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REVIEW

The Sussex signal: insights into leaf dorsiventrality

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ABSTRACT

The differentiation of a leaf – from its inception as a semicircular bulge on the surface of the shoot apical meristem into a flattened structure with specialized upper and lower surfaces – is one of the most intensely studied processes in plant developmental biology. The large body of contemporary data on leaf dorsiventrality has its origin in the pioneering experiments of Ian Sussex, who carried out these studies as a PhD student in the early 1950s. Here, we review his original experiments in their historical context and describe our current understanding of this surprisingly complex process. Finally, we postulate possible candidates for the 'Sussex signal' – the elusive meristem-derived factor that first ignited interest in this important developmental problem.

KEY WORDS: Apical meristem, Leaf dorsiventrality, Adaxial-abaxial, miRNA, Pattern formation

Introduction

In 1949, Ian Sussex moved from New Zealand to the University of Manchester, UK, and started his PhD thesis, studying the shoot apical meristems of potato tubers. This was before the double helix structure of DNA had been uncovered, and long before the rise of developmental genetics. What was known about the angiosperm shoot apex in 1949? As far as genetics was concerned, ploidy chimeras had been used productively to determine the division patterns and fates of meristem cells. Mutants featured hardly at all in plant research at that time and, of the plant hormones, only auxin was well known; cytokinin, gibberellin and abscisic acid were still waiting to be discovered. However, over the preceding 100 years, meristems had been observed in great anatomical detail. It was recognized that the apical meristem has a layered structure and that descendants of the outer cell layer produce the epidermis of the leaves and stem, whereas internal meristem layers give rise to the various internal tissues. Comparative developmental research was also not limited to one or a few model organisms but covered the breadth of the plant kingdom. While most studies were purely descriptive, experimental work had begun by the end of the 19th century. One of the first experiments was the bisection of a shoot apex by a median longitudinal cut, after which the two halves regenerated (Lopriore, 1895). Another study briefly mentioned that, after injuring the center of the apex by pricking it with a needle, regeneration followed in nearly every case (Pilkington, 1929). Surgical manipulations had also been performed, with the goal of understanding the mechanism of phyllotaxis. These experiments, pioneered by Mary Snow (née Pilkington) and her husband Robert, showed that separating a leaf initial from the meristem by a tangential incision could change the position of subsequent leaf primordia (Snow and Snow, 1932a,b). The interpretation of such experiments was that a new leaf forms as far away as possible from the inhibiting influences of previous leaves. Nothing was known about the identity of such inhibiting influences and most publications did not even speculate on their nature.

In 1949, plant developmental biology was still mostly descriptive and comparative, and experimental approaches were relatively few. Some of the most interesting experimental work was being carried out by Claude W. Wardlaw, Professor of cryptogamic botany at the University of Manchester, UK. Wardlaw had followed up on the work of the Snows and performed a series of ingenious experiments on the shoot apex of ferns (Wardlaw, 1947). He became supervisor to the 22-year-old PhD student from New Zealand. Today, supervisors assign specific projects to new students – most likely within the framework of their grant obligations – that ideally introduce them to a variety of skills and concepts, and provide them with secure publications. Why did Wardlaw give his new student a project on potato rather than have him follow up on the exciting work with ferns? As Sussex recalls in his autobiography, Wardlaw did not assign him a project, did not even give him general directions; his advice was simply 'Go away and do something' (Sussex, 1998). Awesome! Sussex followed the advice of his mentor and chose to do experiments, not on the fern apex, but on an angiosperm. He selected the potato tuber because the system seemed ideal for the type of experiments that he wanted to do. The written account of this work on shoot morphogenesis is his doctoral thesis, submitted in October 1952 (Ian M. Sussex, Experimental and analytical studies of morphogenesis in the shoot apex of potato *Solanum tuberosum* L, PhD thesis, University of Manchester, 1952).

Here, we summarize Sussex's early work, which laid the foundation for a new field in plant developmental biology that focused on leaf dorsiventrality. We then provide an overview of the current state of the field, highlighting the molecular and cellular factors that contribute to the establishment, resolution and maintenance of what is now called adaxial-abaxial leaf polarity.

Sussex's early work and the origins of the 'Sussex signal'

Sussex's PhD thesis is remarkable for the quality of both the science and the writing. The first, and by far largest, chapter of the thesis describes the normal development of the potato shoot. It is the solid basis for subsequent work in which normal development is experimentally disrupted. Particularly interesting is the Discussion part at the end of the chapter. It considers the possible interpretations and implications of the experiments presented in the next chapters. For instance, it discusses potential gradients of nutrients and hormones as developmental signals, and even considers that such gradients could occur in opposite directions.

In the second chapter, Sussex repeats the Pilkington puncture experiment (Fig. 1A,B). This work shows that cells adjacent to the puncture become the new center of growth, which continues to form leaves in normal phyllotactic sequence, as if nothing had happened.

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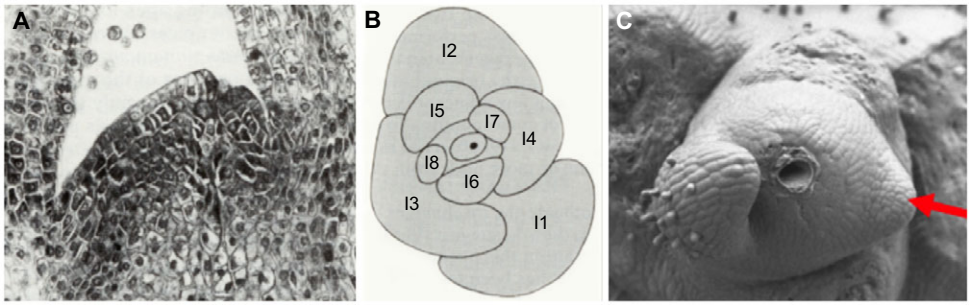

Fig. 1. Leaf development is not affected by stem cell ablation. (A) Cross section through a potato shoot apex after puncture with a fine needle. (B) Diagram of the shoot apex after puncture with a 17-µm-diameter needle, illustrating that leaf development proceeds normally. (C) Initiation of a correctly positioned leaf primordium (red arrow) 3 days after laser ablation of the stem cells. A,B are reproduced from Ian M. Sussex, Experimental and analytical studies of morphogenesis in the shoot apex of potato *Solanum tuberosum* L, PhD thesis, University of Manchester, 1952; C is reprinted with permission from Reinhardt et al. (2003).

These experiments were mostly forgotten, but around the year 2000, advances in stem cell biology led to discussions about the analogies between plant meristems and animal stem cell niches (Weigel and Jurgens, 2002). As a result of these discussions, the apical initials in the shoot meristem are now generally referred to as stem cells. In this new context, it seemed surprising that the removal of all stem cells would have no dire consequences. Indeed, a more precise ablation than was possible in the mid-1900s showed that, after laser ablation of all stem cells, leaf initiation continued without a pause (Reinhardt et al., 2003). Both experimental data and computational modeling indicated that peripheral cells re-acquire stem cell fate and re-establish a functional meristem (Reinhardt et al., 2003; Heisler and Jonsson, 2006) (Fig. 1C). This highlights the remarkable regenerative capacities of plant stem cells compared with their animal counterparts.

In the third thesis chapter, the emphasis shifts from regeneration to morphogenesis. Its Introduction starts from the perspective of function: ‘An organ which displays its maximum assimilating surface to the light, and at the same time possesses a high surface-volume ratio, would be the most efficient for photosynthesis. Such an organ is the dorsiventral leaf’. It then goes on to focus on the developmental perspective: ‘Dorsiventrality, which confers on the mature leaf its special physiological properties, is attained by the primordial leaf, while still enclosed in the apical bud’. This new theme follows from preliminary observations in which some incisions in the apex cause leaves to grow out as tubular (centric, radialized) structures. Sussex begins by asking what the cause of this loss of dorsiventrality could be. He hypothesizes that it could be the interruption of a signal, a hormone moving down from the meristem, or nutrients moving upwards, or a signal moving laterally between leaf primordia.

To discriminate between these hypotheses, he performed a set of precise tangential incisions between the meristem and the position of the incipient primordium (I1), defined as the stage just before the leaf becomes visible as a bulge on the surface of the apical meristem (Reinhardt et al., 2005). It is worth taking a closer look at these experiments, which are summarized in Fig. 2. Panels A and B of this figure, demonstrate the basic phenomenon. The separation of I1 from the apical meristem (A) leads to a centric or radialized primordium (I1c) instead of a normal dorsiventral primordium (I1d). In panel C, the I1 has been separated from the two older primordia, P1 and P2, but a corridor with the meristem is retained, resulting in a normal leaf. Conversely, in panel D, the I1 has been separated from the apical meristem but not from the older primordia leading to the emergence of a radial leaf. Taken together, these experiments point at the apical meristem as the origin of dorsiventrality. The results shown in panels E and F add a further layer of proof. Sussex performs more subtle incisions that induce an adventitious (a) meristem. As long as I1 is in contact with this meristem, it develops as a dorsiventral leaf that is oriented towards the induced meristem. But when the connection between I1 and the adventitious meristem is severed, I1 develops as a radialized organ. From these experiments, the logical conclusion is that a signal moving from the apical meristem towards the incipient leaf induces dorsiventrality. In 2005, Didier Reinhardt performed similar experiments in tomato using infrared laser ablation and drew essentiality the same conclusion as Sussex had done 50 years earlier (Reinhardt et al., 2005). In addition, he showed that superficial incisions can induce radialization (Fig. 3), suggesting that the signal moves through the L1 surface layer.

This leaves open the issue of whether a potential signal from below the primordium is involved. The thesis mentions experiments with incisions made below the primordium that have no effect on dorsiventrality. Even after inserting a piece of mica into the cut to prevent regrafting, the leaf is always dorsiventral. The simple inference from this experiment would be that there is no (nutritional) signal moving upwards. However, Sussex is careful about the interpretation of this experiment, suggesting that a nutritional signal might still move around the incision.

The Discussion section of chapter 3 is vague about the nature of what we now call the Sussex signal. Similarly, the short paper about this work (Sussex, 1951), which was published a year before the thesis was, ends with the conclusion: ‘… the production of centric organs is closely related to a cessation of apical growth, or to an elimination of its effects’. After the lucid statement of the hypothesis in the Introduction, this restraint seems a little odd. It may have been related to the criticism leveled at the newcomer by the established researchers in the field. Each of Sussex’s Nature papers drew a critical response from the Snows, who could not repeat the potato experiments and reported different results in *Epilobium* (Snow and Snow, 1954a,b). In essence, they questioned the idea of a mobile signal and suggested that radialization occurred as a result of lack of space; that is, dorsiventrality is not induced by an external signal but is an intrinsic property of the leaf itself. Sussex responds to these criticisms and, after doing more experiments, concludes that ‘Dorsiventrality is determined not by the subjacent part of the stem, or the adjacent primordia, but by the apical meristem’. However, he does not speculate about the identity of the signal.

Dorsiventrality – or adaxial-abaxial leaf polarity, as it is most often called now – has become a vibrant field of research. The

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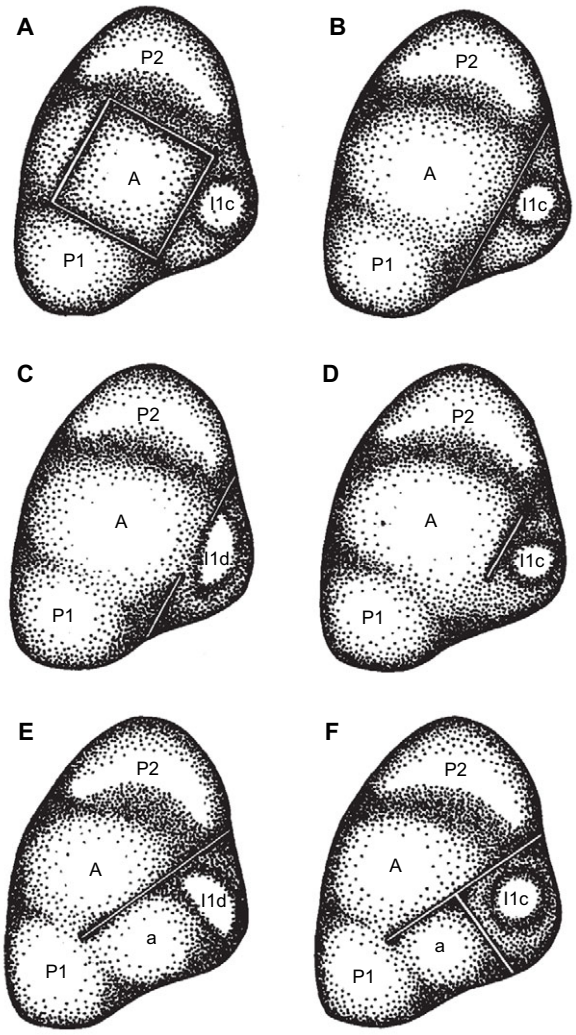

Fig. 2. Dorsiventrality is determined by the shoot apical meristem. Illustrations of the shoot apex after incisions. (A) Four incisions separating the apical meristem (A) from the surrounding leaf primordia (P1 and P2) cause the incipient primordium (I1) to develop into a centric organ (I1c). (B) A single incision separating I1 from the apical meristem causes it to stay centric. (C) A small corridor left open between I1 and the apical meristem is sufficient to establish a dorsiventral organ (I1d). (D) A shorter incision that separates I1 from the apical meristem but maintains the connection between I1 and older primordia causes I1 to develop into a centric organ. (E) When I1 is separated from the apical meristem but develops in contact with an induced adventitious meristem, it will establish dorsiventrality. (F) Incisions that separate I1 from both the apical and the induced adventitious meristem cause I1 to develop into a centric organ. For more details, see the text. Abbreviations: d, dorsiventral primordium; c, centric or radialized primordium; A, main apex; a, induced apex. Reproduced from Ian M. Sussex, Experimental and analytical studies of morphogenesis in the shoot apex of potato *Solanum tuberosum* L, PhD thesis, University of Manchester, 1952.

insightful experiments described by Sussex in his PhD thesis and accompanying publications remain the cornerstone of this field. In the next sections, we discuss how the leaf polarity field has progressed since these early days and provide an overview of its present state.

The molecular genetics of leaf polarity

The first molecular insight into how tissues become polarized along the adaxial-abaxial axis came from characterization of *phantastica* (*phan*) mutants in *Antirrhinum*. Severe *phan* mutant leaves

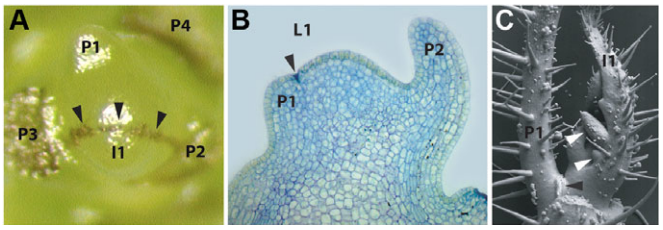

Fig. 3. The Sussex signal may move through the L1 surface layer in tomato. (A) Top view of a tomato shoot apex with a superficial incision (denoted by arrows) separating the meristem from the incipient (I1) primordium. (B) A longitudinal section through the apex shows that the incision ablates only the L1 layer. (C) Such incisions at the P1 stage also causes abaxialization, as can be seen from the absence of leaflets and the presence of abaxial-type trichomes on both sides of the developing primordium. Black arrow, ablation scare; white arrows, leaflets on a normal primordium. Reprinted with permission from Reinhardt et al. (2005).

resemble those observed in the earlier described surgical experiments and show radial symmetry with abaxial cell types encircling central xylem tissue (Fig. 4; Waites and Hudson, 1995). In contrast, weakly phenotypic leaves develop adventitious blade outgrowths on their upper surface that are associated with sectors of cells that have lost adaxial fate and instead have taken on abaxial identity. The range of *phan* phenotypes indicated a role for PHAN in adaxial cell fate specification, and led the authors to propose that extension of the leaf blade results from the juxtaposition of adaxial and abaxial tissues (Waites and Hudson, 1995). The prediction of this novel hypothesis is that a shift towards adaxial fate should also lead to loss of lamina extension. Indeed, in the *Arabidopsis phabulosa-1d* mutant, adaxial characters develop in place of abaxial leaf characters and these adaxialized leaves fail to develop leaf blades (McConnell and Barton, 1998).

The insightful recognition of leaf polarity phenotypes by Waites and Hudson (1995) provided a roadmap that opened up the field, resulting in the rapid identification of an impressive number of genes involved in adaxial-abaxial patterning. *PHAN*, which encodes a MYB transcription factor (Waites et al., 1998), proved to be part of an intricate gene regulatory network required for the acquisition and maintenance of leaf polarity. Integral to the polarity network are two sets of conserved transcription factors that promote either adaxial or abaxial fate and are expressed in complementary domains on the upper and lower side of the developing leaf, respectively (Fig. 5; see Husbands et al., 2009 for more details on individual polarity determinants). In *Arabidopsis*, the LOB domain transcription factor ASYMMETRIC LEAVES2 (AS2) acts in a complex with the PHAN ortholog AS1 to promote adaxial identity (Lin et al., 2003; Iwakawa et al., 2007; Husbands et al., 2015). In addition, members of the CLASS III HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIPIII) family, which in *Arabidopsis* includes *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*) and *REVOLUTA* (*REV*), specify adaxial fate (McConnell et al., 2001; Emery et al., 2003; Juarez et al., 2004a; Itoh et al., 2008). In contrast, members of the KANADI (KAN) and YABBY (YAB) gene families, along with *AUXIN RESPONSE FACTOR3* and *-4* (*ARF3* and *ARF4*), contribute to the specification of abaxial identity (Siegfried et al., 1999; Eshed et al., 2001; Kerstetter et al., 2001; Pekker et al., 2005).

In addition to these transcription factors, small RNAs form central network components (Fig. 5). For example, miR166, which accumulates on the abaxial side of the leaf, guides the cleavage of HD-ZIPIII transcripts, limiting the expression of these adaxial determinants to the top side of primordia (Juarez et al., 2004a;

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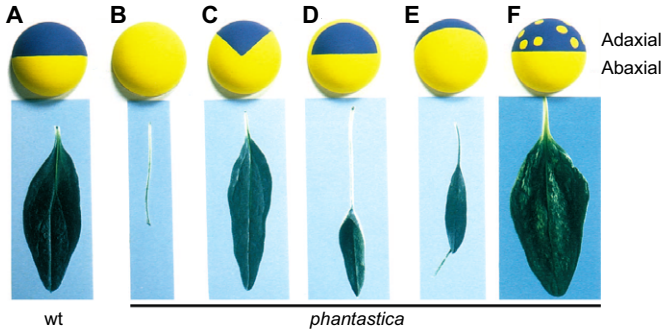

Fig. 4. Blade outgrowth occurs at the boundary between adaxial and abaxial domains. Patterns of adaxial-abaxial identity in early leaf primordia are depicted as spheres above the different leaf morphologies of wild type (A) and *phan* mutants (B-F). (A) Normal leaf primordia are partitioned into adaxial (blue) and abaxial (yellow) domains, with the boundary between these domains driving the flattened outgrowth of the leaf. (B) *phan* leaf primordia that are fully abaxialized develop into radial, needle-like leaves. (C-E) Reduction of the adaxial domain limits blade outgrowth to those parts of the primordium in which an adaxial-abaxial boundary occurs, resulting in leaves with a reduced blade region. (F) If cell fate is unstable and patches of abaxial cells form on the adaxial side of the primordium, outgrowths develop at the ectopic adaxial-abaxial boundaries surrounding these patches. Reprinted with permission from Waites and Hudson (1995).

Nogueira et al., 2009; Yao et al., 2009). Conversely, tasiARF, which is generated through the specialized *TAS3 trans*-acting siRNA pathway (see Chapman and Carrington, 2007 for details on this small RNA pathway) limits expression of the ARF3 and ARF4 targets to a precisely defined domain on the bottom side of developing primordia (Nogueira et al., 2007; Nagasaki et al., 2007; Chitwood et al., 2009; Yifhar et al., 2012; Petsch et al., 2015).

Interestingly, although the individual network components are highly conserved between angiosperms, the extent to which they contribute to adaxial or abaxial cell fate is not. For instance, *Antirrhinum phan* mutants show clear adaxial–abaxial polarity phenotypes, as do tobacco and tomato plants harboring mutations in *phan* orthologs, but *rough sheath2* from maize and *as1* from *Arabidopsis* do not (Timmermans et al., 1999; Tsiantis et al., 1999; Byrne et al., 2000; Kim et al., 2003a; McHale and Koning, 2004). Likewise, mutations affecting tasiARF biogenesis condition a strong abaxializing phenotype in maize, rice and tomato, but cause only subtle polarity defects in *Arabidopsis* (Nogueira et al., 2007; Nagasaki et al., 2007; Chitwood et al., 2009; Douglas et al., 2010; Yifhar et al., 2012; Dotto et al., 2014). The latter difference may be explained, in part, by variation in the spatiotemporal expression of pathway components across species; tasiARF, for example, acts in the incipient primordium in maize and rice but during later stages in *Arabidopsis* leaf development (see Husbands et al., 2009).

The degree to which different plant species depend on each of the network components in adaxial-abaxial leaf patterning may also reflect divergence in the nature or function of downstream targets. The YABBY genes, in this regard, present a most striking example. The function of YABBY genes and their abaxial-specific expression are conserved in *Antirrhinum* and tomato (Golz et al., 2004; Navarro et al., 2004; Kim et al., 2003b). In rice, however, YABBY homologs show a non-polarized pattern of expression (Yamaguchi et al., 2004; Tanaka et al., 2012), and in maize, YABBY expression is limited to the adaxial side of leaf primordia (Juarez et al., 2004b). In addition to promoting abaxial fate, YABBY genes are thought to act downstream from other polarity determinants to direct blade outgrowth at the adaxial-abaxial boundary (Eshed et al., 2004). This latter function appears to be conserved, but its contribution to abaxial fate obviously is not. Moreover, while the function of other polarity determinants in promoting either adaxial or abaxial fate is conserved, their input into the regulation of YABBY genes must have diverged during plant evolution. Many more targets acting downstream of the core polarity network have recently been identified in *Arabidopsis* (Reinhart et al., 2013; Merelo et al., 2013; Iwasaki et al., 2013; Huang et al., 2014; Xie et al., 2015) and it will be interesting to see the extent to which these are conserved.

Cellular properties of the adaxial-abaxial polarity network

Adaxial and abaxial cell fates are mutually exclusive. This was evident from early genetic analyses and explains the replacement of adaxial cell types with their abaxial counterparts in mutants such as *phan*, or the converse effects seen for loss-of-function mutations in genes promoting abaxial identity (e.g. Waites and Hudson, 1995; Timmermans et al., 1998; McConnell and Barton, 1998; Kerstetter et al., 2001; Eshed et al., 2004). Accordingly, adaxial and abaxial determinants employ a series of mutually antagonistic interactions to define opposing cell fates (Fig. 5). For instance, the *Arabidopsis* HD-ZIPIII and KAN proteins have opposite effects on a common set of direct and indirect targets, most notably genes in the auxin pathway (Fig. 5; Reinhart et al., 2013; Merelo et al., 2013; Huang et al., 2014; Xie et al., 2015). Although the precise contributions of auxin to leaf polarity remain unclear, the current data points to a role for auxin signaling at the adaxial-abaxial boundary, where it may coordinate the flattened outgrowth of the leaf blade in conjunction with the YABBY and WOX1 transcription factors known to drive this process (Eshed et al., 2004; Heisler et al., 2005; Wang et al., 2011; Nakata et al., 2012).

A more immediate and direct mechanism of mutual antagonism is achieved by repressive interactions between the polarity determinants themselves (Fig. 5). KAN1 is also a direct repressor of *AS2* (Wu et al., 2008; Merelo et al., 2013; Huang et al., 2014). This regulatory interaction can explain the dynamic pattern of *AS2* expression, which transitions from uniform to adaxialized as the incipient leaf grows out (Fig. 6; Iwakawa et al., 2007; Husbands et al., 2015). The interaction between KAN1 and *AS2* thus

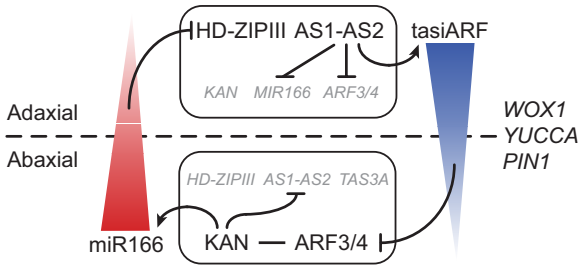

Fig. 5. The core adaxial-abaxial polarity network. The two sets of transcription factors at the core of the polarity network act cell autonomously. These employ antagonistic interactions and positive feedback regulation to define either adaxial or abaxial fate at the cellular level (boxes). Active proteins are indicated in black, repressed genes in gray. The clean separation of adaxial and abaxial identity at the domain level also requires positional information provided in part by opposing gradients of mobile miR166 and tasiARF. Expression of these small RNAs at their source is directly reinforced by core transcription factors, thus stabilizing the system. The core transcription factors also regulate genes at the boundary between the adaxial and abaxial domains, such as the transcription factor WOX1, and genes involved in auxin biosynthesis (*YUCCA*) and auxin transport (e.g. *PIN1*), that may drive outgrowth at the boundary. Only direct regulatory interactions between polarity determinants are shown. See text for more detail.

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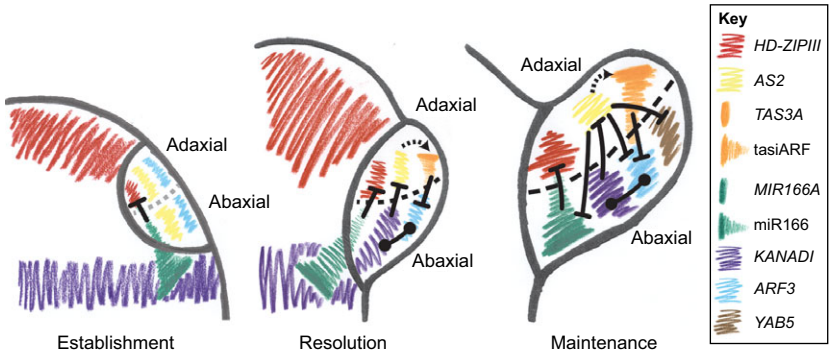

Fig. 6. Model describing the establishment, resolution and maintenance of adaxial-abaxial polarity in *Arabidopsis thaliana*. The incipient *Arabidopsis* primordium is polarized by miR166, which moves into I1 from its site of biogenesis in the internode. This primordium-independent source of mobile miR166 limits HD-ZIPIII activity to the adaxial side, establishing the adaxial-abaxial axis in the incipient primordium (left panel). As the primordium develops, polarity resolves from an externally to an internally patterned process as KAN proteins, which are initially excluded from the primordium at the I1 stage, become active on its abaxial side. KAN proteins then directly repress AS2, possibly in complex with the tasiARF target ARF3, thereby restricting the downstream effects of AS1-AS2 on their direct targets *MIR166A*, *YAB5*, *ARF3* and *TAS3A* to the adaxial side (middle panel). Once resolved, polarity is then stably maintained throughout primordium development by mutually antagonistic interactions and positive feedback regulation of polarity determinants at the cellular level, and opposing miR166 and tasiARF gradients at the domain level (right panel). T-bars denote direct repressive interactions, whereas the dotted arrows denote a non-repressive, possibly protective, interaction. The dumbbell denotes protein-protein interaction. Reprinted with permission from Husbands et al. (2015).

adaxializes AS1-AS2 complex activity, which, in turn, targets the promoters of *MIR166A* and *YAB5*, limiting their expression to the abaxial side. AS1-AS2 also binds the promoters of *ARF3* and the tasiARF precursor *TAS3A*. Interestingly, at *TAS3A*, AS1-AS2 appears to use a 'protective mechanism' to prevent downregulation of *TAS3A* expression by abaxial determinants, most likely ARF3 and KAN1 (Husbands et al., 2015). Indeed, to attain a clean separation of adaxial and abaxial fates, the polarity network must also include positive regulatory interactions that reinforce cell identity (Fig. 5). The direct regulation of *MIR166A* and *MIR166F* by KAN1 (Merelo et al., 2013) may provide another key example of this, especially considering the central role that miR166 plays in leaf polarity.

Mobile signals that polarize the leaf along the adaxial-abaxial axis

While such positive and negative interactions between adaxial and abaxial determinants ensure that the fate of an individual cell is robustly defined, these interactions are insufficient to create a precise separation of adaxial and abaxial identity at the domain level. The transcription factors described above do not move from cell to cell, indicating that their activities across the leaf must be patterned in response to mobile signals that instruct cells of their relative positions. Therefore, a key outstanding question in the field is how the adaxial and abaxial domains are initially established and then stably maintained throughout primordium development. The seminal contribution of Ian Sussex is the hypothesis that a mobile, meristem-borne signal provides positional information that delineates the adaxial side of leaves. Although the precise molecular nature of the Sussex signal remains a topic of much debate (see below), several mobile signals required for the establishment and/or maintenance of adaxial-abaxial polarity have been identified.

For example, two important mobile signals in leaf polarity are the small RNAs miR166 and tasiARF (Fig. 5; Chitwood and Timmermans, 2010). The biogenesis of tasiARF is restricted to the two adaxial-most cell layers of developing primordia (Chitwood et al., 2009; Nogueira et al., 2009). However, tasiARF then moves from this defined source of biogenesis and forms a concentration gradient across the leaf that dissipates abaxially. This gradient is read out as a sharply defined expression domain of its targets, *ARF3* and *ARF4*, on the bottom side of the leaf (Chitwood et al., 2009). Likewise, miR166 accumulates in a gradient across the leaf, but this originates from the abaxial epidermis of leaf primordia and limits the expression of HD-ZIPIII targets to the adaxial side (Juarez et al., 2004a; Nogueira et al., 2009; Yao et al., 2009). The stable division of leaf primordia into discrete adaxial and abaxial domains thus relies on opposing gradients of small RNAs that separate the expression patterns of transcription factors at the core of the polarity network and whose own precursor expression is then reinforced by components of that network (Fig. 5). This novel patterning mechanism suggests that small RNAs have morphogen-like properties (Skopelitis et al., 2012); this scenario is supported by mathematical modeling, which predicts that gradients of mobile small RNAs are uniquely suited to generate sharply defined boundaries of target gene expression (Levine et al., 2007).

The nature of the transcription factors at the core of the polarity network also suggests several additional candidate signals that might provide positional information to drive the establishment and/or maintenance of adaxial-abaxial polarity. One of these is auxin, but whether auxin acts as a positional signal to polarize the leaf or cooperates with factors such as WOX1 to drive outgrowth at the boundary remains to be resolved (Heisler et al., 2005; Wang et al., 2011; Nakata et al., 2012). A second candidate is a lipophilic signal, although the only evidence currently supporting such a signal is the fact that HD-ZIPIII proteins contain a START domain, which in animal systems is known to mediate high affinity binding of lipids (Schrick et al., 2014).

Hierarchical and sequential signaling characterizes adaxial-abaxial patterning

An important outcome of the surgical experiments – both those performed by Sussex and those carried out more recently – is the observation that the acquisition of polarity is gradual. Incisions at the I1 stage result in fully abaxialized leaves, whereas P1 primordia separated from the meristem show correct polarization at their distal tip yet remain abaxialized at their base (Reinhardt et al., 2005). Similar surgical incisions at the P2 stage do not condition defects in adaxial-abaxial patterning, suggesting that positional information from the meristem is only required over a short developmental

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window. This, in turn, predicts that the mechanisms required to maintain polarity are distinct from those required for its establishment and that these need time to resolve.

In line with this, the molecular interactions and expression dynamics of core polarity determinants in *Arabidopsis* have led to a model in which the sequential polarization of polarity factors divides adaxial-abaxial patterning into three phases: establishment, resolution and maintenance (Fig. 6; see Husbands et al., 2015 for details). Central to this model, which fully explains the available genetic data, is the notion that adaxial-abaxial polarity is established or pre-patterned in the incipient primordium by extrinsic signals and is resolved from an externally to an internally patterned process as the primordium develops. This notion is based on the observation that key polarity determinants are asymmetrically localized in the shoot apex prior to organ initiation. These include the HD-ZIPIII and KAN proteins, which localize to the tip of the meristem and the internode, respectively (McConnell et al., 2001; Heisler et al., 2005; Yadav et al., 2014). As a primordium initiates, HD-ZIPIII expression extends from the meristem tip into the incipient primordium. There, HD-ZIPIII expression is restricted to the adaxial side by miR166 (Juarez et al., 2004a; Nogueira et al., 2009; Yao et al., 2009). This small RNA, whose precursors are direct targets of KAN1 (Merelo et al., 2013), is generated in the internode from where it is thought to move into I1 to establish adaxial-abaxial polarity. Subsequently, KAN proteins become active on the abaxial side of the developing primordium (Yadav et al., 2014). This dynamic behavior may underlie the resolution of organ polarity into a primordium-autonomous process. As mentioned above, KAN limits AS1-AS2 activity to the adaxial side, which, in turn, polarizes expression of additional components in the polarity network (Husbands et al., 2015). Once resolved, polarity is then stably maintained throughout primordium development by further positive and negative feedback between the polarity determinants and interdomain signaling involving the opposing tasiARF and miR166 gradients (Fig. 5).

The meristem-borne Sussex signal would, in principal, function during the establishment/resolution phases of adaxial-abaxial patterning, but not subsequently during the primordium-autonomous maintenance phase. The surgical experiments thus provide a possible timeframe for the transition from an externally to an internally patterned process, namely that adaxial-abaxial polarity is primordium-autonomous by the P2 stage of development (Reinhardt et al., 2005). A caveat to this, however, is that much of the molecular analyses were conducted in *Arabidopsis* whereas the surgical experiments were performed in *Solanaceae* and, as mentioned above, the expression dynamics of individual polarity determinants, and therefore their contributions to adaxial-abaxial polarity, can vary from species to species. This applies to the mobile molecules as well; whereas tasiARF in *Arabidopsis* functions in the maintenance of adaxial-abaxial polarity in older primordia, in both maize and rice, this small RNA is required to specify this axis in the incipient leaf (Nogueira et al., 2007, 2009; Nagasaki et al., 2007; Petsch et al., 2015). Consequently, loss of tasiARF activity in maize and rice leads to the formation of fully abaxialized leaves reminiscent of those obtained in surgical experiments. As such, the contribution, or perhaps even the identity, of the Sussex signal may differ between species.

What is the Sussex signal?

It follows from the above that tasiARF qualifies as a candidate for the Sussex signal in maize and rice, but not in *Arabidopsis* (Nogueira et al., 2007, 2009). In *Arabidopsis*, it was proposed that the Sussex signal could be succinate semialdehyde (Toyokura et al., 2011), an intermediate of the stress-induced GABA shunt. However, plants deficient in succinate semialdehyde metabolism are severely compromised as a result of the accumulation of reactive oxygen species (Ludewig et al., 2008), raising the concern that abiotic stresses interfere with polarity. Stress is often raised as a caveat in surgical manipulations, but it is important to realize that concerns about induced stress apply equally to genetic interferences. The extensive control experiments as described in Ian Sussex's PhD thesis (Fig. 2) provide a good example of how to alleviate such caveats.

A further candidate for the polarizing signal in *Arabidopsis* as well as in tomato is auxin. Ectopic auxin signaling on the adaxial side of leaf primordia, achieved via the expression of a constitutively active allele of the ARF transcription factor MONOPTEROS or the application of exogenous IAA, was shown to convert adaxial into abaxial cell fate (Qi et al., 2014). Indeed, following primordium initiation, auxin flows from the adaxial side towards the meristem, creating a local auxin minimum (Heisler et al., 2005). Based on these observations, it was proposed that the surgical experiments might be explained by their effect on auxin distribution, with surgical incisions that perturb the flow of auxin leading to abaxialization (Qi et al., 2014). If so, auxin may constitute a 'reverse Sussex signal'. This is an interesting idea, but whether this is the case or whether auxin acts as a downstream effector of the genuine signal remains to be resolved.

The most attractive candidate for the Sussex signal is the lipophilic molecule predicted to bind the START domain of the HD-ZIPIII transcription factors. Based on their expression patterns and the nature of the first isolated alleles, the HD-ZIPIII transcription factors were originally proposed to perceive this signal (McConnell et al., 2001; Bowman et al., 2002). This idea lost traction after the discovery that HD-ZIPIII patterning is driven by miR166. However, these two regulatory paradigms are not necessarily mutually exclusive. The Sussex signal could be a meristem-derived lipophilic ligand, for example, that is required for HD-ZIPIII activity without providing spatial information, which instead comes from miR166. Importantly, the regulation of HD-ZIPIII members by miR166 in the incipient primordium is conserved, lending support to the idea that HD-ZIPIII proteins might function as receptors of the Sussex signal. In fact, this presents the intriguing possibility that these key adaxial determinants integrate positional information from several sources; a miR166 signal from the internode below that restricts HD-ZIPIII accumulation to the adaxial side, and a lipophilic signal from the meristem above that activates these proteins.

Over the past 20 years, we have seen tremendous progress in the field of leaf dorsiventrality. One thing that is now obvious is that this process is far more complex than envisioned in 1952. Perhaps the most surprising new concept is that the same molecular players drive adaxial-abaxial patterning across angiosperms, but that they are wired in different ways. The key question of how polarity is first established, or what the Sussex signal is, may therefore have more than one answer. Based on the broad regulatory and functional conservation of the HD-ZIPIII transcription factors, we expect that the putative lipophilic ligand of their START domains will be a central component of the Sussex signal. The determination of the molecular identity of this ligand will therefore be a crucial advance in the field.

Ian Sussex's legacy

We need not discuss here the enduring quality of Ian's research; the previous sections will have made it abundantly clear that he was a

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brilliant scientist. He was also an inspiring teacher, and many generations of students and postdocs at Yale University, UC Berkeley, and the Cold Spring Harbor Plant Course can attest to that. Like so many of our colleagues, we have read, re-read and loved *Patterns in Plant Development*, the book that Ian wrote, together with Taylor A. Steeves, which taught us how rich and diverse plant developmental biology can be.

On a more personal note, we quote from his autobiography:

> 'Anyone who has dissected the bud of a vascular plant under a stereomicroscope must surely have been thrilled by the translucent, glistening beauty of the apical meristem and the surrounding leaf primordia. There are subtleties that are lost in the starkness of an SEM image. As well as being thrilled by the appearance of the meristem, one must surely also be awed, thinking, how does it work and how can I find out?'

Isn't such a combination of curiosity and excitement a great way of doing science?

We end with an anecdote that tells us something else about Ian. In the submitted manuscript of Reinhardt et al. (2005), we introduced the term 'Sussex signal'. Reviewer 4 was mostly favorable about the paper but objected to the use of this term as being against botanical tradition. We have good reason to assume that this reviewer was Ian himself. Despite his objections, the term 'Sussex signal' has caught on. Ian did not need to raise his voice to be heard, or as we say in Dutch: Goede wijn behoeft geen krans (good wine needs no praise). He was a brilliant scientist, a great teacher and, so important to those who had the good fortune to know him personally, a kind person.

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Competing interests

The authors declare no competing or financial interests.

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