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Establishing leaf polarity: the role of small RNAs and positional signals in the shoot apex

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The flattening of leaves results from the juxtaposition of upper (adaxial) and lower (abaxial) domains in the developing leaf primordium. The adaxial-abaxial axis reflects positional differences in the leaf relative to the meristem and is established by redundant genetic pathways that interpret this asymmetry through instructive, possibly non-cell autonomous, signals. Small RNAs have been found to play a crucial role in this process, and specify mutually antagonistic fates. Here, we review both classical and recently-discovered factors that contribute to leaf polarity, as well as the candidate positional signals that their existence implies.

Introduction

The flattening of the leaf is an important adaptation that maximizes photosynthesis. The laminar plane of the leaf is composed of distinct cell types within its upper and lower layers (Fig. 1). In many plant species, the upper surface of the leaf develops a thicker cuticle and contains a densely packed layer of palisade mesophyll cells to optimize the capture of light, whereas the underside of the leaf contains stomata and spongy mesophyll cells that function in gas exchange and in the regulation of transpiration (Gifford and Foster, 1989). The extension of the lamina and the differentiation of these distinct cell fates result from dorsoventral (adaxial-abaxial) patterning events that occur during the earliest stages of leaf development (Waites and Hudson, 1995; Bowman et al., 2002).

Unlike animals, plants exhibit indeterminate growth and continuously give rise to new organs, such as leaves, from their shoots. The growing tip of the plant shoot system, the shoot apical meristem (SAM), contains a population of pluripotent stem cells, which divide to replenish themselves and produce daughter cells from which lateral organs arise (for a review, see Kidner et al., 2002). Leaf primordia develop away from the flank of the meristem and consequently possess an inherent asymmetry with respect to the tip of the SAM (Fig. 2). This asymmetry underlies leaf polarity: the upper surface of the leaf, the adaxial side, develops in closer proximity to the SAM than the lower surface of the leaf, known as the abaxial side, and implies that positional signals within the plant apex establish organ polarity (Wardlaw, 1949; Steeves and Sussex, 1989).

The mechanisms that establish adaxial-abaxial patterning in the leaf were first addressed by microsurgery experiments performed over 50 years ago. Incisions separating incipient primordia from the meristem result in centric, abaxialized leaves (Sussex, 1951; Sussex, 1954). The results of the Sussex experiments are consistent with the existence of a positional signal that emanates from the SAM and that specifies adaxial cell fate. They also suggest that abaxial cell fate alone is not sufficient to mediate the outgrowth of the leaf blade along the mediolateral axis. A recent elaboration of the Sussex experiments indicates that the outermost cell layer of the meristem, the L1 layer, is necessary for the hypothetical signal to specify adaxial fate (Fig. 3A-C) (Reinhardt et al., 2005). Surgical incisions made shortly after the appearance of a leaf primordium demonstrate that adaxial-abaxial patterning established at its distal end is unable to spread to proximal regions, which remain radialized after incision (Reinhardt et al., 2005). These results suggest that a sustained meristem-borne signal is required throughout the early development of a leaf to correctly establish its polarity.

Although the identity of the Sussex signal remains unknown, much progress has been made towards understanding the molecular mechanisms that establish adaxial-abaxial polarity downstream of such positional signals. Several families of putative transcription factors are key determinants of adaxial and abaxial cell fate (Table 1). These proteins act in distinct genetic pathways that have redundant and mutually antagonistic roles in the establishment of leaf polarity. Such mutual antagonism between pathways is common in other developmental systems, but adaxial-abaxial polarity in leaves is established by an additional layer of regulation. Recent experiments demonstrate an important role for small regulatory RNAs – microRNAs (miRNAs) and *trans*-acting short interfering RNAs (ta-siRNAs) – in specifying both adaxial and abaxial fates.

Here, we review the diverse pathways now known to establish adaxial and abaxial fates, and suggest a number of putative positional signals that contribute to leaf polarity, including possible candidates for the ever-elusive Sussex signal. Among such candidate positional signals are small RNAs, whose contributions to mutually antagonistic domains of a polar axis reveal a patterning mechanism that is, thus far, unique to leaves.

Adaxial determinants: phantastic and phabulous

The first gene to be identified that is involved in adaxial-abaxial patterning was discovered nearly 50 years after surgical experiments established that a fundamental relationship exists between leaf polarity and the meristem. The *PHANTASTICA* (*PHAN*) gene from *Antirrhinum* encodes a protein with a DNA-binding MYB domain, which is required for adaxial fate (Waites and Hudson, 1995; Waites et al., 1998). Leaves that exhibit a weak *phan* phenotype are seemingly normal but have small, ectopic patches of abaxial tissue on their adaxial side, around which adventitious blade outgrowths develop. More severe *phan* phenotypes show progressively abaxialized leaves that fail to extend along their mediolateral axes, consistent with the idea that laminar expansion in leaves may result from the juxtaposition of adaxial and abaxial tissues (Fig. 3D). At colder temperatures, null alleles of *phan* frequently result in meristem arrest, suggesting that adaxial identity in leaves is necessary for meristem maintenance (Waites et al., 1998). The experiments, in conjunction with evidence from the Sussex experiments for a meristem-borne adaxializing signal, suggest that communication between the meristem and leaf might be bidirectional.

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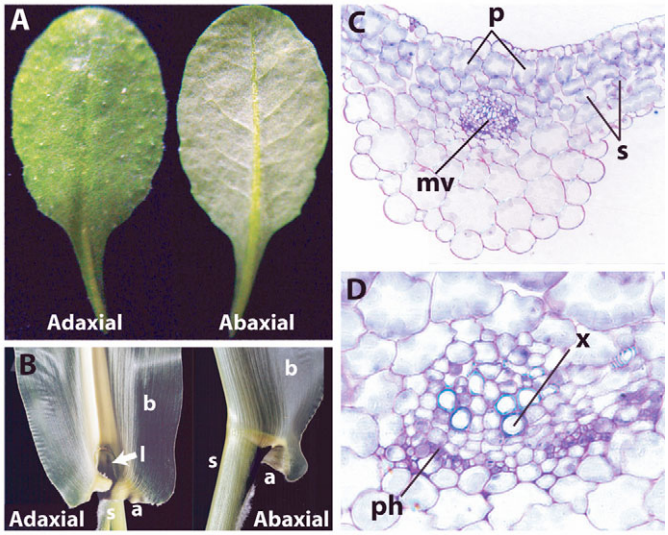

Fig. 1. Adaxial-abaxial leaf architecture. (**A**) The adaxial side of an *Arabidopsis* leaf is dark green and trichome rich, whereas the abaxial leaf surface is matte, grey-green and trichome poor. (**B**) The adaxial and abaxial sides of a maize leaf blade (b) and sheath (s) are separated by the auricle (a) and the ligule (l), an adaxial, epidermal fringe. (**C**) Transverse section of an *Arabidopsis* leaf, showing adaxial palisade cells (p), abaxial spongy mesophyll cells (s) and the central midvein (mv). (**D**) Magnified cross-section of a vascular bundle in an *Arabidopsis* leaf, showing the spatial relationship between adaxial xylem (x) and abaxial phloem (ph). Images C and D, which were first published by Lin et al. (Lin et al., 2003), are reproduced with permission from the American Society of Plant Biologists.

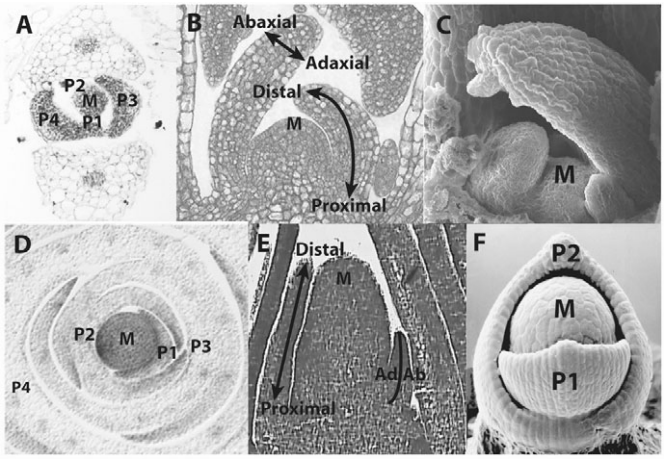

Fig. 2. Meristem architecture. (**A**) Transverse section of an *Arabidopsis* vegetative shoot apex, showing the youngest (P1) and increasingly older (P2 and onward) leaf primordia and meristem (M). Notice the spiral phyllotaxis of leaves around the SAM. (**B**) Longitudinal section of an *Arabidopsis* meristem, showing the proximal-distal and adaxial-abaxial axes of leaf primordia relative to the SAM. (**C**) Scanning electron micrograph (SEM) of an *Arabidopsis* vegetative SAM, showing the spatial relationship of primordia relative to each other and to the meristem (M). (**D**) Transverse section of a maize SAM, showing successively older (P1-P4) leaf primordia encircling the SAM. (**E**) Longitudinal section of a maize SAM, showing the proximal-distal and adaxial-abaxial (Ad/Ab) axes of leaf primordia. Notice the alternate phyllotaxis. (**F**) SEM of a maize SAM with two leaf primordia. Images in C and F kindly provided by C. Kidner and D. Jackson, respectively.

The polarity defects of *phan* mutants in *Antirrhinum* are not indicative of the function of its orthologs in *Arabidopsis* and maize (Table 1). Mutations in *ASYMMETRIC LEAVES 1* (*AS1*) (Byrne et al., 2000) from *Arabidopsis* and *ROUGH SHEATH2* (*RS2*) (Timmermans et al., 1999; Tsiantis et al., 1999) from maize cause no obvious polarity defects. *AS1* and *RS2* are better known for their role in repressing the expression within developing leaves of the *KNOTTED-LIKE HOMEOBOX* (*KNOX*) genes, which are required for meristem maintenance (Schneeberger et al., 1998; Ori et al., 2000; Phelps-Durr et al., 2005). This function is conserved throughout the ARP (AS1, RS2 and PHAN) clade, as *ARP* genes maintain *KNOX* repression in cross-species complementation studies (Theodoris et al., 2003; Harrison et al., 2005). This is not to say that other *ARP* genes, besides *PHAN*, do not contribute to adaxial fate. *arp* mutants in tobacco and the compound-leafed species tomato, pea and *Cardamine hirsuta* develop a range of adaxial-abaxial polarity defects (Kim et al., 2003; McHale and Koning, 2004; Tattersall et al., 2005; Hay and Tsiantis, 2006). *AS1* in *Arabidopsis* may also play a role in leaf polarity, albeit redundantly with other pathways (Fig. 4). The contribution of the ASYMMETRIC LEAVES pathway to polarity becomes evident in plants that constitutively express *ASYMMETRIC LEAVES 2* (*AS2*), a gene whose activity is required for proper *AS1* function (Iwakawa et al., 2002; Byrne et al., 2002). Such plants develop curled leaves, ectopic abaxial outgrowths, vascular defects, and abaxial-to-adaxial transitions in cell fate consistent with an adaxialized phenotype (Lin et al., 2003; Xu et al., 2003).

In *Arabidopsis*, more-prominent contributors to adaxial fate than the ASYMMETRIC LEAVES pathway are the class III *HOMEODOMAIN-LEUCINE ZIPPER* (*HD-ZIPIII*) genes. The *Arabidopsis* HD-ZIPIII family includes *PHABULOSA* (*PHB*), *PHAVOLUTA* (*PHV*), *REVOLUTA* (*REV*), *CORONA* (*CNA*, also known as *ATHB-15*) and *ATHB-8*, all of which encode putative transcription factors with N-terminal homeodomain-leucine zipper motifs followed by a StAR-related lipid transfer (START) domain, which, in animals, is known to interact with lipids (Table 1) (Sessa et al., 1998; Pontig and Aravind, 1999; Schrick et al., 2004). *PHB*, *PHV* and *REV* belong to a single clade (the REV clade) and are the only members of the HD-ZIPIII family that contribute to leaf polarity (McConnell and Barton, 1998; McConnell et al., 2001; Emery et al., 2003; Prigge et al., 2005). Members of the REV clade are expressed in the incipient organ and become adaxially restricted as the primordium emerges (McConnell et al., 2001; Otsuga et al., 2001; Emery et al., 2003). *REV* and *PHB* are also expressed within the tip of the meristem, and *PHB* forms a ray of expression that connects the meristem with predicted sites of leaf initiation (McConnell et al., 2001). These expression patterns suggest a role of the *HD-ZIPIII* genes in meristem function and adaxial patterning in young primordia, and perhaps in coordinating the two processes.

The *HD-ZIPIII* genes were first identified as semi-dominant mutations that result in the formation of adaxialized leaves that develop ectopic, abaxial axillary meristems (McConnell and Barton, 1998; McConnell et al., 2001). In addition, dominant *HD-ZIPIII* mutants possess an enlarged SAM and can partially suppress the meristem defects resulting from mutations in the *KNOX* gene

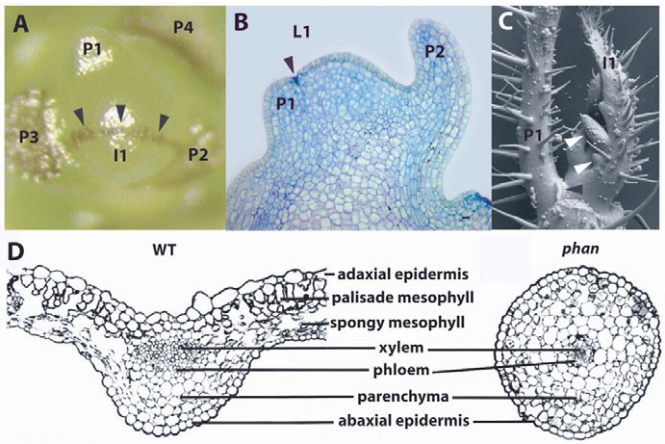

Fig. 3. Surgical and genetically induced leaf-polarity defects. (**A**) Vegetative tomato shoot apex, showing ablation of the L1 layer (black arrowheads), separating the incipient leaf (I1) from the meristem. Leaf primordia of increasing age are shown (P1-P4). (**B**) Longitudinal section of a tomato apex, showing an incision restricted to the outermost (L1) layer of the SAM (black arrowhead), which separates the P1 primordium from the meristem. (**C**) SEM of a tomato apex 1 day after the P1 has been surgically separated from the SAM. The P1 develops as a radial, abaxialized primordium (black arrowhead shows the scar from the ablation). By contrast, a primordium that developed in contact with the meristem (I1) is dorsoventrally flattened and develops leaflet pairs (white arrowheads). (**D**) Transverse sections of *Antirrhinum* leaves from wild-type (WT) and *phan* plants. Relative to wild type, the *phan* leaf is radial and composed of abaxial parenchyma surrounded by abaxial epidermis. Notice that, in the *phan* mutant, vascular polarity is lost and that the xylem no longer lies adaxial relative to normally abaxial phloem. Images A-C are reproduced with permission from Reinhardt et al. (Reinhardt et al., 2005) and D with permission from Waites and Hudson (Waites and Hudson, 1995).

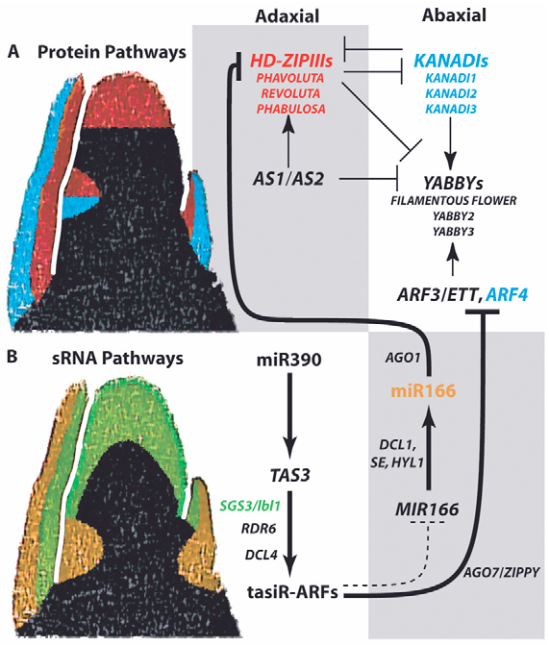

Fig. 4. Genetic interactions that establish adaxial-abaxial polarity in leaves. Pathways are divided into those contributing to adaxial versus abaxial fate and those involving protein components (**A**) versus the biogenesis and activity of small RNAs (sRNAs) (**B**). Direct interactions are distinguished from indirect and putative (dashed line) interactions by bold arrows and lines. On the left are false-colored meristems representing generalized expression patterns across plant lineages. The colors of the expression domains correspond to the colors of the factors that they represent.

SHOOTMERISTEMLESS (*STM*), validating the connection between adaxial fate and meristem identity (McConnell and Barton, 1998). Loss-of-function phenotypes for individual HD-ZIPIII-family members are obscured by redundancy (Prigge et al., 2005). Single *phb*, *phv* and *rev* mutants exhibit no polarity defects, but the importance of the clade as a whole becomes evident in *phv; phb; rev* triple mutants, in which only a single, abaxialized cotyledon develops and in which there is no functional SAM (Emery et al., 2003).

Besides leaf polarity and meristem maintenance, *HD-ZIPIII* genes also contribute to the development and patterning of the vasculature, which consists of water-conducting xylem and nutrient-bearing phloem tissues. Within the shoot, xylem lies internally relative to phloem, an arrangement that is maintained in the leaf, where xylem is positioned adaxially relative to phloem (Fig. 1D, Fig. 3D). *HD-ZIPIII* genes are expressed in the differentiating xylem, and semi-dominant mutations in these genes create amphivasal bundles in which the xylem surrounds the phloem tissue (Baima et al., 1995; Kang et al., 2003; McConnell and Barton, 1998; Emery et al., 2003; Zhong and Ye, 2004). The mechanisms that establish leaf adaxial-abaxial polarity may have co-opted genetic pathways that are involved in shoot patterning, because the seed-plant leaf is thought to have arisen from extensive shoot branching (Zimmerman, 1952). This idea is consistent with the fact that the role of *HD-ZIPIII* genes in leaf polarity is restricted to the REV clade, which is thought to have arisen by gene duplications that occurred just before and during the diversification of the seed plants and angiosperms (Floyd et al., 2006; Prigge and Clark, 2006).

The role of the *HD-ZIPIII* genes in both leaf and shoot patterning is thought to be mediated through the interpretation of a positional signal (Emery et al., 2003). Dominant *HD-ZIPIII* mutations all affect a small, highly conserved region in the START domain. The specificity of the mutations suggests that HD-ZIPIII proteins act via a mechanism similar to that of some metazoan nuclear receptors, requiring steroid signals to function. Such a signal might emanate from the central pith and meristem, and might fulfill the requirements of the Sussex signal, to specify adaxial, central fates non-cell autonomously (McConnell et al., 2001). Although, the existence of loss-of-function *rev* mutations within the START domain still support the concept of lipid signaling (Otsuga et al., 2001), dominant *HD-ZIPIII* mutations have in fact been found to abrogate a miRNA target site, indicating the importance of miRNAs in restricting *HD-ZIPIII* expression and in specifying adaxial-abaxial polarity (Fig. 4) (McConnell et al., 2001; Rhoades et al., 2002; Tang et al., 2003; Juarez at al., 2004a).

Drawing the line: adaxial fate restriction by miRNA166

miRNAs are endogenous small RNAs (~21-24 nucleotides in size) that act in *trans* to regulate the expression of target genes (Table 2). miRNAs are processed by DICER-LIKE1 (DCL1) from long, non-coding RNA polymerase II-dependent primary transcripts (pri-miRNAs) (Kurihara and Watanabe, 2004; Lee et al., 2004; Xie et al., 2005a). The processing of miRNAs in plants is distinct from that in animals. In plants, DCL1 processes the pri-miRNA into a 70-300-

Table 1. Protein determinants of adaxial and abaxial fate

Family	Domains	Notable members	Function	Phenotypes	Expression	References[1]
HD-ZIPIII	Homeodomain (DNA binding) Leucine zipper (protein interaction) START (lipid-sterol binding)	*Arabidopsis*: *PHB*, *PHV*, *REV*, *CNA*, *ATHB-8*	Adaxial determinants Meristem function	GOF: adaxialized leaves, enlarged meristem LOF *rev phv phb*: abaxialized cotyledon, meristem arrest	Adaxial side of leaf primordia, developing xylem and in the meristem	McConnell et al., 2001; Otsuga et al., 2001; Emery et al., 2003
		Maize: *rld1*		GOF: adaxialized leaves	Adaxial side of leaf primordia, developing xylem and in the meristem	Juarez et al., 2004a
ARP	MYB (DNA binding)	*Antirrhinum*: *PHAN*	Adaxial determinants and/or determinacy	LOF: abaxialized leaves, cold-dependent meristem arrest	Uniform within leaf primordia	Waites and Hudson, 1995; Waites et al., 1998
		Arabidopsis: *AS1*		LOF: asymmetrically lobed leaves. Enhanced by mutations in *TAS3* pathway	Uniform within leaf primordia	Li et al., 2005; Garcia et al., 2006; Xu et al., 2006
		Maize: *rs2*		LOF: proximodistal patterning defects	Uniform within leaf primordia	Timmermans et al., 1999; Tsiantis et al., 1999
AS2	LOB (DNA binding)	*Arabidopsis*: *AS2*	Adaxial determinants Determinacy	LOF: asymmetrically lobed leaves. Enhanced by mutations in *TAS3* pathway GOF: adaxialized leaves	Adaxial L1 layer of cotyledons, vegetative expression pattern unknown	Iwakawa et al., 2002; Lin et al., 2003
KANADI	GARP (DNA binding)	*Arabidopsis*: *KAN1*, *KAN2*, *KAN3*	Abaxial determinants	LOF *kan1 kan2*: adaxialized leaves GOF: abaxializes leaves, meristem arrest	Abaxial side of leaf primordia and in phloem	Kerstetter et al., 2001; Eshed et al., 2001; Eshed et al., 2004
YABBY	Zn-finger YABBY (DNA binding)	*Arabidopsis*: *FIL*, *YAB2*, *YAB3*	Laminar outgrowth Abaxial determinants in *Arabidopsis*	LOF *fil yab3*: adaxialized leaves Suppresses ectopic laminar outgrowths of *kan1 kan2* GOF: abaxializes leaves	Abaxial side of leaf primordia, in regions of ectopic laminar outgrowth	Sawa et al., 1999; Siegfried et al., 1999; Eshed et al., 2004
		Maize: *zyb9*, *zyb14*		?	Adaxial side of leaf primordia, in regions of ectopic laminar outgrowth	Juarez et al., 2004b
ARF	B3 (DNA binding) Aux/IAA CTD (protein interaction)	*Arabidopsis*: *ETT*, *ARF4*	Abaxial determinants Auxin signaling	LOF *arf3 arf4*: abaxialized leaves GOF *ARF3*: accelerated vegetative phase change	*ETT*: throughout the meristem and leaf primordia *ARF4*: Abaxial side of leaf primordia and phloem	Pekker et al., 2005

[1]Please refer to text for additional references.

AS1-2, *ASYMMETRIC LEAVES1-2*; *ARF4*, *AUXIN RESPONSE FACTOR4*; *CNA*, *CORONA*; *ETT*, *ETTIN*; *FIL*, *FILAMENTOUS FLOWER*; GOF, gain-of-function; *KAN1-3*, *KANADI1-3*; LOF, loss-of-function; *PHB*, *PHABULOSA*; *PHAN*, *PHANTASTICA*; *PHV*, *PHAVOLUTA*; *REV*, *REVOLUTA*; *rld1*, *rolled leaf1*; *rs2*, *rough sheath2*; *YAB2-3*, *YABBY2-3*; *zyb9*, *ZmYABBY9*; *zyb14*, *ZmYABBY14*.

Table 2. SmallRNA pathway components involved in adaxial-abaxial patterning

Family	Domains	Notable members	Function	Phenotypes	Expression	References[1]
MIR166	miR166	*Arabidopsis*: *MIR165a-b* *MIR166a-g*	Abaxial determinant Restriction of *HD-ZIPIII* transcripts to adaxial domain	GOF *MIR166a, MIR166g*: fasciated meristem, vascular patterning defects *PHB>>MIR165b*: abaxialized leaves, meristem arrest	Mature miR165 accumulates abaxially within leaf primordia	Kidner and Martienssen, 2004; Kim et al., 2005; Williams et al., 2005b; Alvarez et al., 2006
		Maize: *mir166a-m*		?	Mature miR166 accumulates abaxially within leaf primordia	Juarez et al., 2004a
SERRATE	Zn-finger	*Arabidopsis*: *SE*	pri-miRNA processing	LOF: adaxialized leaves, enlarged meristem	Tip of meristem, adaxially within leaf primordia	Prigge and Wagner, 2001; Grigg et al., 2005; Yang et al., 2006
TAS3	tasiR-ARFs miR390 target site	*Arabidopsis*: *TAS3* Maize: *tas3a-d*	Adaxial determinant? Mediates cleavage of *ETT* and *ARF4*	LOF: accelerated vegetative-phase change	?	Allen et al., 2005; Williams et al., 2005a; Adenot et al., 2006; Garcia et al., 2006
SGS3	Zn-finger XS	*Arabidopsis*: *SGS3*	ta-siRNA biogenesis	LOF: accelerated vegetative-phase change. Enhances leaf defects of *as1*, *as2* mutants	?	Peragine et al., 2004; Vazquez et al., 2004b; Allen et al., 2005; Yoshikawa et al., 2005
		Maize: *lbl1*		LOF: radial abaxialized leaves	Tip of meristem, adaxially within leaf primordia	Timmermans et al., 1998; Juarez et al., 2004b; F. Nogueira et al., unpub. data
RdRp	RdRp	*Arabidopsis*: *RDR6*	ta-siRNA biogenesis	LOF: accelerated vegetative-phase change. Enhances leaf defects of *as1*, *as2* mutants	?	Peragine et al., 2004; Vazquez et al., 2004b; Allen et al., 2005; Adenot et al., 2006
HYL1-like	dsRNA-binding	*Arabidopsis*: *HYL1*	pri-miRNA processing Interacts with DCL1 in vitro	LOF: pleiotropic effects, including adaxialized leaves	?	Kurihara et al., 2006; Han et al., 2004; Vazquez et al., 2004a; Yu et al., 2005
		Arabidopsis: *DRB4*	*TAS3*-derived ta-siRNA biogenesis Interacts with DCL4 in vitro	LOF: accelerated vegetative-phase change	?	Hiraguri et al., 2005; Adenot et al., 2006
DCL	PAZ RNaseIII	*Arabidopsis*: *DCL1*	pri-miRNA processing	LOF: pleiotropic effects, including filamentous leaves	Throughout the inflorescence. Vegetative expression pattern unknown	Jacobsen et al., 1999; Park et al., 2002; Reinhart et al., 2002
		Arabidopsis: *DCL4*	ta-siRNA biogenesis	LOF: accelerated vegetative-phase change. Enhances leaf defects of *as1*, *as2* mutants	?	Gasciolli et al., 2005; Xie et al., 2005b; Yoshikawa et al., 2005
AGO	PAZ PIWI	*Arabidopsis*: *AGO1*	miRNA-mediated target-transcript cleavage	LOF: pleiotropic effects, including filamentous organs and meristem defects	Uniform within the meristem and leaf primordia	Bohmert et al., 1998; Baumberger and Baulcombe, 2005
		Arabidopsis: *AGO7*	ta-siRNA-mediated target-transcript cleavage	LOF: accelerated vegetative-phase change. Enhances leaf defects of *as1*, *as2* mutants	?	Hunter et al., 2003, 2006; Adenot et al., 2006; Fahlgren et al., 2006

[1]Please refer to the text for additional references.

AGO1, *ARGONAUTE1*; *AGO7*, *ARGONAUTE7*; *DCL1*, *DICER-LIKE1*; *DCL4*, *DICER-LIKE4*; *DRB4*, *dsRNA BINDING PROTEIN4*; *HYL1*, *HYPONASTIC LEAVES1*; *lbl1*, *leafbladeless1*; *SE*, *SERRATE*; *SGS3*, *SUPPRESSOR OF GENE SILENCING3*; *RDR6*, *RNA-DEPENDENT RNA POLYMERASE6*; GOF, gain-of-function; LOF, loss-of-function.

nucleotide stem-loop intermediate (the pre-miRNA), which is immediately processed again into a mature miRNA duplex (Park et al., 2002; Reinhart et al., 2002; Kurihara and Watanabe, 2004; Kurihara et al., 2006). The mature miRNA becomes incorporated into a complex with ARGONAUTE1 (AGO1), which mediates the cleavage or translational repression of target transcripts (Vaucheret et al., 2004; Baumberger and Baulcombe, 2005; Qi et al., 2005). Plant miRNAs and their targets frequently show near-perfect complementarity, facilitating their prediction using in silico approaches (Rhoades et al., 2002). Significantly, many known miRNAs regulate transcription factors or other genes that coordinate crucial steps during plant development (for a review, see Jones-Rhoades et al., 2006). This is evident in mutations that affect the miRNA-biogenesis machinery, which yield pleiotropic effects, including defects in meristem function and adaxial-abaxial patterning (Bohmert et al., 1998; Jacobsen et al., 1999; Schauer et al., 2002; Han et al., 2004; Vazquez et al., 2004a; Yu et al., 2005).

The *HD-ZIPIII* genes are targets of miR165 and miR166 (Rhoades et al., 2002; Tang et al., 2003). Ancient conservation of the miR166 sequence and its target site within the *HD-ZIPIII* genes throughout all land plants, including the liverworts and hornworts, demonstrates the importance of miR166-mediated *HD-ZIPIII* regulation (Floyd and Bowman, 2004; Floyd et al., 2006). In both *Arabidopsis* and maize, *HD-ZIPIII* genes and miR166 display complementary expression patterns; *HD-ZIPIII* genes are expressed within the meristem and on the adaxial side of developing leaves, while miR166 accumulates on the abaxial surface of young leaf primordia (Juarez et al., 2004a; Kidner and Martienssen, 2004). In maize, the peak of miR166 accumulation is observed immediately below the incipient leaf, and a gradient of weaker miR166 expression extends into the abaxial domain of the young primordia (Juarez et al., 2004a). This complementary expression of *HD-ZIPIII* genes and miR166 suggests that it may act as a polarizing signal that establishes organ polarity by spatially restricting *HD-ZIPIII* expression to the adaxial side of developing primordia. Such an hypothesis is consistent with dominant *HD-ZIPIII* alleles arising from mutations abrogating the miR166 target site (McConnell et al., 2001; Emery et al., 2003; Zhong and Ye et al., 2004). Although the original dominant alleles disrupt both the START domain and the miR166 target site, transgenic plants expressing miR166-insensitive versions of *HD-ZIPIII* genes that harbor silent mutations have similar phenotypes (Emery et al., 2003; Mallory et al., 2004). These mutations reduce the susceptibility of *HD-ZIPIII* transcripts to miRNA-directed cleavage, resulting in the misexpression of mutant transcripts on the abaxial side of developing leaves, a phenomenon not seen for wild-type alleles of other *HD-ZIPIII* members in the same plant (Juarez et al., 2004a). This contrasts with plants that overexpress wild-type *HD-ZIPIII* transcripts that display an essentially wild-type phenotype, presumably due to the elimination of ectopic *HD-ZIPIII* expression by miR166-mediated cleavage (McConnell et al., 2001).

The fact that ectopic expression of the *MIR165* and *MIR166* genes disrupts vascular patterning and phenocopies loss-of-function *hd-zipIII* mutants similarly demonstrates the importance of miR166-mediated restriction of *HD-ZIPIII* transcripts (Kim et al., 2005; Williams et al., 2005b; Alvarez et al., 2006). Interestingly, miR166 also seems to regulate *HD-ZIPIII* expression at the transcriptional level. Dominant *PHB* alleles are hypomethylated relative to wild type, suggesting an unknown miRNA-mediated transcriptional silencing mechanism mediated through nucleic acid binding with the nascent transcript (Bao et al., 2004). Such an epigenetic mark may provide a cellular memory of positional cues that are perceived earlier in primordium development, as exemplified by the hypomethylation of *PHB* loci in undifferentiated tissues compared with differentiated tissues in wild-type plants.

Recently, additional evidence has demonstrated the importance of miR166-mediated restriction of *HD-ZIPIII* expression. The zinc-finger protein SERRATE (SE) coordinately regulates meristem activity and leaf polarity through the *HD-ZIPIII* genes (Grigg et al., 2005). SE is a general regulator of miRNA levels that, similar to the dsRNA-binding protein HYPONASTIC LEAVES 1 (HYL1), affects the processing of pri-miRNA transcripts into mature miRNAs (Table 2) (Yang et al., 2006; Lobbes et al., 2006; Kurihara et al., 2006). Despite its pleiotropic effects on plant development, specific *se* mutants (*se-2* and *se-3*) resemble dominant *PHB* mutants, and the defects of *se-3* are suppressed by loss-of-function *hd-zipIII* mutations, consistent with the involvement of miR166-mediated repression in *HD-ZIPIII* gene expression. Moreover, a reduction in DNA methylation at the *PHB* locus is observed in *se-3* mutants, similar to the hypomethylation of the dominant *PHB* alleles (Grigg et al., 2005).

Determinants down under: KANADIs and YABBYs

Besides miR166, other abaxial determinants in *Arabidopsis* include the KANADI (KAN) and YABBY gene families (Table 1, Fig. 4A). The *KANADI* genes (*KAN1-KAN4*) encode putative transcription factors that contain a MYB-like GARP DNA-binding domain. KAN-family members are expressed abaxially within the cotyledons and leaf primordia; within the stem, *KAN* expression is found in the developing phloem, complementary to *HD-ZIPIII* expression (Kerstetter et al., 2001; Emery et al., 2003; Eshed et al., 2004). Although *kan1* mutants exhibit indications of adaxialization early in development (Kerstetter et al., 2001), polarity defects in mature leaves only appear in *kan1; kan2* double mutants and are enhanced in a *kan3* background (Eshed et al., 2001; Eshed et al., 2004). *kan1; kan2* mutants develop narrow leaves with blade outgrowths that surround ectopic sectors of adaxial tissue, similar to those found in *phan* mutants. The outgrowths lend further support to the idea that the juxtaposition of adaxial and abaxial tissues is necessary for laminar expansion (Eshed et al., 2001; Eshed et al., 2004).

The boundary between adaxial and abaxial domains is necessary to coordinate the proper outgrowth and patterning of the leaf (Sussex, 1951; Waites and Hudson, 1995). The definition and maintenance of the boundary may in part be established through a mutual antagonism between adaxial and abaxial determinants, which is best exemplified by the opposing activities of the KANADI and HD-ZIPIII-family members (Eshed et al., 2001; Kerstetter et al., 2001; McConnell et al., 2001; Emery et al., 2003). The adaxialized phenotype of *kan1; kan2* mutants is accompanied by the ectopic expression of *HD-ZIPIII* genes. Likewise, gain-of-function *HD-ZIPIII* alleles resemble the phenotypes that result from the loss of *kan* activity (Eshed et al., 2001; McConnell et al., 2001; Emery et al., 2003). The importance of preventing the dominance of one domain over the other is further demonstrated by plants that constitutively express *KAN1* and that undergo meristem arrest, perhaps owing to a lack of *HD-ZIPIII* activity, which is necessary for meristem maintenance (Kerstetter et al., 2001).

In *Arabidopsis*, the YABBY family, which consists of six members, also specifies abaxial fate. At least three *YABBY* genes – *FIL*, *YAB2* and *YAB3* – are similarly expressed on the abaxial side within primordia and cotyledons (Sawa et al., 1999; Siegfried et al., 1999). *YABBY* gene expression is mislocalized in *kan1; kan2* mutants, indicating that the *YABBY* genes act partially downstream of the *KANADI*s, and ectopic *YABBY* gene expression is sufficient to

specify abaxial fate in *Arabidopsis* (Sawa et al., 1999; Siegfried et al., 1999; Eshed et al., 2001). However, in maize, *YABBY* genes are expressed adaxially and act downstream of the *REV* homolog *rolled leaf1* (Juarez et al., 2004b). This discrepancy may in part be explained by an additional role for *YABBY* genes in specifying blade outgrowth. The ectopic outgrowths of *Arabidopsis kan1; kan2* mutants are lost in a *fil; yab3* background, and *fil* expression concentrates within the ectopic blades (Eshed et al., 2004). Similarly, maize *yabby* expression becomes restricted to the actively-growing margins of primordia as they mature. Because the contribution of *YABBY* genes to polarity is not conserved between lineages, their conserved function may be to mediate laminar expansion (Juarez et al., 2004b).

Putting polarity in-phase: ta-siRNAs and adaxial identity

A screen for suppressors of ectopic *KAN* expression revealed *ETTIN* [*ETT*, also known as *AUXIN RESPONSE FACTOR3* (*ARF3*)] as an additional contributor to abaxial fate in *Arabidopsis* (Fig. 4A) (Pekker et al., 2005). Single *ett* mutants have no obvious leaf-polarity defects, but resemble *kan* loss-of-function mutants in an *auxin response factor4* (*arf4*) background. Although the abaxial localization of *ARF4* within primordia is consistent with its role in abaxial identity, *ETT* is expressed more ubiquitously throughout the meristem and primordia.

ETT and *ARF4* are both regulated by a recently discovered, plant-specific small RNA class known as the trans-acting short-interfering RNAs (ta-siRNAs). ta-siRNAs are derived from non-coding *TAS* transcripts, which are initially targeted for cleavage by specific miRNAs (Allen et al., 2005). However, unlike most miRNA-directed cleavage products, *TAS* cleavage fragments are stabilized and converted into dsRNAs through the activities of the zinc-finger protein SUPPRESSOR OF GENE SILENCING 3 (SGS3) and RNA-DEPENDENT RNA POLYMERASE 6 (RDR6), respectively (Yoshikawa et al., 2005). DICER-LIKE 4 (DCL4) then processes these dsRNAs into 21 bp ta-siRNAs, which guide the cleavage of target mRNAs, similar to the action of miRNAs (Allen et al., 2005; Gasciolli et al., 2005; Xie et al., 2005b; Yoshikawa et al., 2005).

In *Arabidopsis*, the transcripts of three gene families – TAS1, TAS2 and TAS3 – are processed into ta-siRNAs (Vazquez et al., 2004b; Peragrine et al., 2004; Allen et al., 2005). Because ta-siRNAs are generated with a 21-nucleotide phasing that starts from the miRNA cleavage site, the sequences of the ta-siRNAs and their potential targets can be predicted using computational approaches (Allen et al., 2005). The *TAS3*-derived ta-siRNAs, ta-siR2141 and ta-siR2142 (referred to hereafter as tasiR-ARFs), regulate the expression of *ETT* and *ARF4* through cleavage, thus implicating tasiR-ARFs as important adaxial determinants (Allen et al., 2005; Williams et al., 2005a).

The contribution of the ta-siRNA pathway to adaxial-abaxial patterning in *Arabidopsis*, however, is not immediately apparent. Null mutations in *RDR6*, *SGS3* and *DCL4* block ta-siRNA biogenesis, but lead to phenotypes that are associated with accelerated vegetative-phase change – such as early leaf elongation, downward curling of leaf margins and precocious abaxial trichome production – rather than defects in leaf polarity (Table 2) (Peragine et al., 2004; Yoshikawa et al., 2005; Xie et al., 2005b). The loss of *TAS3*-derived ta-siRNAs is probably the cause of such phenotypes, because leaves from hypomorphic *rdr6* mutants that specifically lack *TAS1*- and *TAS2*-derived ta-siRNAs, but not *TAS3*-derived ta-siRNAs, seem normal (Adenot et al., 2006). Consistent with these findings, loss-of-function mutations in *TAS1* and *TAS2* cause no obvious developmental defects in *Arabidopsis*, unlike *tas3* mutants, which exhibit phenotypes similar to other ta-siRNA-pathway mutants (Adenot et al., 2006). Additionally, *argonaute 7* [*ago7*, also known as *zippy* (*zip*)] and *double-stranded RNA-binding protein4* (*drb4*) mutants specifically affect the accumulation of *TAS3*-derived ta-siRNAs and lead to leaf morphology defects (Adenot et al., 2006; Fahlgren et al., 2006). The evidence suggests that a unique RNAi pathway, sub-specialized for the biogenesis and action of a small subset of ta-siRNAs, contributes to leaf development.

Evidence that the ta-siRNA pathway in *Arabidopsis* interacts with components involved in leaf polarity is suggested through mutants compromised for both the ta-siRNA and ASYMMETRIC LEAVES pathways. The leaves of double mutants are severely lobed and elongate, and the expression levels of *FIL*, miR165 and miR166 are elevated in double mutants relative to wild-type plants (Li et al., 2005; Garcia et al., 2006; Xu et al., 2006). Single mutants for components in either pathway have normal levels of *FIL*, indicating the cooperative repression of an abaxial determinant by the ASYMMETRIC LEAVES pathway and the tasiR-ARF pathway, via *ETT*. However, the consequences of such repression on leaf polarity remains unknown, as *as1* defects are enhanced in the double mutants, but the leaves of such plants largely retain adaxial-abaxial polarity (Garcia et al., 2006).

Other evidence illustrates that the role of *ETT* in adaxial-abaxial patterning is surprisingly complex and multifaceted. *ETT* alleles resistant to tasiR-ARF cleavage (*ETTmut*) recapitulate the phase-change phenotypes of ta-siRNA-pathway mutations and develop precocious abaxial trichomes, a phenotype that is inconsistent with the predicted abaxializing phenotype of a leaf-polarity defect (Fahlgren et al., 2006; Hunter et al., 2006). Given the uniform expression of *ETT* throughout the meristem and primordia, and the abaxial restriction of *ARF4* (Pekker et al., 2005), the ability of *ETT* to specify abaxial fate may depend on the presence of *ARF4*. It is conceivable that, in the absence of *ARF4, ETT* has a neutral effect on leaf polarity, but not on vegetative-phase change. Additionally, *ETT* may be subject to translational repression that might restrict ETT protein to the abaxial domain.

The localization of tasiR-ARF activity within the meristem and leaf primordia is key to fully understanding the role of the ta-siRNA pathway in leaf polarity. A genetrap that lies upstream, and in the anti-sense orientation, of the *TAS3* locus expresses adaxially (Garcia et al., 2006), but whether this genetrap reflects the expression pattern of the sense *TAS3* precursor or the accumulation of tasiR-ARFs within leaf primordia remains to be shown. If tasiR-ARFs act adaxially, a more direct role for ta-siRNAs in leaf polarity might be inferred from the expression of a tasiRARF-resistant *ARF4* allele in the adaxial domain.

In maize, the contribution of the ta-siRNA pathway to leaf polarity is more evident than in *Arabidopsis*. The leaves of maize *leafbladeless1* (*lbl1*) mutants are centric and abaxialized, with defects in vascular patterning (Timmermans et al., 1998). The cloning and sequencing of *lbl1* has revealed that it encodes the maize *SGS3* homolog (F.N., unpublished data). Interestingly, tasiR-ARFs are conserved throughout both monocot and dicot lineages (Allen et al., 2005; Williams et al., 2005a), and, predictably, their biogenesis is compromised in *lbl1* mutants (F.N., unpublished data). Relative to wild-type plants, expression of the *HD-ZIPIII* family members *rld1* and *phb* are reduced in *lbl1* mutants, and elevated levels of *HD-ZIPIII* expression in *Rld1-O* mutants, which carry a miR166-insensitive allele of *rld1*, suppress the polarity defects of *lbl1* (Juarez et al., 2004b). The ta-siRNA pathway in maize thus contributes to leaf polarity by non-redundantly

regulating the accumulation of *HD-ZIPIII* transcripts on the adaxial side of the developing leaf. The limited contribution of the *Arabidopsis* ta-siRNA pathway to leaf polarity suggests a variable dependence on individual polarity pathways between plant lineages; just as the *phan* mutation illustrates a key role for the ASYMMETRIC LEAVES pathway in polarity in *Antirrhinum* relative to *Arabidopsis* (Waites and Hudson, 1995). The differing contributions of these pathways between plant lineages may arise from variation in the downstream targets that they regulate (e.g. *YABBY* genes) (Juarez et al., 2004b) or in the time during leaf development at which they act.

Acting *in trans*: candidate positional signals in adaxial-abaxial patterning

The seminal contribution of the Sussex experiments to the study of leaf polarity is the hypothesis that a mobile, meristem-borne signal specifies the adaxial fate of leaves. Although the Sussex signal remains unknown, circumstantial evidence suggests non-cell-autonomous activity for several polarity determinants – both those of adaxial and abaxial fates.

In situ hybridizations of miR166, an abaxial determinant, in maize reveal a gradient of accumulation, reminiscent of a mobile signal (Juarez et al., 2004a). Whether such a gradient derives from the movement of miR166 itself or from the movement of a secondary signal remains to be determined. The results from experiments on artificial miRNAs and from studies using sensors of miRNA activity suggest that miRNAs largely act cell autonomously (Parizotto et al., 2004; Alvarez et al., 2006; Schwab et al., 2006). However, at least in one instance, the non-cell autonomy of miRNAs over small cellular distances has been implied (Schwab et al., 2006). Formal proof that miRNAs themselves (rather than their activity) can act non-cell autonomously within specific developmentally relevant contexts remains to be shown.

The notion that miRNAs might act as mobile signals is interesting, considering that their targets – the *HD-ZIPIII* genes – might themselves be regulated by other positional signals. Even though the original semi-dominant *HD-ZIPIII* alleles result from the abrogation of the miRNA-binding site (McConnell et al., 2001; Emery et al., 2003), loss-of-function *rev* alleles, with conservative mutations in the START domain downstream of the miR166 target site (Otsuga et al., 2001), are consistent with a role for lipids in regulating HD-ZIPIII function and, perhaps, in contributing to the Sussex signal.

Just as the *HD-ZIPIII* genes implicate the involvement of candidate signals with seemingly opposing activities – miR166 and a lipid signal – *ETT* and *ARF4* suggest other putative polarizing signals, namely auxin and the tasiR-ARFs, that might convey positional information within the shoot apex to the newly initiated leaf (Fig. 5). The control of gene expression by ARF transcription factors is modulated by auxin signaling (for a review, see Woodward and Bartel, 2005). The role of *ETT* and *ARF4* in abaxial identity is compelling, considering the abaxial localization of the putative auxin influx carrier AUX1 (Reinhardt et al., 2003). Although the mechanisms by which *ETT* and *ARF4* contribute to abaxial fate are unknown and might involve auxin-independent functions, one hypothesis is that they may interpret auxin gradients present in the leaf (Pekker et al., 2005). If so, auxin could conceivably serve as a secondary signal that directs a gradient of miR166 accumulation on the abaxial side of incipient primordia in maize (Juarez et al., 2004a), because a number of *MIR166*-family members contain ARF-binding sequences within their promoters. The idea that auxin exists as gradients in plants is not unprecedented; such gradients have been found within the cambium of trees and correlate with the differential expression of potential downstream genes (Uggla et al., 1996; Moyle et al., 2002; Bhalerao and Bennett, 2003). However, the presence of auxin gradients in leaves and a direct contribution of auxin to adaxial-abaxial patterning has yet to be shown.

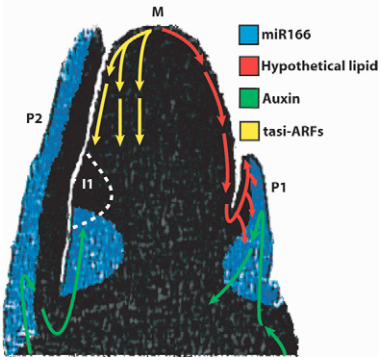

Fig. 5. Hypothetical signals specifying leaf polarity in the meristem. miR166 (blue) accumulates most strongly below the incipient leaf (I1, white dashed line) and in a graded pattern in I1 and older primordia (P1, P2). Such a gradient might be formed by the movement of miR166 itself or may be directed by a secondary mobile signal. A hypothetical lipid signal (red), possibly required for HD-ZIPIII activity, is predicted to act in the meristem (M). If such a lipid contributes to the Sussex signal, it might be restricted to the L1 layer, as shown. The distribution of auxin within the primordia and stem (green), as inferred from the localization of AUX1 and PIN1 (Reinhardt et al., 2003), could contribute to abaxial fate through either *ETT* and/or *ARF4* or perhaps *MIR166*. Notice that the distributions of auxin within the leaf are speculative. tasi-ARFs (yellow) might also regulate leaf polarity non-cell autonomously, possibly by moving out of the meristem tip, where *SGS3/lbl1* is expressed.

Unlike miR166, a more palpable connection to mobility exists for ta-siRNAs. ta-siRNAs are created through a distinct branch of the RNAi machinery that includes *DCL4* and *RDR6* (Allen et al., 2005; Gasciolli et al., 2005; Xie et al., 2005b; Yoshikawa et al., 2005), factors required for systemic silencing via mobile siRNAs (Himber et al., 2003; Dunoyer et al., 2005; Voinnet, 2005). If the non-cell autonomy of siRNAs depends on factors that are specific to their biogenesis, similar factors may mediate the movement of tasiR-ARFs. The tasiR-ARF biogenesis machinery – which uniquely involves miR390, AGO7 and DRB4 – might also exist to limit tasiR-ARF production, as mature tasiR-ARFs scarcely accumulate in vivo despite an abundance of precursor transcripts (Allen et al., 2005; Williams et al., 2005a). If tasiR-ARFs themselves move, then their dose would implicitly affect their range of activity, an important variable if a balance between adaxial and abaxial fates is to be struck. The conservation of the tasiR-ARF pathway might reflect a requirement to differentially regulate the activity of these small RNAs relative to other ta-siRNAs, with the result of properly patterning leaves and maintaining the meristem.

Conclusion

Cell fate in plants is largely determined by positional information. However, despite their inferred importance, plant signals with known developmental roles are lacking. The few well-known examples include CLAVATA3, which regulates meristem size (Fletcher et al., 1999), the mobile transcription factor SHORT-

ROOT, which is required for proper radial patterning (Nakajima et al., 2001), movement of the *FLOWERING LOCUS T* (*FT*) transcript, perhaps as a component of 'florigen' (Huang et al., 2005), and auxin, whose transport underlies a range of developmental processes (for a review, see Woodward and Bartel, 2005).

The identification of *ETT* and *ARF4* as contributing to the adaxial-abaxial polarity of leaves, as well as the more classical HD-ZIPIII pathway, suggests possible identities of the positional signals required to pattern the leaf. Unexpectedly, small RNAs are among such candidates, the opposing activities of which contribute to the establishment of both the adaxial and abaxial domains of the leaf – an example unprecedented in developmental biology. The restriction of *HD-ZIPIII* activity and adaxial fates by miR166, and the further demarcation of the adaxial-abaxial boundary by the tasiR-ARF pathway in maize, suggests that upstream mechanisms exist that establish a balance between mutually antagonistic determinants within the leaf. Also possible is the confluence and integration of multiple positional signals; for example, the possible convergence of the tasiR-ARF pathway and auxin signals on *ETT* and *ARF4* to regulate downstream factors, such as miR166 and *FIL*. As the contributions of different polarity pathways differs widely between plants lineages – for example, the prominence of the HD-ZIPIII, ARP and ta-siRNA pathways to adaxial fate in the Eurosids (*Arabidopsis*), Euasterids (*Antirrhinum* and the Solanaceae), and monocots (maize), respectively – it is possible that different signals are present in different species, or that they affect different downstream targets or act to varying degrees. Identifying functional positional signals, exploring their interactions and understanding how the patterning of leaves is determined by their movement remain the major challenges facing the study of leaf polarity.

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