistent with their role in promoting organ polarity, all three genes have overlapping expression patterns, first throughout the incipient organ primordia and then in the adaxial domain of emerging organ primordia (McConnell *et al.* 2001; Otsuga *et al.* 2001). In addition to having a role in polarity, these genes also promote SAM and FM initiation and in conjunction with another member of this family, *CORONA*/*ATHB15* (*CNA*), in the patterning of the developing embryo (Emery *et al.* 2003; Prigge *et al.* 2005). *PHB*, *PHV* and *CNA* also function together in meristem maintenance (Green *et al.* 2005; Prigge *et al.* 2005) and together with *REV* and the last remaining member of the *HD-ZIPIII* family, *ATHB8*, in vascular development in leaves and the stem (Zhong *et al.* 1997; Baima *et al.* 2001; Emery *et al.* 2003; Prigge *et al.* 2005).

In addition to having a DNA binding homeodomain and a leucine zipper domain, the HD-ZIPIIIs have a highly conserved START domain. This domain is also found in animal nuclear receptors where it binds to small hydrophobic ligands, such as steroids leading to their activation (Ponting and Aravind 1999; Schrick *et al.* 2004). Based on the presence of this domain, it has been suggested that a lipid produced in the SAM may activate the HD-ZIPIII proteins in a dose-dependent manner within the adaxial domain of initiating leaves (McConnell *et al.* 2001). Candidates for this lipid include sterols, as these are known to have a role in plant development (Jang *et al.* 2000; Schrick *et al.* 2000, 2002). However, as the only patterning defects associated with sterol biosynthetic mutants are limited to the vasculature, there is presently some doubt over whether sterols could really be the ligands for the HD-ZIPIII proteins (Willemsen *et al.* 2003).

Recent work has shown that HD-ZIPIII protein activity is regulated by *LITTLE ZIPPER*s (*ZPR*s). These encode proteins that are composed almost entirely of a leucine zipper that has extensive sequence similarity to the zipper domain present in the PHB, PHV and REV proteins (Wenkel *et al.* 2007). *In vitro* studies have shown that ZPRs are capable of dimerizing with REV, and as a consequence preventing REV from binding DNA elements found in promoters of putative *HD-ZIPIII* regulated genes. Subsequent analysis has also shown that overexpression of *ZPR*s in plants causes a partial loss of adaxial cell identity, suggesting that ZPRs may repress the activity of HD-ZIPIII proteins. Furthermore, based on the finding that *HD-ZIPIII*s promote *ZPR* expression, it is thought that the *ZPR*s may function in a negative feedback loop by regulating the abundance of active HD-ZIPIII homodimers. According to this model, the hydrophobic ligand could regulate adaxial polarity by altering the balance of active and inactive HD-ZIPIII dimers (Wenkel *et al.* 2007). Clearly the next step is to identify the elusive ligand and determine whether it functions in the way suggested by this model.

Tales from the underside

Several different classes of transcription factors regulate abaxial cell identity. The GARP-like *KANADI*s (*KAN1-4*) are expressed in the abaxial domain of developing organs and the stem, in a pattern that is broadly complementary to the *HD-ZIPIII*s (Kerstetter *et al.* 2001; Emery *et al.* 2003; Eshed *et al.* 2004; McAbee *et al.* 2006). While loss-of-function mutations in any one of these genes causes subtle changes to cell identity, leaves of double or triple mutants are severely adaxialised (Eshed *et al.* 2001; Kerstetter *et al.* 2001; Eshed *et al.* 2004). Loss of abaxial cell identity from leaves of these *kan* mutant lines correlates with expanded *HD-ZIPIII* expression, suggesting that KANs repress *HD-ZIPIII* activity. Consistent with this role, ectopic *KAN* expression in developing leaves causes a loss of adaxial cell identity and *HD-ZIPIII* expression (Emery *et al.* 2003; Eshed *et al.* 2004). The antagonism between these gene families appears mutual, as leaves of gain-of-function *HD-ZIPIII* mutants are completely adaxialized, presumably reflecting a loss of *KAN* activity (McConnell and Barton 1998; McConnell *et al.* 2001).

AUXIN RESPONSE FACTORs have also been implicated in the control of abaxial cell identity as *ETTIN* (*ETT*; also known as *ARF3*) was recently identified in a screen for mutants that suppress the patterning defects associated with ectopic *KAN* activity (Pekker *et al.* 2005). Although the *ett* mutant does not have a leaf phenotype by itself, when combined with mutations in the closely related *ARF4* gene, leaves of the double mutant develop *kan*-like polarity defects (Pekker *et al.* 2005). Consistent with redundancy between these genes, their expression patterns overlap in the abaxial domains of lateral organs. While these observations are consistent with *ETT* and *ARF4* functioning downstream of the *KAN*s, neither gene is capable of rescuing the *kan* mutant phenotypes when overexpressed. Collectively these results suggest that *ETT* and *ARF4* promote abaxial cell identity in parallel with *KAN*s rather than downstream of them (Pekker *et al.* 2005).

Micro-managing leaf polarity

An exciting recent development has been the realisation that several small non-coding RNAs, called microRNAs (miRNAs) and trans-acting short-interfering RNAs (ta-siRNAs) play an important role in polarity determination. miRNAs are small endogenous non-coding RNAs (~21 nucleotides) that regulate a range of developmental process in plants and animals (Jones-Rhoades *et al.* 2006). miRNA duplexes are generated in the nucleus following RNA polymerase II-dependent transcription of miRNA precursor genes and the subsequent processing of their transcripts by DICER-LIKE 1. Following export of these miRNA duplexes into the cytoplasm, mature miRNAs are incorporated into a silencing complex (RNA-Induced Silencing Complex; RISC). In the case of plants, complementary base pairing between the miRNA in the RISC complex and the binding site in target mRNAs results in transcript degradation (Schwab *et al.* 2005).

As plant miRNAs show high sequence complementarity to binding sites in target mRNAs, it is possible to predict genes that are regulated by miRNAs (Rhoades *et al.* 2002). Of the numerous plant miRNA that target transcription factors, miR165 and miR166 were found to be complementary to a short stretch of the *HD-ZIPIII* START domain (Rhoades *et al.* 2002; Tang *et al.* 2003). The importance of these miRNA target sites was immediately apparent, as previous studies had mapped gain-of-function mutations to this region of the *PHB* and *PHV* genes (McConnell *et al.* 2001). As a result of these mutations, *phb-1d* and *phv-1d* transcripts accumulate ectopically in mutant organs resulting in a dose-dependent loss of abaxial cell identity, vascular patterning defects and the formation of ectopic auxiliary meri-

stems around the base of the leaf (McConnell and Barton 1998; McConnell *et al.* 2001). Similar mutations affecting *REV* and *CNA* were subsequently identified and in each case vascular patterning defects and a loss of adaxial cell identity were apparent in these mutant lines (Emery *et al.* 2003; Kim *et al.* 2005; Ochando *et al.* 2006). In addition, *in vitro* experiments showed that mutations in the miRNA target site rendered *HD-ZIPIII* transcripts insensitive to miRNA-mediated cleavage (Tang *et al.* 2003; Mallory *et al.* 2004). These observations support a model of polarity establishment in which miR165/166 function as negative regulators of *HD-ZIPIII*s, possibly by preventing transcripts of these genes from accumulating in the abaxial domain of organs. Finding that the *Arabidopsis* miR165 and maize miR166 accumulate in the abaxial domain of lateral organs and the SAM is consistent with this scenario (Juarez *et al.* 2004; Kidner and Martienssen 2005). Furthermore, plants expressing elevated levels of either miR165 or miR166 have polarity, vascular and SAM defects that mirror loss-of-function *HD-ZIPIII* mutants, providing compelling support for this model of *HD-ZIPIII* regulation (Emery *et al.* 2003; Kim *et al.* 2005; Prigge *et al.* 2005; Williams *et al.* 2005b; Alvarez *et al.* 2006; Jung and Park 2007; Zhou *et al.* 2007).

In situ hybridisation revealed that the expression of the maize miR166 is highest in the region of the meristem below the incipient primordia and then decreases as it extends into developing organs (Juarez *et al.* 2004). This expression pattern gives the impression that miR166 moves from the SAM into the abaxial domain of the leaf along a concentration gradient. Given that miR165/166 play an important role in polarity establishment, it has been suggested that these miRNAs may in fact be the long sought after 'Sussex' signal (Juarez *et al.* 2004; Kidner and Martienssen 2005). Movement of RNAs, particularly small regulatory RNAs has been well documented in plants (Kehr and Buhtz 2008) and thus proposing miRNAs are mobile is not without precedent. Also, supporting this possibility is the finding that small RNAs with homology to *Arabidopsis* miRNAs are found in the sap of pumpkin and *Brassica napus* (Yoo *et al.* 2004; Buhtz *et al.* 2008) and that a miRNA induced by phosphate starvation is capable of moving from the shoot to roots across a graft junction (Pant *et al.* 2008). There is also at least one documented case of an artificially constructed miRNA displaying limited cell-cell movement (Schwab *et al.* 2006).

In spite of this, there is still an ongoing debate about whether miRNAs in general and miR165/166 in particular are capable of movement (Chitwood *et al.* 2007). In a recent study the non-autonomous effects of miR165 and miR166 were directly tested in *Arabidopsis* flowers (Alvarez *et al.* 2006). As expected, ectopic expression of these miRNA in petals and stamens caused abaxialisation of these structures, but had no discernable effect on the development of the adjacent carpel. Thus based on this test, miR165/166 do not appear to move between adjacent whorls of the flower. At least two other lines of evidence cast doubt on the role of miRNAs as a polarising signal. The first is that promoters of *HD-ZIPIII* genes, where examined, tend to reflect the pattern of transcript accumulation suggesting that *HD-ZIPIII*s are largely under transcriptional rather than post-transcriptional control (Baima *et al.* 2001; Kang and Dengler 2002; Alvarez *et al.* 2006). Second, analysis of several miRNA165/166 promoters (there are two miR165 and five miR166 loci in the *Arabidopsis* genome, (Reinhart *et al.* 2002)) has found that they are active in a variety of organs including leaves (Jung and Park 2007). Assuming that the maize miR166 promoter is similarly active in leaves, the observed gradient of expression may simply reflect a difference in promoter activity between organs and the SAM, or that miR166 is less stable in leaves than in the SAM. Given the conflicting data surrounding miRNA movement, it is perhaps too early to conclude that miR165/166 are the elusive 'Sussex' signal.

Intriguingly another class of small RNAs, ta-siRNAs, have recently been implicated in the regulation of organ polarity. ta-siRNAs are plant specific 21 nucleotide RNAs that are derived from the cleavage of endogenous non-coding *TAS* transcripts by specific miRNAs. However, unlike miRNA cleavage products, the *TAS* RNA is not degraded but instead converted into double stranded RNA through the activity of *SUPPRESSOR OF GENE SILENCING* (*SGS3*) and *RNA-DEPENDENT RNA POLYMERASE* (*RDR6*). The double stranded RNA is then recognised by the DICER-LIKE4 protein and cleaved into smaller ta-siRNA duplexes. Once diced, one of the siRNA strands enters an ARGONAUTE1 (AGO1) or ARGONAUTE7/ZIPPY (AGO7/ZIP) containing RISC complex, where it guides the degradation of specific mRNAs following pairing between the target sequence in the mRNA and the siRNA (Jones-Rhoades *et al.* 2006).

Given the sequence of the *TAS* genes and the fact that ta-siRNAs are produced in sequential blocks of 21 nucleotides from the start of miRNA cleavage site, it is possible to predict the sequence of ta-siRNAs and their target mRNAs (Allen *et al.* 2005). This analysis led to the discovery that ta-siRNAs generated from the *Arabidopsis TAS3* gene target *ARF2*, *ETT* and *ARF4* (Allen *et al.* 2005; Williams *et al.* 2005a). Subsequent analysis of loss-of-function mutants defective in ta-siRNA biogenesis (such as *sgs3*, *rdr6* or *zip*) showed elevated expression of *ETT* and *ARF4*, confirming that these genes are indeed targets of tasiR-ARFs (Peragine *et al.* 2004). Somewhat surprisingly however, these mutants do not have prominent organ polarity defects, but instead prematurely develop trichomes (leaf hairs) on the abaxial surface of leaves (Hunter *et al.* 2003; Peragine *et al.* 2004; Xie *et al.* 2005; Yoshikawa *et al.* 2005). As formation of trichomes on the abaxial surface of *Arabidopsis* leaves is typically initiated following the formation of the fourth or fifth leaf, tasiR-ARFs presumably function in a pathway that determines the timing of juvenile to adult leaf phase transitions. Similar defects are apparent in lines over-expressing a tasiR-ARF resistant *ETT* transgene showing that the phenotype of *sgs3*, *rdr6* and *zip* mutants is dependent on ectopic accumulation of *ETT* (Fahlgren *et al.* 2006; Hunter *et al.* 2006).

Unlike their *Arabidopsis* counterpart, mutations in the *SGS3* orthologue of maize called *leafbladeless1* (*lbl1*), result in an abaxialisation of leaves and a reduction in *HD-ZIPIII* expression (Nogueira *et al.* 2007). The lack of similar polarity defects in *Arabidopsis* may indicate that other pathways, in addition to the tasiR-ARFs, are involved in regulating abaxial cell identity. Indeed when mutants in the ta-siRNA pathway are combined with those affecting activity of the *AS1-AS2* pathway, subtle leaf polarity defects are observed (Li *et al.* 2005; Garcia *et al.* 2006; Xu *et al.* 2006).

As much of the molecular machinery involved in the biogenesis of mobile siRNAs is shared with ta-siRNA pathway (Voinnet 2005), it has been suggested that tasiR-ARFs may also be mobile and thus capable of functioning as the 'Sussex' signal (Nogueira *et al.* 2007). Again the evidence supporting this proposal is far from conclusive, especially as expression of the *Arabidopsis TAS3* and the maize tasiR-ARFs may actually be limited to the adaxial domains of organs (Garcia *et al.* 2006; Nogueira *et al.* 2007).

Auxin – A ubiquitous signal in leaf development

In addition to its role in regulating phyllotaxy, auxin also functions with the *AS1-AS2* pathway to prevent *KNOX* genes from being expressed in developing leaves (Hay *et al.* 2006). Enhanced lobbing caused by increased *KNOX* misexpression occurs when *as1* plants are combined with mutations that disrupt polar auxin transport or auxin signalling. In addition ectopic stipules, which are normally restricted to the base of the leaf, arise in the sinus of each lobe suggesting the presence of ectopic leaf/SAM boundaries (Hay *et al.* 2006). Supporting the functional overlap in these two pathways is the observation that ectopic *BREVIPEDICELLUS* (*BP*) expression is detectable in mutants defective in polar auxin transport and auxin signalling as well as in

plants treated with the polar auxin transport inhibitor TIBA (Hay *et al.* 2006).

The role of auxin in regulating cell fate is not limited to *KNOX*, but also includes the control of organ polarity in *Arabidopsis*. For instance, *HD-ZIPIII* expression in the SAM, incipient organ primordia and pro-vasculature largely overlaps with cells expressing PIN1, raising the possibility that auxin flow and *HD-ZIPIII* expression are linked (Baima *et al.* 2001; Otsuga *et al.* 2001). This possibility is further strengthened by the observation that PIN1 protein distribution is altered in *phb phv rev* embryos, such that the characteristic subcellular reversals in PIN1 distribution marking the formation of the cotyledons does not occur in these lines (Izhaki and Bowman 2007). A failure to undergo reversals presumably leads to an accumulation of auxin in the apical region of the embryo and as a consequence, the replacement of the SAM with a single radial cotyledon (Emery *et al.* 2003; Izhaki and Bowman 2007). As *HD-ZIPIII*s antagonise *KAN*s, the defects in *hd-zipIII* mutant embryos may simply be an indirect consequence of ectopic *KAN* activity. Consistent with this scenario, PIN1 reversals and bilateral symmetry are restored in *hd-zipIII*s mutants lacking *KAN* activity (Izhaki and Bowman 2007). A more direct link between *HD-ZIPIII*s and auxin comes from the observation that expression of an *HD-ZIPIII* family member, *ATHB8*, is responsive to auxin (Baima *et al.* 1995) and that expression of other family members is associated with vascular differentiation, a process that is regulated in part by auxin (Ohashi-Ito *et al.* 2005). Finally, analysis of *PIN1* and *REV* expression in the SAM and developing floral bud primordia show that *REV* expression lags slightly behind PIN1, giving the impression that *REV* expression responds to the flow of auxin rather than directing it (Heisler *et al.* 2005). Having *HD-ZIPIII* activity responsive to auxin is an attractive scenario as auxin moves through the L1 layer and could therefore be considered a candidate for the 'Sussex' signal.

However, arguing against such a simple hierarchical arrangement is the finding that vascular defects in *rev* mutants are correlated with changes in auxin transport and a reduction in *PIN* expression (Zhong and Ye 2001). Thus at present, the possibility that *HD-ZIPIII*s regulate auxin movement cannot be excluded. The recent characterisation of two closely related *APETALA2*-domain transcription factors, *DORNROSCHEN* (*DRN*) and *DRN*-like (*DRNL*) provides another tentative link between *HD-ZIPIII*s and auxin. This study showed that the embryo patterning defects arising from the loss of *DRN* and *DRNL* activity are associated with altered auxin movement and/or responses (Chandler *et al.* 2007). As HD-ZIPIIIs both physically and genetically interact with DRN/DRNL, it is possible that these genes regulate auxin responses together during embryo development (Chandler *et al.* 2007).

The second observation linking auxin to polarity comes from the recent discovery that *kan1 kan2 kan4* plants have leaves arising from the seedling hypocotyl (Izhaki and Bowman 2007). As the presence of such leaves correlate with more basal accumulation of PIN1 in *kan* mutant embryos, it has been proposed that *KAN*s may regulate the timing and/or position of auxin maxima during embryogenesis (Izhaki and Bowman 2007). This hypothesis is further supported by the observations that ectopic expression of the *KAN*s alters PIN1 expression in *phb phv rev* embryos (as described above). Interestingly, the role of *KAN*s in regulating auxin movement does not seem to be confined to embryonic development. For instance, the presence of ectopic leaf blades on the abaxial surface of *kan* mutant leaves has been interpreted as *de novo* leaf primordia (Eshed *et al.* 2001, 2004) as their formation is associated with the presence of ectopic auxin maxima (Izhaki and Bowman 2007). Moreover, both loss and gain-of-function *kan* mutants affect vascular patterning, a process that is driven in part by auxin (Eshed *et al.* 2001; Kerstetter *et al.* 2001; Emery *et al.* 2003). Collectively then, these observations suggest that the *KAN*s function to restrict the flow of auxin in various tissues of the plant. How this is achieved is presently not well understood, but one attractive scenario is that *KAN*s directly regulate *PIN* expression (Izhaki and Bowman 2007).

An alternative possibility is that *KAN*s are part of the complex feedback mechanism that regulates the expression and/or subcellular distributions of PIN proteins (Vieten *et al.* 2007). It is now well established that auxin regulates *PIN* expression and protein localisation through the AUX/IAA-ARF pathway (Vieten *et al.* 2005; Sauer *et al.* 2006), in addition to directly altering the stability of PIN proteins in the cell (Paciorek *et al.* 2005; Abas *et al.* 2006). Given that *KAN*s promote abaxial cell identity in parallel with two *ARF*s - *ETT* and *ARF4* (Pekker *et al.* 2005), it is possible that these proteins function together to regulate the flow of auxin during leaf development (Izhaki and Bowman 2007). One consequence of regulating auxin flow is that *KAN*s and *ARF*s indirectly control genes that are responsive to auxin. Candidates for these genes include miR165/miR166 that target the *HD-ZIPIII*s, as auxin response elements have been identified in the promoters of their precursors (Chitwood *et al.* 2007). Although this model is plausible, it should be pointed out that despite PIN1 accumulating on the abaxial side of developing primordia, there is no direct evidence for an abaxial gradient of auxin or for that matter any evidence that auxin regulates the activity of *ETT* and *ARF4* in leaves. In addition, auxin response elements identified in the promoters of miRNA precursor genes have not been shown to be functional.

Thus while there is little doubt that auxin is functioning as a signal during leaf development, its not yet possible to conclude whether it's varied functions also include being the elusive 'Sussex' signal regulating adaxial cell identity. Given the complexity of pathways regulating auxin movement, it may take some time to resolve where auxin fits in the hierarchy of signals regulating organ polarity.

Pore Signals – Role of receptors in epidermal patterning

In recent years much progress has been made in understanding how endogenous and environmental signals influence the development of stomata, tiny epidermal pores that regulate both the uptake of CO_2 for photosynthesis and the loss of water vapour into the atmosphere. Their suitability for study arises from the fact that it is easy to identify mutants that either have too few or too many stomata, or which have changes in stomatal spacing.

Stomatal development is characterised by a stereotypic series of asymmetric and symmetric cell divisions that begins with an epidermal meristemoid mother cell (MMC) dividing into daughter cells of unequal size (**Fig. 4A**; see Bergmann and Sack 2007). The smaller of these two cells, called a meristemoid, may either undergo further rounds of cell divisions (amplifying cell divisions) or directly differentiate into an oval guard mother cell (GMC). The GMC then undergoes a single symmetric cell division to form the two guard cells that surround the stomatal pore. Adjacent daughter cells will either differentiate into epidermal pavement cells or divide asymmetrically to form additional meristemoids. Although stomatal densities vary according to the position of the epidermis and organ type, one or more non-stomatal cells almost always separate stomata from each other (Sachs 1991). The one-cell-spacing pattern arises because a signal produced by a stoma or a stomatal precursor determines the plane of cell division in neighbouring MMCs (Geisler *et al.* 2000). As a result, the cell arising next to the stoma is always the larger daughter cell, ensuring that the new meristemoid is furthest from existing stomata.

Short-range signals determine stomatal spacing

Several *Arabidopsis* mutants in which the pattern of stomatal spacing is disrupted have recently been identified.

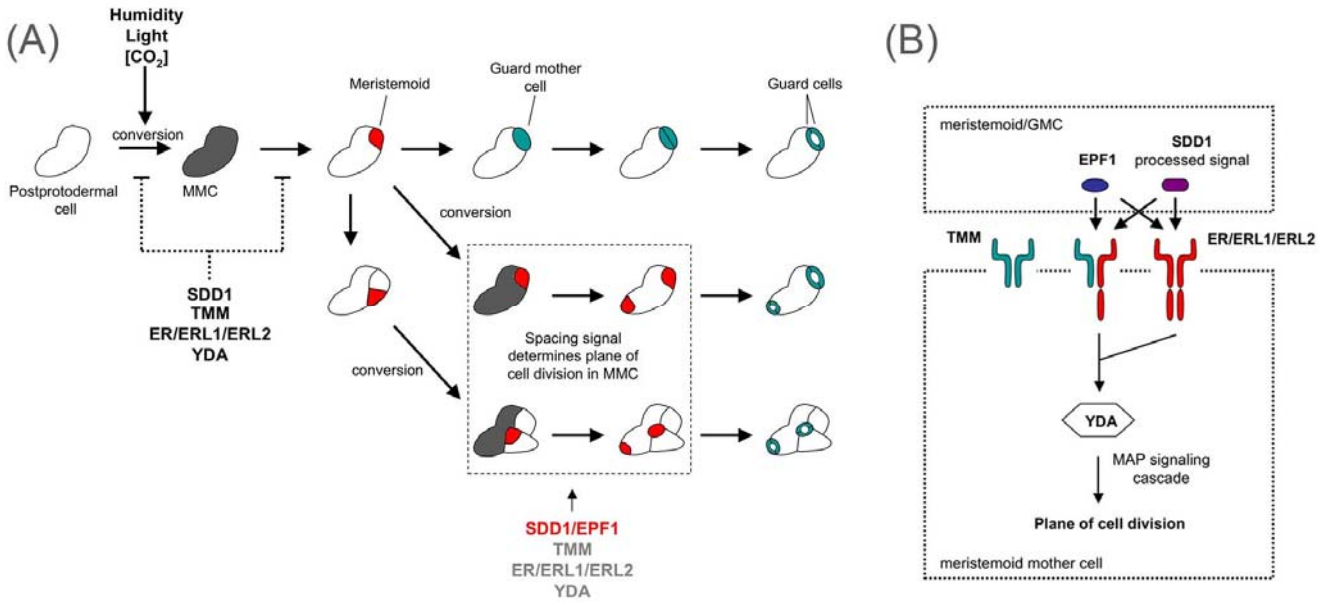

Fig. 4 Genetic pathways regulating signalling during stomatal development in *Arabidopsis*. (**A**) The conversion of a postprotodermal cell into a meristemoid mother cell (MMC) is influenced by a number of environmental factors ranging from humidity to the concentration of atmospheric CO_2. In addition, a small family of receptor-like kinases (ER, ERL1, ERL2), a receptor-like protein (TMM), a putative ligand generated by the SDD1 encoded protease and a MAP kinase pathway are all likely to function together to either limit MMC formation or the asymmetric cell division that occurs once a MMC has formed. The MMC divides asymmetrically to form a small meristemoid (red cell) and a larger daughter cell. The meristemoid then either becomes a guard mother cell (GMC) and divides symmetrically to form the guard cells (top pathway) or undergoes several rounds of asymmetric divisions before differentiating into a GMC (bottom pathway). In addition, the large daughter cell may also be converted into a MMC. In this case the plane of cell division occurring in this new MMC cell is determined by signals received from the adjacent meristemoid. The signal generated by the meristemoid may either be the small EPF1 protein or a ligand generated by SDD1. Defects in these genes or those involved in perceiving the signal – TMM, ER, ERL1, ERL2 and YDA – result in aberrant spacing of stomata leading to clustering. Redrawn from (Bergmann and Sack 2007). (**B**) Molecular basis of stomatal spacing signalling. The LRR-receptor-like kinase ER, ERL1 and ERL2 are shown in red on the surface of the MMC cell. They possess an extracellular LRR domain and an intracellular kinase domain. *TMM* encodes a protein with a LRR domain but lacks the internal kinase domain. It is proposed that TMM may dimerise with members of the ER family, forming active signalling complexes on the surface of the MMC. It is also possible that ER family members dimerise between themselves, although at present there is no experimental evidence to support such interactions. These signalling complexes presumably recognise ligands secreted by the adjacent meristemoid. Candidates for the ligand include the small secreted EPF1 protein (blue oval) and the unidentified protein produced following SDD proteolysis (purple box). Once activated, the membrane bound signalling complex triggers a MAP kinase signalling cascade that includes the MAPKKK protein YDA.

One of these, *too many mouths* (*tmm*), not only has more stomata than normal but also forms clusters of stomata with no intervening cells (Yang and Sack 1995; Geisler *et al.* 1998). These defects arise from an increased recruitment of cells into the stomatal developmental pathway and a failure to correctly orient the division of MMCs (Geisler *et al.* 2000). Subsequent cloning of *TMM* showed that it is related to a large class of leucine-rich repeat receptor-like kinases (LRR-RLKs) that have an extracellular Leucine Rich Repeat domain, a transmembrane domain and a cytoplasmic kinase (Nadeau and Sack 2002). Unlike these proteins however, TMM lacks the cytoplasmic domain and thus presumably cannot initiate an intracellular signalling response (Nadeau and Sack 2002). Instead these truncated proteins, called LRR receptor-like proteins, are thought to function by pairing with LRR-RLKs leading to the formation an active multimeric signalling complex (Torii 2004).

While the LRR-RLKs interacting with TMM has not been unambiguously identified, likely candidates include members of ERECTA (ER) family of LRR-RLKs. *ER* and the closely *ERECTA-LIKE 1* (*ERL1*) and *ERECTA-LIKE 2* (*ERL2*) genes have diverse roles in plant development that also include regulating stomatal patterning (Shpak *et al.* 2005). Although both *TMM* and members of the *ER* family are expressed in the same tissue (Nadeau and Sack 2002; Shpak *et al.* 2005), there is at present no data to support the view that these proteins physically interact *in vivo*. In addition, analysis of the *tmm* and *er erl1 erl2* triple mutant phenotypes shows that these proteins have opposing roles in some tissues of the plant, which argues against a simple model of interaction. Consequently, further work is required to show that these proteins function together in the regulation of stomatal spacing.

Given that TMM is activated by a ligand, it is likely that mutations in this ligand or in the pathway producing the ligand will result in a phenotype that is similar to *tmm* plants. One such mutant, *stomatal density and distribution* 1 (*sdd1*) affects the activity of a subtilisin-like serine protease, suggesting that SDD1 may proteolytically process the ligand that is recognised by TMM (Berger and Altmann 2000). Consistent with this possibility, SDD1 is expressed and secreted from meristemoids and GMCs, cells predicted to be the source of the spacing signal (von Groll *et al.* 2002). More telling is the finding that *SDD1* causes a reduction of stomata formation when over-expressed and that this phenotype is dependent on TMM activity. Although these data point to *SDD1* being in the same pathway as *TMM*, subtle differences between the *sdd1* mutant phenotype and those of *tmm* and the *er/erl* mutants suggest that *SDD1* may regulate an overlapping but distinct pathway involved in stomatal spacing.

A potential TMM ligand was recently identified in a screen designed to test the role of secreted peptides in plant development. Of the 153 peptides that were over-expressed in plants, one caused a severe reduction in stomatal density (Hara *et al.* 2007). Mutations in the gene encoding this peptide, called *EPIDERMAL PATTERNING FACTOR 1* (*EPF1*), have increased stomatal density and display stomatal clustering identical to that seen in *tmm* and the *er erl1erl2* mutants. Although the reduction in stomatal density resulting from *EPF1* over-expression is dependent on TMM activity, it apparently does not require SDD1, suggesting that the EPF1 peptide is probably not the target of SDD1 proteolysis (Hara *et al.* 2007). These observations raise the intriguing possibility that two distinct signalling pathways may be in-

volved in regulating stomatal spacing or that TMM is activated by other ligands requiring SDD1 processing (**Fig. 4B**).

How perception of the EPF1/SDD1 signal by the MMC influences the plane of cell division is currently not known, although it has been suggested that a MAP kinase signalling cascade may be involved (Bergmann *et al.* 2004). This is based on the finding that mutations in a member of the mitogen-activated protein kinase kinase kinase (MAPKKK) family called *YODA* (*YDA*) have phenotypes that are similar to *tmm* and *er erl1 erl2* mutants (Bergmann *et al.* 2004; Shpak *et al.* 2005). Consistent with the hypothesis that these genes function in a common pathway is the finding that a constitutively active YDA suppresses the *tmm* phenotype, showing that YDA is likely to function downstream of TMM (Bergmann *et al.* 2004). However these observations do not rule out the possibility that YDA is part of a parallel pathway generating the spacing signal. Future studies are therefore needed to establish whether TMM and/or members of the ER family physically and biochemically interact with YDA.

MAPKKKs function by sequentially activating MAP kinase kinases (MAPKKs) and MAP kinases (MAPKs) in a signalling cascade. In a recent report it has been shown that *Arabidopsis* plants defective in two MAPKKKs (MKK4/MKK5) or two MAPKs (MPK3/MPK6) produce organs with clustered stomata (Wang *et al.* 2007). Previous work has shown that MKK4 and MKK5 function upstream of MPK3 and MPK6, suggesting that they are all components of the same signalling cascade (Ren *et al.* 2002; Liu and Zhang 2004). YDA has been placed at the head of this cascade based on two lines of evidence. The first shows that the *yda* phenotype can be suppressed when the tobacco orthologue of the MKK4/MKK5, NtMEK2, is expressed in the epidermis (presumably because signalling has been restored) and the second shows that MPK3 and MPK6 are both activated in plants expressing a constitutively active YDA (Wang *et al.* 2007). Given the number of MAP kinases in the plant genome, and the fact that they function in a wide range of processes, it is possible that other MAPKKs and MAPKs are also involved in transducing positional information in the epidermis. The next step in elucidating stomatal patterning will be the identification of genetic pathways targeted by the MAP kinase cascade, as these ultimately regulate the orientation of cell division in MMC.

Long range signals in stomatal development

Levels of atmospheric CO_2, light intensity and humidity all have a major impact on stomatal density. For instance when levels of CO_2 are elevated, stomatal density decreases and *vice versa*. Given this link, botanists have used the stomatal index of fossilised leaves to infer the likely atmosphere concentration of CO_2 in prehistoric times (Woodward 1987; Beerling 2002). Interestingly, perception of both CO_2 concentration and light intensity occurs in mature leaves and is then relayed systemically to developing leaves (Lake *et al.* 2001; Thomas *et al.* 2004). While the nature of this signal and how it is relayed through the plant are presently not known, characterisation of the *HIGH CARBON DIOXIDE 1* (*HIC1*) gene of *Arabidopsis* suggests that it may be a component of this signalling network (Gray *et al.* 2000). This is based on the observation that stomatal development in *hic1* mutant leaves is unresponsive to elevated concentrations of CO_2. Intriguingly *HIC1* is involved in long-chain fatty acid synthesis that is required for the formation of the plant cuticle, suggesting that surface covering of the leaf is either involved in CO_2 perception or transmission of the systemic signal (Gray *et al.* 2000). Other candidates for the signal include the plant hormones ethylene, ABA, gibberellins and cytokinins, which have all been implicated in responses to environmental cues. Indeed recent work has shown that stomatal development is influenced by several of these hormones, although whether these hormones are functioning as a long-range signal or more locally remains to be tested (Saibo *et al.* 2003; Kazama *et al.* 2004).

CONCLUSION

Positional cues play an important role in regulating cell fate decisions in both plant and animal development. While the developmental programs operating in both kingdoms have separate evolutionary origins, current evidence suggests that there are extensive mechanistic similarities in the perception and generation of such signals. For instance, the plant hormone auxin promotes pattern formation along a concentration gradient, acts directly on a single cell and regulates gene expression in a dose-dependent manner; features that are shared with animal morphogens. However despite these similarities, there is still some doubt about whether auxin can be considered a true morphogen. This is largely due to the difficulty of showing that auxin has all of the properties expected of a morphogen in any one experimental system. For example, recent studies have used PIN1 subcellular localisation to infer that auxin gradients play a major patterning role in the SAM of higher plants. Consistent with this scenario, disrupting auxin movement within the apex of a plant either pharmacologically or genetically results in altered cell identity. While these data support the idea of an auxin gradient, presence of such a gradient has not been experimentally confirmed. In addition, it is currently not known how auxin regulates cell fate within the SAM. It is possible that auxin promotes leaf formation in conjunction with other morphogenic signals or even downstream of such signals. Regardless of these ambiguities, it is clear that auxin is the closest thing a plant has to a morphogen.

Finding that receptors and their ligands are involved in epidermal patterning during leaf development further supports the argument that animals and plants share common signalling mechanisms. Although there are only a few known cases of receptor/ligand combinations involved plant development, this list will undoubtedly grow as the function of the many uncharacterised receptors in the *Arabidopsis* genome are examined in detail. In addition, finding that a MAP signalling cascade lies downstream of the receptors involved in epidermal patterning, suggests that the similarity between plant and animal signalling extends to the intracellular transduction of signals.

The characterisation of pathways involved in adaxial/abaxial patterning has uncovered components of a signalling pathway that may be unique to plants. Early surgical experiments suggested that a meristem derived signal –the 'Sussex' signal- promotes adaxial cell identity in adjacent organ primordia. Recent studies in *Arabidopsis* and maize have identified several small non-coding RNAs (siRNAs/miRNAs) that have the potential to function as this signal. However, as data supporting movement of these small RNAs during organ formation is equivocal, further work is required to resolve their role in polarity determination. An alternative, but not necessarily mutually exclusive possibility is that the meristem signal is a hydrophobic molecule such as a sterol. This hypothesis is based on the similarity between the HD-ZIPIIIs and the highly conserved START sterol-binding domain present in animal nuclear receptors. Identifying the ligand that interacts with the HD-ZIPIIIs may well provide a further mechanistic link to animal signalling pathways.

A major challenge for the future is to understand how these signalling pathways are integrated into a cohesive network that produces lateral organs of particular size and shape according to their position and identity. This will require the development of techniques that can monitor the activities of these signalling pathways more accurately than is possible with current technology. As these techniques are developed, our understanding of plant development is set to take another leap forward.

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