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Molecular Mechanisms of Leaf Morphogenesis

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ABSTRACT

Plants maintain the ability to form lateral appendages throughout their life cycle and form leaves as the principal lateral appendages of the stem. Leaves initiate at the peripheral zone of the shoot apical meristem and then develop into flattened structures. In most plants, the leaf functions as a solar panel, where photosynthesis converts carbon dioxide and water into carbohydrates and oxygen. To produce structures that can optimally fulfill this function, plants precisely control the initiation, shape, and polarity of leaves. Moreover, leaf development is highly flexible but follows common themes with conserved regulatory mechanisms. Leaves may have evolved from lateral branches that are converted into determinate, flattened structures. Many other plant parts, such as floral organs, are considered specialized leaves, and thus leaf development underlies their morphogenesis. Here, we review recent advances in the understanding of how three-dimensional leaf forms are established. We focus on how genes, phytohormones, and mechanical properties modulate leaf development, and discuss these factors in the context of leaf initiation, polarity establishment and maintenance, leaf flattening, and intercalary growth.

Key words: leaf, lateral organ, morphogenesis, meristem, blastozone, shoot

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INTRODUCTION

Seed plants have two different modes of organ growth: stems and roots undergo radial symmetric growth in one direction; by contrast, appendages such as leaves, lateral branches, and flowers, as well as lateral roots, undergo lateral growth in multiple directions (Bowman et al., 2002; Golz and Hudson, 2002). The various appendages occur in startling variety across the plant kingdom and some of them may have common origins. For example, floral organs, such as sepals, petals, stamens, carpels, and other structures, are considered modified leaves that arose through "metamorphosis" (von Goethe, 1790; Bell, 1991; Cronk, 2009), and leaves of ferns and seed plants appear to have been initially derived from a cleaved or branched shoot (Zimmermann, 1952; Sanders et al., 2011). Thus, leaves serve as an ideal system to understand the plasticity of organ morphogenesis in developmental and evolutionary contexts. Moreover, leaves are exposed to a variety of environmental factors such as light, water, temperature, microbes, and insects, and leaves use this environmental information to integrate internal and external signals. Therefore, a deeper understanding of leaf development contributes to our overall comprehension of plant biology, and this understanding can be used to improve crop production.

In this review, we briefly introduce the basic structures and events involved in typical leaf development. Furthermore, we detail the mechanisms of leaf morphogenesis, including the genetic and mechanical regulatory frameworks that contribute to leaf initiation, leaf polarity determination, and leaf outgrowth and flattening in the bifacial, flat-formed leaves of eudicots such as *Arabidopsis* (*Arabidopsis thaliana*) and tomato (*Solanum lycopersicum*). We also emphasize recent findings that have improved our understanding of leaf development. As most of the findings were made in *Arabidopsis*, and usually can be generalized, we use *Arabidopsis* gene names in this review unless otherwise specified.

AN OUTLINE OF LEAF DEVELOPMENT: STRUCTURE, MORPHOGENESIS, AND POSSIBLE ORIGIN

Leaf development originates from the shoot apical meristem (SAM), which harbors a stem cell niche that is the source of all above-ground organs, during the post-embryonic development of plants. The SAM can be divided into different functional domains, including a central zone (CZ) containing pluripotent stem cells that organize and renew the meristem, and a peripheral zone (PZ) from which lateral organs, such as leaves, initiate (Figure 1A) (Barton, 2010). Leaf morphogenesis in eudicots occurs in four stages (Figure 2). First, the founder cells that are

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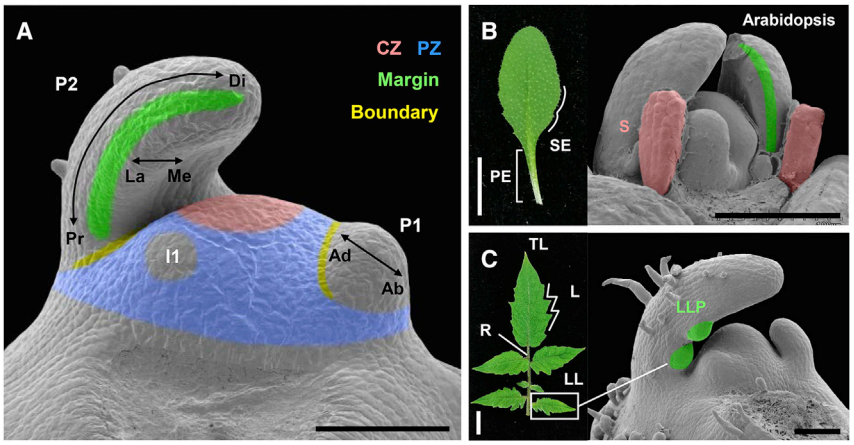

Figure 1. Morphology of Simple and Compound Leaves.

(A) The organization of the shoot apex of tomato. Leaf primordia initiate from the peripheral zone (PZ), which surrounds the central zone (CZ) of the shoot apical meristem (SAM). The oldest incipient primordium (I1) marks the future initiation site, which cannot be distinguished in appearance from the SAM at this stage. Primordia are named according to the order of initiation; the youngest primordium that proliferates from the SAM is designated P1, the second youngest is P2, etc. A boundary forms following primordia initiation; this boundary separates the SAM and lateral organs. Leaf asymmetries are established in three axes marked by arrows: adaxial–abaxial axis (Ad–Ab), proximal–distal axis (Pr–Di), and medio—lateral axis (Me–La). The margin, covering the juxtaposition of the adaxial and abaxial leaf domains, initiates leaf blade outgrowth.

(B) Morphology of a simple leaf from *Arabidopsis*. Left: the mature rosette leaf displays serrations (SE) along the leaf margins and a petiole (PE) at the leaf base. Right: stipules (S) are generated beside young leaf primordia. Note that the leaf margins (green) of simple leaves are continuous.

(C) Morphology of a compound leaf from tomato. Left: the compound leaf is composed of a terminal leaflet (TL) and several lateral leaflets (LL) attached to a central rachis (R). Lobes (L) can be found in the margins of leaflets. Right: the lateral leaflet develops from the lateral leaflet primordium (LLP) along the discontinuous leaf margins.

Black scale bars, 100 μm. White scale bars, 1 cm.

designated to develop into leaves are recruited from the PZ of the SAM (Figure 2A). Second, distal growth occurs after the initiation of the leaf primordium, and the adaxial–abaxial and proximal–distal axes are established (Figures 1A and 2B). Third, the leaf blade, otherwise known as the lamina, initiates at the site neighboring the margin, along the medio–lateral axis, separating the blade from the petiole (Figures 1A and 2C). Finally, intercalary growth occurs throughout the entire leaf blade, which results in the overall expansion of leaf area in multiple directions (Figure 2D) (Poethig and Sussex, 1985; Donnelly et al., 1999; Ichihashi et al., 2011; Nakata et al., 2012). These steps may temporarily overlap with each other. For example, the formation of the leaf blade (i.e., growth in the medio–lateral axis) can occur while the adaxial–abaxial and proximal–distal axes are being established. In this review, we dissect the mechanisms and regulators of leaf growth through these stages.

In many eudicots, the leaf blade is attached to the stem by a petiole (Figure 1B). Many eudicots, such as *Arabidopsis*, also generate stipules near the leaf base that protect developing young leaves and serve as a source of the phytohormone auxin during early leaf development (Figure 1B) (Aloni et al., 2003). Despite variation in size and shape, leaves are traditionally classified as simple or compound based on the number of blades (Figure 1B and 1C). Simple leaves have a single blade, the margins of which are continuous and can be smooth, serrated, or lobed; compound leaves have several separate blades known as leaflets, which are attached to a common rachis (Bar and Ori, 2015; Efroni et al., 2010; Runions et al., 2017). Genetic evidence shows that the initiation of leaflet primordia during compound leaf development resembles the initiation of simple leaf primordia (Hasson et al., 2010). We refer the reader to recent reviews on compound leaves for a more comprehensive view (Blein et al., 2010; Bar and Ori, 2015).

The ability of compound leaves to initiate leaflet primordia from their lateral sides suggests that a leaf, in particular a compound leaf, can be considered a "partial shoot" (Arber, 1950). Indeed, different models have proposed that leaves originate from shoot-identical structures such as branches (De Candolle, 1868; Zimmermann, 1952; Sanders et al., 2011). Consistent with this, fossil evidence reveals that the earliest vascular plants were composed of leafless, dichotomously branched axes (Figure 3A) (Giesen and Berry, 2013). The widespread appearance of flattened leaf blades in the Late Devonian is thought to have arisen in response to a dramatic (~90%) decline in atmosphere carbon dioxide (CO_2), since high CO_2 levels may cause large photosynthesizing leaf blades to reach lethal temperatures (Beerling et al., 2001; Beerling and Fleming, 2007). Zimmermann's telome theory speculates that the leaves of seed plants evolved from a three-dimensional lateral shoot branch of early vascular land plants, which subsequently transformed into a two-dimensional planar branch system, and finally formed leaf blades via the fusion of branches (Figure 3B–3E) (Zimmermann, 1952; Beerling and Fleming, 2007). However, the telome theory has long lacked a developmental mechanism (Sanders et al., 2007; Boyce, 2010).

SPECIFICATION OF PRIMARY LEAF CELLS AND INITIATION FROM THE SAM

Hormonal Regulation during Leaf Initiation (Phyllotaxis)

Leaves initiate from PZ cells that are amenable to differentiation. Auxin plays a key role in specifying those cells, as only the PZ cells overlapping periodic auxin maxima are designated to develop into leaves. Auxin maxima are established by auxin efflux carrier PINFORMED1 (PIN1)-dependent polar auxin transport (PAT) (Reinhardt et al., 2000, 2003). Indeed, leaf formation is blocked in tomato shoot apices treated with the PAT inhibitor *N*-1-naphthylphthalamic acid (NPA) (Reinhardt et al., 2000). Floral primordium formation is also blocked in *Arabidopsis pin1* mutants (Okada et al., 1991). External application of auxin restores primordia formation in *pin1* mutants and NPA-treated

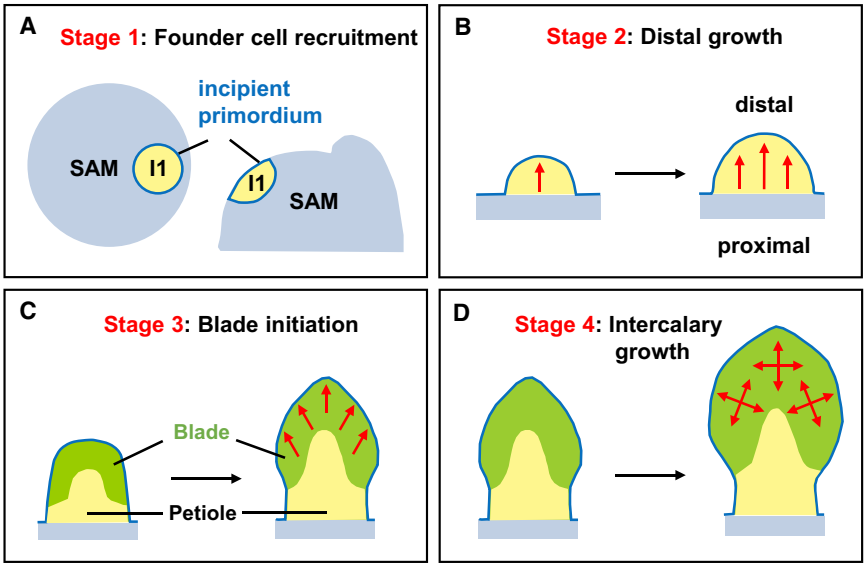

Figure 2. Schematic Illustration of Leaf Morphogenesis in Eudicots.

(A) The founder cells are recruited from the peripheral zone of the SAM at the site of the incipient leaf primordium before initiation. I1: the oldest incipient leaf primordium. Left, top view; right, front view.

(B) Immediately following initiation, the leaf primordium grows predominantly in the distal direction.

(C) Subsequently, the blade and petiole regions are specified, and leaf growth and expansion occur largely at the margins during this stage.

(D) Finally, along with the termination of marginal meristem activity, cell proliferation and expansion occur in the entire blade, which leads to both distal and lateral leaf expansion.

Modified from Nakata and Okada (2013).

shoot apices, indicating a pivotal role for auxin in primordium initiation (Reinhardt et al., 2000). Detailed analysis of subcellular PIN1 localization indicated that PIN1-mediated PAT in the surface layer of the SAM leads to auxin accumulation at leaf initials (Reinhardt et al., 2003; Heisler et al., 2005). Computational models that assume positive feedback between auxin and PIN1 can recapitulate phyllotaxis, the regular patterns of leaf and flower arrangement around the SAM. Some models assume that PIN1 proteins are oriented toward neighboring cells with higher auxin concentrations (Jönsson et al., 2006; de Reuille et al., 2006; Smith et al., 2006), whereas other models orient PIN1 in the direction of higher tensile stress (Heisler et al., 2010). Additional models may also explain phyllotaxis (Stoma et al., 2008; Abley et al., 2016), and more experimental data are clearly required to describe the molecular mechanism underlying the positive feedback between auxin and PIN1. The stabilization of auxin convergence also involves auxin drainage through internal tissues (Bayer et al., 2009; Deb et al., 2015; Bhatia et al., 2016). PAT in internal tissues forms an additional layer of regulation that enables feedback from lateral organs to influence SAM size (Shi et al., 2018). An organ primordia-expressed CLAVATA3/ENDOSPERM SURROUNDING REGION-related (CLE) peptide forms another, parallel feedback loop to the SAM (Je et al., 2016). It should be noted that even in strong *Arabidopsis pin1* mutants, leaf initiation is less compromised than floral primordium initiation, as shown by the naked-inflorescence phenotype of *pin1*, which produces few or no flowers. Thus, additional regulatory mechanisms exist, which are not likely to involve other PIN proteins (Guenot et al., 2012).

As discussed above, auxin and PIN1 function together to generate a pattern, which requires environmental and metabolic signals in the SAM as inputs. At a minimum, light and sugar signals are required to enable lateral organ formation in the SAM; these signals converge on the TARGET OF RAPAMYCIN (TOR) kinase, a central growth regulator (Pfeiffer et al., 2016; Li et al., 2017). Light activates auxin biosynthesis in the shoot apex, and auxin mediates light-dependent activation of TOR (Li et al., 2017). Moreover, cells relay the light signal through cytokinin signal transduction pathways (Yoshida et al., 2011; Pfeiffer et al., 2016). Light regulates leaf initiation by activating cytokinin signaling and affecting efflux-dependent auxin gradients (Yoshida et al., 2011). Based on the role of light, sugar energy, and TOR in promoting cell growth and division, it is tempting to speculate that active growth and cell-cycle progression are necessary for the auxin/PIN1 pattern generator to function.

The auxin/PIN1 pattern generator requires additional feedback loops that stabilize phyllotaxis. For example, auxin influx carriers stabilize phyllotactic patterning (Stieger et al., 2002; Bainbridge et al., 2008), and these factors participate in both simple leaf morphogenesis and the regulation of leaflet initiation in compound leaves. The genes encoding the auxin influx carriers AUX1 and LIKE-AUX1 2 (LAX2) are direct targets of MONOPTEROS (MP), an auxin-responsive transcription factor (Robert et al., 2015). Auxin, acting through MP, also activates the expression of *MP* and *PIN1* (Bhatia et al., 2016; Krogan et al., 2016), forming multiple feedback loops leading to robust regulation. Another unique feedback regulatory mechanism involves the cytokinin signaling inhibitor ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6 (AHP6) (Besnard et al., 2014). Auxin activates *AHP6* expression through MP in young primordia, and AHP6 moves between cells to generate inhibitory fields that prevent premature outgrowth of the primordia.

How then does auxin specify primordium cells? Recent work in tomato indicates that expression of the ethylene response factor-type transcription factor LEAFLESS (LFS) is induced by auxin maxima in the PZ, and is necessary for leaf initiation (Capua and Eshed, 2017). The *lfs* mutants, as the gene name suggests, fail to produce cotyledons and leaves and grow as a naked pin, a phenotype that resembles the pin-like shoots induced by NPA. However, *lfs* pins also fail to initiate leaf primordia following auxin microapplication. *LFS* is the single tomato ortholog of the *Arabidopsis DORNRÖNSCHEN* (*DRN*) and *DRN-LIKE* (*DRNL*) genes, whose expression patterns overlap with auxin response maxima in both vegetative and inflorescence

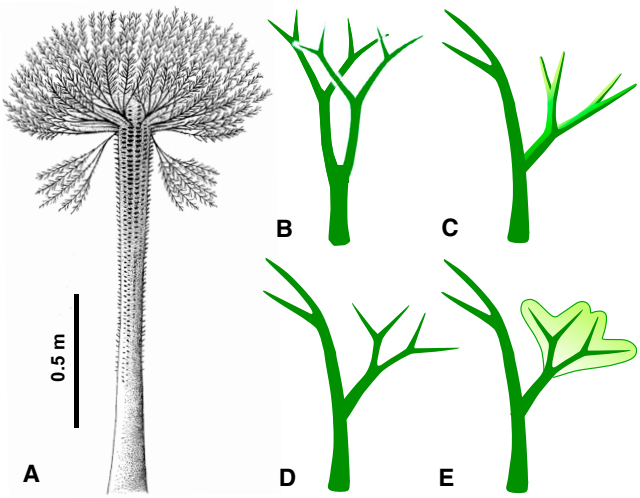

Figure 3. Evolutionary View of Leaf Morphogenesis Based on the "Telome Theory."

(A) Reconstruction of the early tree *Calamophyton* from the Middle Devonian.

(B) Leaves evolved from three-dimensional shoot branches of early vascular plants.

(C) One branch overgrew and formed the main stem, while the other branch became the three-dimensional lateral branch (overtopping).

(D) Neighboring lateral branches became arranged into a single plane (planation).

(E) Lateral growth and fusion between branches led to the formation of a flattened leaf blade (webbing).

(A) is reproduced from Giesen and Berry (2013). **(B)** to **(E)** are modified from Beerling and Fleming (2007).

SAMs (Chandler et al., 2011; Seeliger et al., 2016; Capua and Eshed, 2017). Compared with *DRN*, the expression of *DRNL* correlates more with leaf primordia (Chandler et al., 2011). DRN and DRNL function redundantly in cell-cycle progression and promote G_1–S transitions in the SAM (Seeliger et al., 2016). Based on the role of auxin in leaf initiation and the expression pattern and function of *LFS*/*DRNL*, it is tempting to hypothesize that auxin maxima induce cells to develop into leaf primordia by activating *LFS*/*DRNL* expression, which then promotes cell division.

Roles of Transcription Factors during Leaf Initiation and Separation from the SAM

During leaf initiation, the expression of *SHOOT MERISTEMLESS* (*STM*), which encodes a class I KNOTTED-LIKE HOMEOBOX (KNOX1) transcription factor, is downregulated in the cells of the developing leaf (Figure 4) (Long et al., 1996). KNOX1 transcription factors maintain the SAM cell fate in *Arabidopsis* and maize (Barton and Poethig, 1993; Kerstetter et al., 1997). The maintenance of the repressed state of *KNOX1* genes in the leaf primordium depends on ARP MYB domain transcription factors, which are named after ASYMMETRIC LEAF1 (AS1) from *Arabidopsis*, ROUGH SHEATH2 (RS2) from maize, and PHANTASTICA (PHAN) from *Antirrhinum* (Figure 4) (Timmermans et al., 1999; Tsiantis et al., 1999; Byrne et al., 2000). A repressor complex consisting of AS1 and AS2, a LATERAL ORGAN BOUNDARIES (LOB) domain protein, directly binds to the promoters of the *KNOX1* genes *BREVIPEDICELLUS* (*BP*) and *KNAT2* to repress their expression (Guo et al., 2008). In addition, auxin works together with ARPs to repress the expression of *BP* in *Arabidopsis* (Hay et al., 2006). In addition to *ARP* genes, *KNOX2* genes antagonize *KNOX1* genes to promote leaf development (Furumizu et al., 2015). Similar to ARPs, ectopic expression of KNOX2 and its heterodimeric partner BELL suppresses SAM activity.

During compound leaf development, at least in tomato and *Cardamine hirsuta*, *KNOX1* expression is restored within leaf primordia and leads to the formation of separate leaflets (Hay and Tsiantis, 2006). Recent work also identified the crucial role of a novel homeobox gene, *REDUCED COMPLEXITY* (*RCO*), in compound leaf development by repressing growth between leaflets and thus promoting leaflet separation (Vlad et al., 2014). *RCO* is present in *Cardamine hirsuta* and various *Capsella* species that have compound leaves or deeply serrated leaf margins, but was evolutionarily lost in *Arabidopsis*, which has simple leaves (Sicard et al., 2014; Vlad et al., 2014).

When leaf development initiates at the PZ, a boundary region partitions the new leaf primordium from the SAM on the adaxial side (Figure 1A) (Wang et al., 2016). This boundary region forms the future leaf axil. Formation of a boundary is accompanied by a reduced frequency of cell division and a low growth rate (Kwiatkowska and Dumais, 2003). The CUP-SHAPED COTYLEDON (CUC) NAC-domain transcription factor genes *CUC1*, *CUC2*, and *CUC3* are specifically expressed in boundary positions (Figure 4) (Aida et al., 1999; Vroemen et al., 2003; Hibara et al., 2006). Mutations of *CUC* genes lead to varying degrees of shoot organ fusion phenotypes, indicating that boundary formation is crucial for organogenesis. Expression of *CUC1* and *CUC2* is negatively regulated by the miR164 family of microRNAs (miRNAs), which promote mRNA cleavage (Laufs et al., 2004; Mallory et al., 2004; Baker et al., 2005). Notably, KNOX1 proteins are also strongly expressed in the boundary region and promote boundary formation. KNOX1 proteins, such as STM, and CUC proteins regulate each other to promote boundary specification (Spinelli et al., 2011; Balkunde et al., 2017). *CUC* expression is negatively affected by auxin maxima, as shown by genetic and expression analyses (Aida et al., 2002; Bilsborough et al., 2011). PIN1 is also necessary for organ positioning, separation, and outgrowth; the expression domains of *CUC1* and *CUC2* expand to the periphery in double mutants of *PIN1* and *PINOID* (*PID*) (Vernoux et al., 2000; Furutani et al., 2004).

Mechanical Forces Involved in Leaf Primordium and Boundary Formation

In addition to hormones and transcription factors, mechanical forces are also important for leaf morphogenesis (Sampathkumar et al., 2014; Traas, 2017). The interplay between turgor pressure and cell wall mechanics can determine the direction and rate of cell expansion and thus affect pattern formation in plants (Cosgrove, 2005; Dumais, 2007). Over the last few years, it has become clear that cells in the CZ of the SAM have stiffer cell walls than those in the PZ (Milani et al., 2011; Kierzkowski et al., 2012). In addition, organ outgrowth is accompanied by an increase in cell wall elasticity (Peaucelle et al., 2011). Local application or expression of expansins, extracellular proteins that increase cell wall

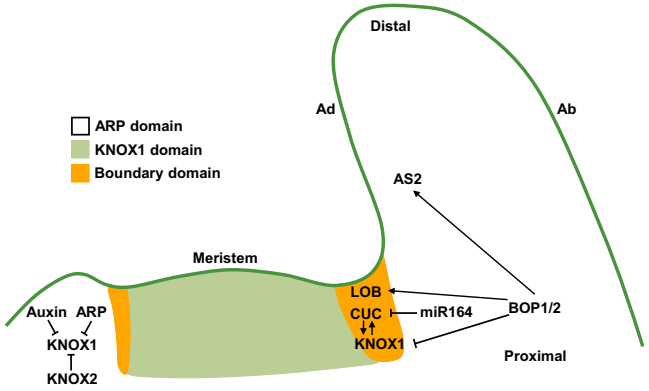

Figure 4. Regulatory Networks during Leaf Initiation.
Leaf initiation requires the repression of *KNOX* gene expression by a local auxin response maximum in the incipient leaf primordium. Moreover, expression of boundary-specific genes leads to the formation of a boundary domain that separates the leaf primordium from the SAM. The establishment of proximal–distal polarity and petiole specification is regulated by the expression of *BOP* genes at the leaf base. Arrows indicate positive/promoting regulation, whereas lines with perpendicular end bars indicate negative/repressing regulation.
Ab, abaxial; Ad, adaxial; ARP, abbreviated from ASYMMETRIC LEAF1, ROUGH SHEATH2, and PHANTASTICA; AS2, ASYMMETRIC LEAF2; BOP, BLADE ON PETIOLE; CUC, CUP-SHAPED COTYLEDON; KNOX, KNOTTED-LIKE HOMEOBOX; LOB, LATERAL ORGAN BOUNDARIES; miR164, microRNA 164.

extensibility *in vitro*, induces leaf primordia at aberrant positions (Fleming et al., 1997; Pien et al., 2001). Alterations of pectin in cell walls mediated by pectin methylesterases also correlate with organ initiation (Peaucelle et al., 2008).

It has long been known that cell wall properties can be regulated by auxin (Rayle and Cleland, 1992). During primordium formation in the shoot apex, auxin not only reduces cell wall stiffness but also affects wall anisotropy through the regulation of cortical microtubule dynamics (Sassi et al., 2014). The boundary region is mechanically distinct from the SAM and the primordium (Hamant et al., 2008). The expression of *STM* and *CUC3* in the boundary region is induced by mechanical stress, which is at least partially auxin independent (Landrein et al., 2015; Fal et al., 2016).

POLARITY ESTABLISHMENT AND MAINTENANCE DURING LEAF OUTGROWTH

Following founder cell recruitment, a young leaf primordium starts distal growth and proliferates from the flank of the SAM. During this process, it becomes asymmetric along the adaxial–abaxial, medio–lateral, and proximal–distal axes (Figure 1A). Asymmetric distribution of cell and tissue types along these axes underlies the final leaf form. As it is an ideal system to understand organ patterning, leaf polarity has been an active field of study for decades.

Adaxial–abaxial polarity allows further establishment of medio–lateral polarity (Waites and Hudson, 1995; Guan et al., 2017), and has been of primary interest in studies aiming to understand leaf polarity. The adaxial–abaxial difference becomes visible soon after leaf initiation, suggesting that an adaxial–abaxial prepattern is established prior to leaf initiation (Hagemann and Gieissberg, 1996; Husbands et al., 2009). Recent work in *Arabidopsis* confirmed prepatterned expression of adaxial–abaxial polarity genes at the PZ prior to leaf primordium formation (Caggiano et al., 2017; Yu et al., 2017). Time-lapse imaging showed that *REVOLUTA* (*REV*), which encodes an HD-ZIPIII transcription factor defining adaxial cell fate (Emery et al., 2003), is expressed in the oldest incipient primordium (I_1) and that *REV*-expressing cells form the adaxial domain. Weak *REV* expression also extends into the SAM. By contrast, expression of *KANADI1* (*KAN1*), which encodes an abaxial domain-promoting protein (Kerstetter et al., 2001), forms a ring surrounding the PZ. *KAN1*-expressing cells become the abaxial domain after leaf initiation. Thus, a prepattern of adaxial and abaxial gene expression is established prior to leaf initiation. Ectopic *KAN1* expression may lead to leaves with reversed or mixed adaxial–abaxial polarity, supporting the idea that polarity is prepatterned by *HD-ZIPIII* and *KAN* gene expression (Caggiano et al., 2017). Although the adaxial–abaxial prepattern is maintained during leaf development, it is not absolute. The *REV* domain expands whereas the *KAN1* domain contracts (Yu et al., 2017), indicating that the adaxial–abaxial prepattern shifts dynamically, at least during early leaf development.

The Sussex Signal

Classical microsurgical experiments suggested that an SAM-derived signal, termed the "Sussex signal", promotes adaxial cell fate (Sussex, 1951). When an incipient leaf is separated from the SAM, it develops into an abaxialized radially symmetric leaf (Reinhardt et al., 2005). Although extensive control experiments led Sussex to conclude that the signal is derived from the SAM, independent experiments from other researchers in different species suggested that the flanking regions between (incipient) primordia were also possible sources. Lateral incisions flanking a primordium, which do not block communication with the SAM, lead to similar polarity defects (Snow and Snow, 1959; Shi et al., 2017). Therefore, the Sussex signal may not be entirely derived from the center of the SAM.

Enormous effort has been dedicated to understanding the molecular identity of the Sussex signal. Although it is speculated that wounding associated with surgical manipulation interferes with polarity (Caggiano et al., 2017), such caveats have been addressed by performing extensive control experiments (Kuhlemeier and Timmermans, 2016). Lipophilic molecules are attractive candidate signals because adaxially expressed HD-ZIPIII transcription factors have a START domain predicted to bind lipophilic ligands (McConnell et al., 2001; Kuhlemeier and Timmermans, 2016); however, this hypothesis remains to be tested.

More recent work has shown that auxin transport in the epidermis may explain the results of microsurgical experiments (Qi et al., 2014; Shi et al., 2017). As mentioned above, PIN1-mediated auxin flow forms auxin maxima that define leaf primordia. Following primordium initiation, auxin flows from the organ boundary back to

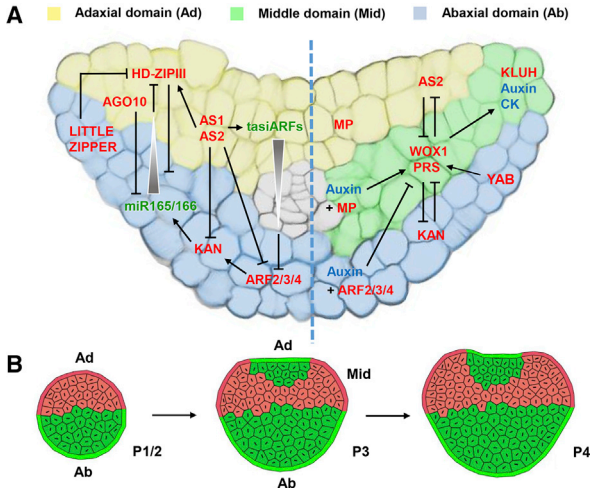

Figure 5. Regulatory Networks during the Establishment of Leaf Polarity.

(A) Left: diverse regulators contribute to the establishment of leaf adaxial–abaxial polarity. Right: interactions between adaxial and abaxial factors contribute to the specification and arrangement of the middle domain. Red, genes; blue, phytohormones; green, small RNAs. AGO, ARGONAUTE; ARF, AUXIN RESPONSE FACTOR; AS, ASYMMETRIC LEAF; CK, CYTOKININ; KAN, KANADI; miR165/166, microRNA 165/166; MP, MONOPTEROS; PRS, PRESSED FLOWER; tasiARFs, *trans*-acting short interfering RNAs targeting *ARF2/3/4*; WOX1, WUSCHEL-RELATED HOMEOBOX1; YAB, YABBY.

(B) Growth simulation of leaf transverse sections showing domains with mechanical heterogeneities caused by different levels of cell-wall pectin methyl-esterification. Green represents low pectin methyl-esterification and high elasticity, and red represents high pectin methyl-esterification and low elasticity. Note that mechanical heterogeneities exist between the adaxial (ad) and abaxial (ab) domains of young leaf primordia, and further exist between all three domains after the middle domain (mid) becomes apparent in P3 and older leaves. Modified from Qi et al. (2017).

the SAM (Heisler et al., 2005; Bayer et al., 2009). The splitting of auxin fluxes transports auxin away from the boundary to the SAM and into the primordium center, making the boundary region an auxin minimum (Wang et al., 2014a, 2014b). Probably because of the reduced number of auxin-supplying cells and substantially reduced auxin levels in the boundary cells, the adaxial domain becomes a transient low-auxin zone (Qi et al., 2014), while PAT converges in the lateral regions (Shi et al., 2017). The blocking of local auxin transport by applying PIN inhibitors can recapitulate microsurgical results (Qi et al., 2014), suggesting that incisions abolish PAT; this then interferes with polarity patterning.

How the Sussex signal is reconciled with the adaxial–abaxial prepattern remains unknown. Given the short developmental window in which microsurgical or PIN inhibitor microapplication interferes with polarity (Sussex, 1951; Reinhardt et al., 2005; Qi et al., 2014; Shi et al., 2017), the Sussex signal may participate in the establishment of the adaxial–abaxial prepattern. Alternatively, it may interfere with the translation of the prepattern into further patterning events. In fact, a recent study showed that auxin signaling activates the middle domain in between the adaxial and abaxial domains (Guan et al., 2017; Shi et al., 2017), supporting the idea that translating the adaxial–abaxial prepattern into leaf blade expansion in the medio–lateral axis requires auxin movement.

Maintenance of Adaxial–Abaxial Polarity

Following its establishment, adaxial–abaxial polarity is maintained and further strengthened via domain-specific expression and mutual repression of adaxial- and abaxial-promoting genes, which encode transcription factors and small RNAs (Figure 5A, left half). Most of these transcription factors fall into distinct classes and act in conserved and partially redundant pathways.

Adaxial cell fate is promoted by the above-mentioned REV and related HD-ZIPIII transcription factors PHAVOLUTA (PHV) and PHABULOSA (PHB) (McConnell et al., 2001; Emery et al., 2003). In addition to their weak expression in the SAM, all three HD-ZIPIII transcription factors are predominantly expressed on the adaxial side. The adaxial expression of *HD-ZIPIII* genes is restricted by miR165 and miR166, which mediate the cleavage and degradation of *HD-ZIPIII* mRNAs (Tang et al., 2003). Mature miR165/166 accumulate in a gradient on the abaxial side of leaf primordia, and the expression pattern of these miRNAs is complementary to that of the *HD-ZIPIII*s in *Arabidopsis* and maize (Juarez et al., 2004a). In *Arabidopsis*, *MIR165A* and *MIR166A* are expressed in the abaxial epidermis of leaf primordia (Yao et al., 2009; Tatematsu et al., 2015). Thus, there is a spatial difference between miR165/166 activity and *MIR165/166* transcription. Strikingly, the small RNA-to-target ratio is finely tuned, resulting in the sharp *HD-ZIPIII* expression boundary observed in the adaxial domain (Skopelitis et al., 2017). Besides this, REV, PHV, and PHB physically interact with the HD-ZIPII transcription factors HAT3 and ATHB4, which are downstream targets of REV, to inhibit miR165/166 expression (Bou-Torrent et al., 2012; Merelo et al., 2016). The spatial distribution of HD-ZIPIII proteins may also rely on miRNA binding by Argonaute proteins (AGOs). In *Arabidopsis*, the adaxial- and SAM-expressed AGO10, also known as PINHEAD/ZWILLE (Moussian et al., 1998; Lynn et al., 1999), specifically sequesters and degrades miR165/166 and thus indirectly upregulates HD-ZIPIII expression (Liu et al., 2009; Ji et al., 2011; Zhu et al., 2011; Zhou et al., 2015). These multi-dimensional bidirectional repressive circuits may lead to robust adaxial domain identity.

HD-ZIPIII expression is sufficient to define adaxial cell fate. Gain-of-function *HD-ZIPIII* mutants, which allow *HD-ZIPIII* genes to escape inhibition by miR165/166, form adaxialized leaves, whereas loss-of-function *phb phv rev* triple-mutant plants form abaxialized leaves and exhibit loss-of-SAM phenotypes. In addition to miRNA regulation, HD-ZIPIII function is also inhibited by LITTLE ZIPPERs, adaxially expressed short proteins (microProteins) that heterodimerize with HD-ZIPIII proteins to inhibit their transcriptional regulatory activity (Wenkel et al., 2007; Kim et al., 2008).

Adaxial cell fate is also promoted by AS2, a LOB-domain (LBD) transcription factor (Iwakawa et al., 2002; Xu et al., 2002; Lin et al., 2003). *AS2* expression is initially uniform in the leaf primordium and becomes restricted to the adaxial side as the leaf primordia grow (Iwakawa et al., 2007). AS2 forms a protein complex with the ARP protein AS1 (Xu et al., 2003), which is

more uniformly distributed in leaf primordia. In *Arabidopsis*, *as1* and *as2* mutants have no obvious polarity defects but show asymmetric laminar growth (Byrne et al., 2000; Semiarti et al., 2001), as do maize *rs2* mutants (Timmermans et al., 1999; Tsiantis et al., 1999). Nevertheless, *Arabidopsis as1* and *as2* mutants are very sensitive to alterations of leaf patterning; in an *as1* or *as2* background mutations in a large number of genes, including those encoding ribosomal proteins, receptor-like kinases, chaperones, and epigenetic regulators, may lead to polarity defects (Machida et al., 2015). In contrast, *arp* mutants in other species, such as in *Antirrhinum*, tobacco, and tomato, have obvious adaxial–abaxial polarity phenotypes (Waites et al., 1998; Kim et al., 2003b; McHale and Koning, 2004).

A group of antagonistic transcription factors and small RNAs define abaxial cell fate. KAN1, and possibly additional KAN family transcription factors, are expressed in the abaxial domain prior to leaf initiation, as shown by time-lapse live imaging (Yu et al., 2017). Loss-of-function *kan1* and *kan2* mutants exhibit adaxialization, whereas *KAN1* and *KAN2* overexpression causes abaxialization and SAM termination (Eshed et al., 2001; Kerstetter et al., 2001; Emery et al., 2003). Therefore, *KAN* genes promote abaxial cell fate, and inhibit both adaxial and SAM cell fates.

In *Arabidopsis*, three redundant repressive AUXIN RESPONSE FACTORs (ARFs), ETTIN (ETT, also known as ARF3), ARF4, and ARF2, are also expressed in the abaxial domain and promote abaxial cell fate (Pekker et al., 2005; Guan et al., 2017). Mutation of these *ARF* genes leads to a phenotype similar to that of *kan* mutants. In fact, ARF and KAN proteins may form complexes (Kelley et al., 2012), which is supported by genetic analysis. *ETT* and *ARF4* expression is not altered in *kan1 kan2* double mutants, but mutation of *ETT* can suppress the *KAN1* overexpression phenotype in *Arabidopsis* (Pekker et al., 2005).

In addition, *trans*-acting short interfering RNAs (ta-siRNAs), whose biogenesis is triggered by miR390, target *ETT*, *ARF2*, and *ARF4* expression (Allen et al., 2005; Fahlgren et al., 2006; Hunter et al., 2006; Marin et al., 2010). The ta-siRNAs are derived from AGO7/miR390-mediated cleavage of their precursors, encoded by the non-protein-coding gene *TAS3*. The ta-siRNAs (collectively termed tasiR-ARFs) are generated in the adaxial domain, and expression of the *ARF* genes they target is restricted to the abaxial domain. The resulting miR390–TAS3–ARF pathway is highly conserved in species including mosses, grasses, and eudicots (Nagasaki et al., 2007; Axtell and Bowman, 2008; Douglas et al., 2010). Similar to the restriction of *HD-ZIPIII* expression by miR165/166, the mobile adaxially formed tasiR-ARFs also generate sharply defined expression boundaries of ARFs restricted to the abaxial domain (Skopelitis et al., 2017).

Additional mutual antagonism exists between regulators of the adaxial and abaxial domains. The adaxially localized AS1–AS2 complex negatively regulates *ETT* and *ARF4* expression at the transcriptional, post-transcriptional, and epigenetic levels (Iwasaki et al., 2013). The AS1–AS2 complex also directly inhibits the expression of *MIR166A* and *ETT* in the adaxial domain (Husbands et al., 2015) and binds the promoter of the ta-siRNA precursor *TAS3A* to maintain its expression (Husbands et al., 2015). In the abaxial domain, KAN1 binds to the promoter regions of *MIR166A* and *MIR166F*. However, KAN1 does not regulate the expression of *MIR166A* and downregulates the expression of *MIR166F* (Merelo et al., 2013), implying complicate regulations exist.

The YABBY (YAB) family of transcription factors also contributes to adaxial–abaxial polarity. In *Arabidopsis*, FILAMENTOUS FLOWER (FIL, also known as YAB1), YAB2, and YAB3 redundantly promote abaxial identity (Sawa et al., 1999; Siegfried et al., 1999). They are expressed on the abaxial side of lateral organs in *Arabidopsis*, and mutation of multiple *YAB* genes lead to abaxialization (Stahle et al., 2009; Sarojam et al., 2010). YAB proteins function by dimerizing with LEUNIG family transcriptional corepressors (Stahle et al., 2009), although this complex can also function as a transcriptional activator (Bonaccorso et al., 2012). *YAB* expression extends into the middle domain between the adaxial and abaxial domains, as discussed below. Notably, *YAB* gene expression is not polarized in rice (Yamaguchi et al., 2004), and is adaxialized in maize (Juarez et al., 2004b), suggesting functional divergence. In fact, it has been proposed that the primary function of YAB is to promote leaf identity and to repress meristematic cell fate (Sarojam et al., 2010).

Whereas most of these adaxial–abaxial regulatory network components are highly conserved across angiosperms, the contribution of each component may vary. Although mutants in the miR390–TAS3–ARF pathway display relatively subtle leaf polarity defects, mutations of their counterparts in maize, rice, and tomato can lead to strong polarity phenotypes (Nagasaki et al., 2007; Nogueira et al., 2007; Douglas et al., 2010; Yifhar et al., 2012). Differences in the timing of tasiR-ARF expression may explain this difference in phenotypic severity. Although tasiR-ARF is expressed on the abaxial side of incipient primordia in maize, it is detected at a later stage of primordium development in *Arabidopsis*. Similarly, AS1–AS2 pathway mutants display polarity phenotypes of different magnitudes in different species, as mentioned above.

Upstream transcription factors act through downstream targets to alter local cell proliferation, growth, and expansion, leading to three-dimensional shape changes and functional specification. Compared with our understanding of the interactions between adaxial and abaxial regulators, we know relatively little about the downstream effects of these transcription factors; however, recent genome-wide studies have begun to deepen our current understanding (Brandt et al., 2012; Merelo et al., 2013; Reinhart et al., 2013; Huang et al., 2014; Xie et al., 2015). Such genome-wide analyses suggest that adaxial-promoting factors, such as HD-ZIPIII, and abaxial-promoting factors, such as KAN, have opposite effects on common pathways.

Among other functions, adaxial–abaxial polarity regulates de-methyl-esterification of cell wall pectins, which correlates with wall mechanics (Qi et al., 2017). The abaxial domain has de-methyl-esterified pectin and high elasticity, whereas the rest of the leaf primordium has methyl-esterified pectin and low elasticity. During leaf growth, pectin in the adaxial domain is de-methyl-esterified, leading to increased elasticity. Only the middle domain maintains methyl-esterified pectin and low elasticity. Numerical simulations have established that mechanical heterogeneity is sufficient to produce leaf primordium asymmetry, which

is supported by experiments (Figure 5B). Thus, mechanical heterogeneity within leaf tissue, which is regulated by polarity signals, may underlie leaf shape asymmetry.

Establishment of Proximal–Distal Polarity

The proximal–distal axis is naturally established when distal growth begins from a young leaf primordium. In contrast to adaxial–abaxial polarity, how proximal–distal polarity is established remains largely unknown. Along the proximal–distal axis, the switch from cell proliferation to differentiation exhibits a basipetal gradient, as discussed in more detail in the next section. Here we focus on genes restricting growth at the proximal end.

In *Arabidopsis* and many other species, leaf petioles lack blades, suggesting that blade formation is inhibited in the proximal end. The *Arabidopsis BLADE ON PETIOLE1* (*BOP1*) and *BOP2* genes encode redundant proteins containing BTB/POZ domains and ankyrin repeats (Ha et al., 2004), and in *bop1* and *bop2* mutants, consistent with the mutant name, a blade forms on the petiole (Ha et al., 2003; Hepworth et al., 2005; Norberg et al., 2005). *BOP1* and *BOP2* are expressed in the proximal region, and directly activate expression of two *LBD* genes, the adaxially expressed *AS2* and the boundary-expressed *LOB* (Ha et al., 2007; Jun et al., 2010). In addition, *KNOX1* expression is repressed by BOP1 and BOP2 (Figure 4) (Norberg et al., 2005; Ha et al., 2007). In tomato, the BOP–KNOX1 module is a central part of the gene-regulatory network regulating leaf shape diversity (Ichihashi et al., 2014).

LEAF FLATTENING AND EXPANSION

WOX Transcription Factors

The meristem defines a region where a group of cells acquire the ability to self-renew and maintain pluripotency, or the capacity to differentiate into multiple specific cell types. Despite being located at different places in the plant body, the maintenance of an undifferentiated state in various meristems occurs via similar mechanisms, in which the WUSCHEL-RELATED HOMEOBOX (WOX) transcription factors play central roles (Haecker et al., 2004; Aichinger et al., 2012). In the *Arabidopsis* SAM, *WUSCHEL* (*WUS*), the founding member of the WOX family, is expressed in the organizing center and promotes stem cell identity in the CZ (Laux et al., 1996; Mayer et al., 1998). In the root apical meristem of *Arabidopsis*, *WOX5* is expressed in the quiescent center and promotes columella stem cell fate in the underlying cell layer (Sarkar et al., 2007). Stem cell fate in the vascular procambium, which generates phloem and xylem tissues, is promoted by the localized expression of *WOX4* in both *Arabidopsis* and tomato (Hirakawa et al., 2010; Ji et al., 2010). Most WUS-clade WOX proteins can compensate for WUS function to different degrees, suggesting functional conservation (Dolzblasz et al., 2016). Moreover, in these three distinct meristems, WOX activities are all regulated by small related peptides belonging to the CLE family via their interaction with diverse leucine-rich repeat receptor-like kinases (LRR-RLKs) (Katsir et al., 2011).

Although fossil evidence indicates that the leaves of seed plants evolved from ancestral shoot systems, no anatomical feature typical of meristems is present in leaves (Foster, 1936; Hagemann and Gieissberg, 1996). In addition, unlike indeterminate shoot growth, leaves exhibit determinate growth, indicating that the potential leaf meristem, if it exists, has only transient meristematic activity. Nevertheless, the leaf marginal and submarginal regions maintain active cell divisions during early leaf development and are responsible for lamina initiation. Later, cells in this region cease to divide, and cell expansion and differentiation lead to lamina expansion.

Here we use the term blastozone to describe the leaf region with organogenetic potential (Hagemann and Gieissberg, 1996; Bar and Ori, 2015); this region is also termed the leaf meristem (Ichihashi and Tsukaya, 2015; Alvarez et al., 2016; Tsukaya, 2018). Within the leaf blastozone, cell proliferation and cell differentiation often occur concurrently, making its zonation less obvious compared with that of the SAM. Nevertheless, examination of a typical *Arabidopsis* leaf reveals that there is at least a marginal zone and a plate zone. Previous studies suggested that the potential leaf blastozone is localized in the leaf margin, and thus it is named the marginal blastozone or marginal meristem (Avery, 1933; Hagemann and Gieissberg, 1996). However, other studies suggested that marginal meristem activity contributes little to protracted growth of the leaf blade, and that blade outgrowth in angiosperms is sustained by active cell proliferation in a broader ‘‘plate meristem’’ region that covers the entire adaxial–abaxial junction (Schüepp, 1918; Waites and Hudson, 1995; Donnelly et al., 1999).

Despite its intercalary nature, the leaf blastozone expresses *WOX* genes, which are key for sustaining stem cells, in a variety of plant species. The *WOX* genes are expressed at the adaxial–abaxial junction. In *Arabidopsis* leaves, both *WOX1* and *PRESSED FLOWER* (*PRS*)/*WOX3* are expressed at the adaxial–abaxial boundary layer, where they redundantly promote leaf blade outgrowth. The *wox1 prs* double mutant possesses leaves that are much narrower than those of wild-type, indicating that *wox1 prs* blade outgrowth along the medio–lateral axis is compromised (Vandenbussche et al., 2009; Nakata et al., 2012).

Recent work has also identified conserved functions of WOX1 orthologs in regulating leaf blade outgrowth in various eudicots, such as LAM1 in *Nicotiana*, MAEWEST in *Petunia*, STENOFOLIA (STF) in *Medicago*, and LATHYROIDES in garden pea (McHale and Marcotrigiano, 1998; Vandenbussche et al., 2009; Tadege et al., 2011; Zhuang et al., 2012; Lin et al., 2013). Single mutant lines deficient in each WOX1 ortholog display narrow-leaf phenotypes similar to that of *Arabidopsis wox1 prs*. By contrast, leaf blade outgrowth in several monocots is controlled by PRS orthologs. For example, maize and rice double mutants carrying defects in *NARROW SHEATH 1* and *2* and *NARROW LEAF 2* and *3*, respectively, exhibit similar leaf margin deletion phenotypes (Scanlon et al., 1996; Nardmann et al., 2004; Ishiwata et al., 2013). WOX1 and PRS belong to the WUS clade, which arose from recent evolutionary events (van der Graaff et al., 2009). The narrow-leaf phenotype of the *Nicotiana lam1* mutant can be rescued by the transgenic expression of either *WOX1* or *PRS*, as well as other *WUS*-clade members of *Arabidopsis*, at the adaxial–abaxial boundary layer, which demonstrates that the function of a WUS-clade member is required for leaf blade outgrowth (Lin et al., 2013). Nevertheless, expression analyses have revealed that WOX1 and PRS orthologs are located in

distinct leaf domains in all the aforementioned monocots and eudicots. *WOX1* orthologs are expressed along the entire adaxial–abaxial junction, presumably overlapping with the plate meristem region (Vandenbussche et al., 2009; Tadege et al., 2011; Nakata et al., 2012; Zhuang et al., 2012; Lin et al., 2013). In contrast, the expression of *PRS* orthologs in the marginal meristem is more restricted (Nardmann et al., 2004; Nakata et al., 2012; Ishiwata et al., 2013). It is hypothesized that spatial differences in leaf meristematic activities may explain differences in the timing and placement of lateral leaf outgrowths between monocots and eudicots (Ishiwata et al., 2013).

Medio–Lateral Polarity Establishment and Leaf Blade Initiation

Genetic and molecular evidence suggests that adaxial–abaxial polarity underlies the establishment of medial–lateral polarity and leaf blade lateral growth. Wild-type leaf blade growth is anisotropic; in a transverse section, cell division is mostly perpendicular to the medial–lateral axis, leading to a flattened leaf. In many mutants with either adaxialized or abaxialized leaves, leaf blade expansion is significantly compromised. Such leaves appear to be radial structures with isotropic growth in all directions, resembling the *Nicotiana lam1* mutant in which the WOX transcription factor is non-functional.

Based on the specific expression of *WOX1* and *PRS* in leaves, a three-domain theory has been proposed, whereby a ''middle domain'' resides in the junction between the adaxial and abaxial domains (Figure 5A, right side) (Nakata et al., 2012). Auxin mediates the designation of the middle domain by the adaxial and abaxial domains. The activator *ARF* gene *MP* is expressed in the adaxial and middle domains (Guan et al., 2017), and the repressor *ARF* genes *ETT*, *ARF2*, and *ARF4* are expressed in the abaxial domain (Pekker et al., 2005). The adaxial domain has lower auxin levels than the abaxial domain, leading to a high auxin response specifically in the middle domain (Qi et al., 2014; Guan et al., 2017). Furthermore, *MP* may be a direct target of adaxially expressed HD-ZIPIIIs (Müller et al., 2016). Thus, auxin acts as a positional cue, specifying the location of the middle domain based on the adaxial and abaxial domains.

Complex regulatory networks mediate the interactions between the middle domain and the adaxial and abaxial domains (Figure 5A, right side). Loss of *WOX1* and *PRS* expression in *wox1 prs* mutant plants results in expansion of adaxial and abaxial identity gene expression into the margins and replacement of the rows of typical elongated marginal cells by adaxialized cell types, indicating that WOX1 and PRS repress adaxial and abaxial identities in the margins of the middle domain (McHale and Marcotrigiano, 1998; Nakata et al., 2012). STF, the WOX1 ortholog in *Medicago*, directly represses the adaxial factor *AS2* in the leaf margin by recruiting TOPLESS family corepressors (Zhang et al., 2014). In turn, *PRS* is repressed by AS2 in the adaxial domain (Alvarez et al., 2009). In the abaxial domain, middle domain identity is repressed by two classes of abaxial proteins, namely KAN1 and KAN2 and the repressors ETT, ARF2, and ARF4 (Nakata et al., 2012; Guan et al., 2017). Members of the YAB family that are expressed in both the middle and abaxial domains can only activate *WOX1* expression in the middle domain, indicating that KAN and ARF are epistatic to YAB in the regulation of *WOX* expression in the abaxial domain (Nakata et al., 2012).

Within the leaf blastozone, WOX proteins can promote cell proliferation. In *kan1 kan2* and *ett arf2 arf4* mutants, where ectopic expression of *WOX1* and *PRS* occurs in the abaxial domain of leaf primordia, protrusions form on the abaxial surface of leaves (Pekker et al., 2005; Nakata et al., 2012; Guan et al., 2017). This morphological change can be phenocopied by artificially expressing *WOX1* in the abaxial domain (Nakata et al., 2012). Indeed, WOX1 and PRS promote the expression of *KLUH* in the marginal regions (Nakata et al., 2012). *KLUH* encodes a cytochrome P450 CYP78A5 monooxygenase and prolongs the duration of cell proliferation in many organs (Anastasiou et al., 2007). The *kluh* mutants have smaller organs due to premature arrest of cell proliferation, whereas *KLUH* overexpression results in prolonged cell proliferation and larger organs (Anastasiou et al., 2007).

WOX transcription factors may promote blade growth via modulation of the auxin/cytokinin ratio in leaves (Tadege et al., 2011). The WOX1 orthologs STF in *Medicago* and LAM1 in *Nicotiana* affect auxin levels, and the bladeless phenotype of *lam1* leaves can be partially rescued by coapplication of auxin and cytokinin (Tadege et al., 2011). Moreover, auxin biosynthesis factors, such as the YUCCA (YUC) flavin monooxygenase-like enzymes, are required for leaf margin development and blade outgrowth in *Arabidopsis* (Wang et al., 2011). It remains to be seen whether WOX transcription factors regulate auxin levels in the marginal and middle domains through YUC-regulated auxin biosynthesis.

Recent work has also revealed that the development of leaf domains into three-dimensional shapes is related to differential methyl-esterification of cell wall pectins, which leads to mechanical heterogeneity at the organ level (Qi et al., 2017). Furthermore, experiments and simulations show that a middle domain with lower cell wall elasticity than those of adaxial and abaxial domains is required for the formation of asymmetry in leaf primordia.

Leaves exhibit considerable morphological diversity within and between species. Recent findings have started to show how the leaf development framework is rewired to produce the amazing diversity of leaf shape among species and cultivars (Tsukaya, 2014, 2018). Among these diverse leaf shapes, unifacial leaves, in which the leaf blade contains only an abaxialized leaf blade, have repeatedly evolved in several divergent monocot families. Some unifacial leaves have flat lamina that do not have an adaxial–abaxial junction or middle domain. In Juncus species, a *YAB* gene promotes *WOX* gene expression in margin-like regions in flattened blades, suggesting conserved roles of *YAB* and *WOX* genes in the patterning of unifacial leaves (Yamaguchi et al., 2010).

Regulation of Marginal and Plate Blastozones during Intercalary Growth

After leaf blade initiation, cell proliferation and differentiation occur in the entire leaf blade region, a process known as

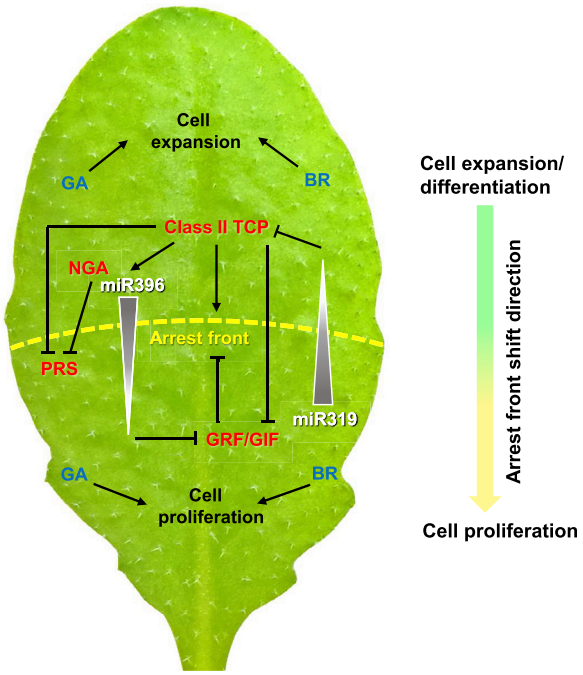

Figure 6. Regulation of Marginal and Intercalary Leaf Growth.
The switch from cell proliferation to differentiation follows a basipetal gradient during leaf development, a process that is promoted by the miR319–*TCP* module and repressed by the miR396–*GRF* module. The *TCP* and *GRF* expression domains are complementary to the gradients of miR319 and miR396, respectively. TCP activates miR396 and thus forms a feedback loop regulating the shift of the cell-cycle arrest front. TCP and NGA repress leaf blastozone activity at the margins. Gibberellins and brassinosteroids promote both cell proliferation and expansion during intercalary leaf growth. Red, genes; blue, phytohormones; green, small RNAs. BR, brassinosteroids; GA, gibberellins; GIF, GRF-INTERACTION FACTOR; GRF, GROWTH-REGULATING FACTOR; miR319, microRNA 319; miR396, microRNA 396; NGA, NGATHA; PRS, PRESSED FLOWER; TCP, TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR.

intercalary growth (Nakata and Okada, 2013). In *Arabidopsis* and other model species, the switch from cell proliferation to differentiation follows a basipetal gradient during leaf development, whereby cell proliferation in the distal end of leaves ceases while cells in the proximal end continue proliferating (Figure 6) (Nath et al., 2003; Ori et al., 2007; Nelissen et al., 2012). Detailed analysis of the dynamics of the cell proliferation "arrest front" have revealed that the boundary between the proliferating and non-proliferating domains appears rapidly, and remains at a constant distance from the leaf base for several days before rapidly disappearing (Kazama et al., 2010; Andriankaja et al., 2012). In agreement with these observations, leaf blastozone activity, which is partially reflected by *PRS* expression, is gradually restricted to the overall marginal region in young leaves, and further restricted to the proximal marginal domain in older leaves of *Arabidopsis* (Alvarez et al., 2016). However, leaf growth polarity can differ in different species, with more growth arising from either the proximal or the distal end, with no apparent polarity or bidirectional dispersion (Das Gupta and Nath, 2015).

Two classes of miRNA/transcription factor modules play dominant and antagonistic roles in sustaining the marginal and plate blastozone activities during intercalary growth (Tsukaya, 2018). The class II TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR (TCP) transcription factors repress marginal meristem activity and thus promote the switch from cell proliferation to cell differentiation. Inactivation of TCP function in *Arabidopsis*, or inactivation of the orthologs CINCINNATA (CIN) in *Antirrhinum* and LANCEOLATE (LA) in tomato, results in overproliferation at the leaf margin, which leads to expanded, crinkly leaves or leaflets with serrated margins (Nath et al., 2003; Ori et al., 2007; Efroni et al., 2008). The indeterminate cell proliferation in leaf margins of *Arabidopsis tcp* mutants is likely due to the prolonged activity of WOX1 and PRS, together with sustained mitotic activity caused by upregulation of *CYCB* expression (Alvarez et al., 2016; Bresso et al., 2018). Moreover, the *tcp* serrated marginal pattern is associated with upregulation of several *CUC* genes, which are repressed by TCP transcription factors in wild-type leaves (Koyama et al., 2017). The expression of class II TCP transcription factors is restricted to the distal regions of leaves via post-transcriptional repression by miR319/JAW (Palatnik et al., 2003; Ori et al., 2007). Mutations that disturb this regulation result in smaller leaves with precocious differentiation of leaf margins and the loss of leaflet generation, a phenotype that is likely due to the ectopic expression of TCP transcription factors in the proximal ends of leaves (Palatnik et al., 2003; Ori et al., 2007; Efroni et al., 2008). Prolonged marginal growth and leaf serration are also observed when the functions of four NGATHA (NGA) transcription factors are lost, whereas *NGA3* overexpression reduces marginal growth, indicating the redundant roles of NGAs, and TCPS, in controlling the switch between leaf margin expansion and differentiation (Alvarez et al., 2009; Trigueros et al., 2009). Together, NGA and TCP transcription factors redundantly inhibit *WOX* expression to terminate the leaf blastozone in *Arabidopsis* (Alvarez et al., 2016).

In contrast to TCPs and NGAs, the GROWTH-REGULATING FACTORs (GRFs) function in a broader region covering the plate blastozone, where they delay the transition from proliferation to differentiation during intercalary growth (Kim et al., 2003a; Rodriguez et al., 2010). GRFs physically interact with GRF-INTERACTING FACTOR1/ANGUSTIFOLIA3 (GIF1/AN3), GIF2, and GIF3, which act redundantly with GRFs in promoting cell proliferation by modulating the levels of cyclins (Horiguchi et al., 2005; Lee et al., 2009; Rodriguez et al., 2010; Debernardi et al., 2014). AN3 interacts with chromatin-remodeling complexes, providing a molecular mechanism for the synergistic activities of GRF–GIF complexes (Debernardi et al., 2014). Several *GRF* genes are post-transcriptionally repressed by miR396 (Jones-Rhoades and Bartel, 2004). Mutations in different *GRF* genes or overexpression of miR396 reduce leaf size, whereas constitutive expression of miR396-resistant *GRF* genes results in enlarged leaf area (Kim et al., 2003a; Kim and Lee, 2006; Rodriguez et al., 2010). In *Arabidopsis*, the restriction of *GRF* expression toward the proximal end of leaves by miR396 is partially determined by TCP4; TCP4 enriched at the distal end directly activates miR396 to inhibit the expression of *GRF* targets (Rodriguez et al., 2010; Schommer et al., 2014). In addition, TCP4 represses the expression of *GIF1* and *GRF*

genes that are not direct targets of miR396, via unknown mechanisms (Rodriguez et al., 2010). Thus, miR319–*TCP* and miR396–*GRF* modules coordinate to balance marginal and overall leaf growth through regulation of cell proliferation (Figure 6).

In addition to the regulation provided by miRNA–transcription factor modules, hormonal control is also involved in intercalary growth. Gibberellins (GAs) and brassinosteroids (BRs) promote leaf growth by increasing cell proliferation and expansion. Constitutive activation of GA signaling or overexpression of GA biosynthetic enzymes leads to larger leaves, whereas a block in GA signaling reduces leaf size (Huang et al., 1998; Achard et al., 2009). The positive role of GAs in cell proliferation likely involves the repression of cell-cycle inhibitors, including KIP-RELATED PROTEIN 2 (KRP2) and SIAMESE (Achard et al., 2009). Overexpression of the BR receptor-encoding gene *BRASSINOSTEROID INSENSITIVE1* or the BR biosynthesis gene *DWARF4* also results in larger leaves (Choe et al., 2001; Gonzalez et al., 2010; Zhiponova et al., 2013). Mutants deficient in BR biosynthesis or signaling display reduced leaf size and lower *CYCB* expression, indicating that BR controls the exit from mitosis (Zhiponova et al., 2013). Moreover, BR signaling represses *PEAPOD1* (*PPD1*) and *PPD2*, which encode transcription factors that limit meristemoid cell proliferation (Gonzalez et al., 2015). Auxin has a less clear role in leaf growth, with contradictory observations showing that auxin has both positive and negative effects on leaf cell proliferation and expansion (Wang and Guo, 2015; Saini et al., 2017). Since the auxin signal is key for development of the vasculature and thus for providing nutrients for leaf cells, it may be difficult to separate the direct effect of auxin on cell proliferation/expansion from its indirect effect on cellular metabolism.

The transition from cell proliferation to cell expansion during leaf development occurs gradually and many cells proliferate and expand. Transcriptional analysis has uncovered increased expression of genes involved in the cell cycle and photosynthesis during the transition from cell proliferation to cell expansion, suggesting that the differentiation of the photosynthetic machinery may be important for the onset of cell expansion (Andriankaja et al., 2012). Compared with the process of cell division, cell expansion may provide a faster and economical way to control leaf size, and also to increase leaf biomass (Vanhaeren et al., 2017).

Endoreduplication, replication of the genome without subsequent mitosis, often occurs during the transition between cell division and cell expansion (Beemster et al., 2005; Breuer et al., 2010). The resulting increased ploidy level can trigger cell enlargement (Melaragno et al., 1993; Sugimoto-Shirasu and Roberts, 2003), which occurs in many cases only after the exit from mitosis (Tsukaya, 2013). How cell division and expansion are coordinated to regulate the final leaf morphology remains to be elucidated.

Variation in leaf margin morphology is also common even within a species. Many species, including *Arabidopsis*, repetitively generate leaf margin protrusions, termed serrations. The CUC–PIN module, which functions in the SAM to generate primordia, also operates in the leaf margin (Bilsborough et al., 2011). The auxin/PIN1 pattern generator, as a positive feedback loop, forms auxin maxima that develop into margin protrusions. *CUC2* is expressed in the sinus region between the auxin maxima because auxin represses *CUC2* expression. The combination of these positive and negative feedback loops explains how leaf margin patterning occurs.

The junction region separating the leaf blade and the petiole may also contribute to leaf shape variation. In a mature *Arabidopsis* leaf, a proliferating region is maintained in this junction after both the blade and the petiole have differentiated. The proliferating region supplies cells to both the blade and the petiole (Ichihashi et al., 2011).

CONCLUSIONS AND FUTURE PERSPECTIVES

Thanks to the powerful molecular genetics of model plants, many genes regulating leaf development have been identified since the 1990s. Research over the past 20 years has vastly enhanced our understanding of the gene-regulatory networks that function in different spatiotemporal domains and has established the interactions among domains. Despite these advances, a full understanding of leaf formation and patterning remains to be achieved. Currently, transcription factors dominate the regulatory landscape, yet details of their regulatory roles within protein complexes are sparse, and many of their downstream factors remain to be identified. Among other processes, coordination of cell growth and division is necessary to enable appropriate patterning and growth. Leaf development requires strict control of the orientation of cell division, by mechanisms that remain largely unknown. Biomechanics has emerged as a link between biochemical signals and three-dimensional biological shape (Hamant and Traas, 2010; Sampathkumar et al., 2014), and we have begun to appreciate the biomechanical regulation of leaf patterning. We expect recent breakthroughs in dynamic imaging and quantitative image analysis, single-molecule biochemistry, and single-cell genomics to further advance the field (Bencivenga et al., 2016; Husbands et al., 2016; Li et al., 2016; Prunet et al., 2016; Palovaara et al., 2017). Additionally, well-characterized gene regulatory networks may need to be reconsidered, using new regulatory logic and better spatiotemporal resolution data, to understand the robustness and dynamics of development. The power of computational modeling to integrate information to gain a better understanding of plant development has been illustrated in recent years (Roeder et al., 2011; Whitewoods and Coen, 2017; Ubbens et al., 2018).

This review has focused mostly on *Arabidopsis* leaf development, but a true understanding of leaf development requires knowledge of other species whose leaf shapes significantly differ from those of *Arabidopsis*, especially grasses and species with compound leaves such as tomato and *Cardamine hirsute*. It is essential to understand how species-specific spatiotemporal activation and/or reiteration of conserved patterning processes, as well as newly evolved regulatory interactions, lead to the diverse leaf shapes that we see in nature.

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