# A New Development: Evolving Concepts in Leaf Ontogeny

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## Abstract

Elucidation of gene regulatory networks (GRNs) underlying aspects of leaf development in multiple model species has uncovered surprisingly plastic regulatory architecture. The meticulously mapped network interactions in one model species cannot now be assumed to map directly onto a different species. Despite these overall differences, however, many modules do appear to be almost universal. Extrapolating findings across different model systems will demand great care but promises to reveal a rich tapestry of themes in GRN architecture and regulation. The purpose of this review is to approach the field of leaf development from the perspectives of the evolution of developmental systems that orchestrate leaf development.

## Contents



## 1. INTRODUCTION

In spite of ancestral origins from branching-shoot systems, the variety of leaf forms among the quarter-million living plant species indicates diversity in underlying developmental systems. These systems show many highly conserved modules of gene regulation, surprising convergences, and remarkable variation. The tools available to study biology are evolving with ever-increasing rapidity, allowing these developmental systems to be investigated with high resolution at the molecular, tissue, phenotypic, genetic, population, and evolutionary levels. The increasing sophistication of conceptual frameworks also allows previously gained knowledge to be seen in new light, further enhancing our understanding of the complex developmental processes in plants. The focus of this review is on the evolution of systems underlying the recruitment and elaboration of a handful of cells in the shoot apical meristem (SAM) into a complex three-dimensional photochemical reactor—the leaf!

The field of plant development has progressed tremendously over the past century, evolving from an observational science of morphology and anatomy to a strongly genetic and molecular field allowing elucidation of the genetic underpinnings of development. Now, in tune with the rest of biology, it is incorporating the established field of graph theory, an area of mathematics that deals with variables (nodes) and their interconnections (edges), to bring the flood of genomic and transcriptomic data generated by rapidly expanding sequencing capabilities into unified systems-level investigational approaches. The regulatory network of interactions between genes, RNAs, proteins, metabolites, and environmental signals can be treated as graphs, networks of nodes with edges representing the nature and direction of regulatory interactions. This allows the gene regulatory networks (GRNs) to be treated as computational problems, thus chipping away at the "irreducible complexity" of developmental biology.

Plants are an ideal system in which to study development and the GRNs that orchestrate developmental programs. Primary morphological development in most animals is an almost-discrete episode in the embryonic stage of the life cycle, whereas development in plants is an ongoing process because the SAM can grow indefinitely, continuously producing lateral organs, each of which must develop from a small number of founder cells into a complete

organ. Plants are also resilient to perturbations in development, which allows for the study of mutations causing severe patterning defects. Such mutations are often lethal in metazoans. Phenomena common in plants such as heterochrony and phase transition also allow for the study of the regulation of developmental GRNs. The study of development as well as the study of the evolution of development are now being brought into the systems fold as problems that can be addressed by studying the developmental GRNs and their evolution.

## 2. NETWORK EVOLUTION

In the era of "omics," leaf development can now begin to be understood at the level of its deepest molecular underpinnings. Comparative morphology and ontogeny have yielded to comparisons in DNA primary sequence, whole genomes, transcriptional profiles, and epigenomes. The explosion in sequencing technologies and parallel growth in methods of analysis have had a profound impact on evolutionary and developmental biology. The study of evolution of developmental GRNs gives a context in which to understand the evolution of the developmental processes that they direct. The unification of evolution, development, and genomics allows us to understand the evolution of networks of genes and how these have shaped leaf development over evolutionary time.

### 2.1. Developmental Systems Drift

One of the most striking revelations about the evolution of GRNs has been that the changes in wiring that occur in evolution are, in large part, not adaptive. This is particularly significant in light of the plasticity of the GRNs regulating aspects of leaf development. A theoretical framework that both explains and predicts the shifting regulatory landscape over evolutionary time, called developmental systems drift (DSD), was put forth in 2001 (116). Within the paradigm of DSD, nonadaptive shifting of regulatory connections accumulates over time, changing the GRN interconnections and network, thereby resulting in diversity in the regulatory wiring underlying developmental processes. This process is stochastic and occurs in the absence of evolutionary pressure (116).

The scope of DSD in developmental biology has recently been shown in exquisite detail in the rewiring of signaling pathways that lead to the induction of a conserved structure, the nematode vulva, between two species, *Caenorhabditis elegans* and *Pristionchus pacificus*, which are separated by 400 million years (124). Among the novel regulatory differences detailed in this study was that the conserved protein LIN-17/Fz has opposing roles in vulva induction between *P. pacificus* and *C. elegans*, acting as an antagonist in the former and an agonist in the latter, illustrating that conserved components can adopt drastically different roles in the context of different GRN wiring.

The implications of DSD on the evolution of novelty in leaf development and form are extremely nontrivial. Evolution is often described as navigating a hypothetical phenotype space toward local maxima, with sequence space underlying this. In this scenario mutations move an individual either "uphill" toward local fitness maxima or "downhill" away from these maxima, with selection acting always to move the population uphill (90) (**Figure 1*a***). Occupying a local maximum in this view essentially means a population is stuck, unable to traverse fitness valleys to reach higher local fitness maxima. DSD may allow evolution to find paths to higher local maxima without necessitating unlikely sojourns through fitness valleys (52, 94). Although selective pressures can act to maintain a given outward phenotype, stepwise acquisition of neutral or nearly neutral changes to network topology are not directly selected (**Figure 1*a***). This array of interrelated network topologies is known as a neutral network (27), where selection does not favor or disfavor transition between equally fit GRN topologies and is analogous to the process of genetic drift. The consequence of a random walk through these neutral networks is a continually changing "phenotypic neighborhood" (128) (**Figure 1*b***). Although the "morphospace" can be thought

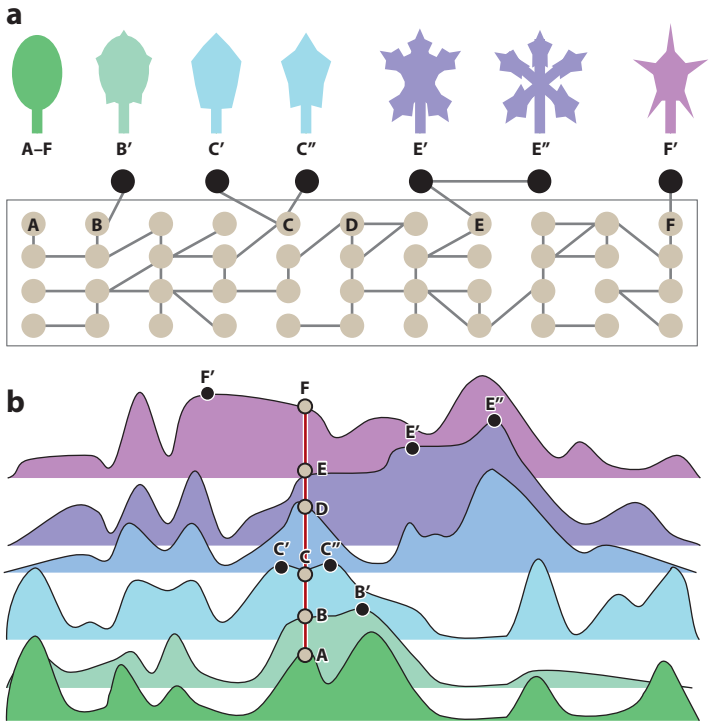


**Figure 1**

(*a*) Random walk through a neutral network of mutational steps without phenotypic consequences. Nodes represent gene regulatory networks (GRNs) with related topologies. Different points within the neutral network are within mutational range of distinct regions of the morphospace, but developmental output is identical for GRN topologies within the neutral network. (*b*) Representation of the shifting topology of the accessible fitness landscape around various points in the neutral network. Fitter solutions may come into "range" as the GRN topology accumulates neutral changes. The red line indicates the same fitness level. Beige nodes exist within the neutral network with no fitness consequence. Black nodes exist outside the neutral network and represent increased fitness.



of as an arbitrarily large set of all possible morphologies, the portion of that space accessible to an organism by mutation is much more limited. The shifting network topologies operating underneath a developmental process, over time, allow access to a much greater portion of the morphospace and, thus, fitter morphological solutions than are accessible from any one point in the neutral network (83) (**Figure 1*b***).

## 2.2. Evolvability

The functionality of the GRN can be anchored by a highly conserved subnetwork of genes that may be less subject to evolutionary rewiring (29). The incredibly important role leaves play in the survival of a plant puts limitations on the plasticity of the systems that regulate their development (14). Severe perturbation can lead to loss of a functional organ, and thus the possibility space must generally be explored in small steps rather than large jumps. This results in a balancing act between evolving GRN architectures that are robust enough to withstand mutational, environmental, and stochastic perturbations without being so canalized that they cannot adapt to shifting environments (27, 83). Bursts of evolutionary diversity within lineages in short spans of geological time such as the Cambrian explosion have been suggested to be the result of highly flexible GRN architectures within lineages (47). Conversely, highly canalized GRN architectures have been implicated as reasons for long epochs of morphological stagnation within a group of organisms (47), illustrating the trade-off between evolvability and robustness in GRN architectures. Mutations, leading to changes in expression levels and expression domains, or the addition and deletion of network connections at the protein-protein interaction (PPI), *cis*-regulation, and posttranscriptional levels provide the raw material for selective and neutral changes in GRN regulation.

## 2.3. Transcription

In plants, there is evidence of stochastic shifts in gene expression from comparisons of the transcriptomes of related species. Recent work compared transcript abundances in fully expanded rosette leaves from 14 members of the Brassicaceae with various degrees of evolutionary divergence, ranging up to 40 million years (18). The analysis showed that transcriptional variation between species was an accumulation of stochastic neutral changes over evolutionary time and that transcriptome divergence correlates positively with evolutionary distances. This illustrates the difficulty in comparing transcript levels in evolutionary studies, because expressional differences may

be neither adaptive nor functionally relevant. The majority of transcriptional differences may originate from DNA sequence changes in the *cis*-regulatory sequences of genes (132). Changes in the expression of regulatory genes, however, can be propagated to downstream genes. Even neutral changes in transcription factor (TF) abundances could prime an organism for a consequential change in another. Although occupancy of DNA-binding sites may be relatively insensitive to the moderate changes in concentration of its TF and may be dependent on appropriate thresholds being reached (13, 24), many plant TFs function as heterodimers and have a large number of possible dimerizing partners. Variations in relative stoichiometries of these proteins could have functionally relevant transcriptional and regulatory consequences (119).

## 2.4. Duplication

Genome duplication is believed to be an important source of starting material for evolutionary novelty. When a gene is duplicated, each copy inherits all or some of the network connections of the original gene (25). Plant evolution is rife with duplication of genes, segments, and entire genomes (1). Following the duplication of a whole genome, the initially duplicated genome will begin stochastically losing one or the other copy of some of the duplicated genes (66). Neo- and subfunctionalization can also occur (11), and fractionation of functionalities between each copy of a recently duplicated pair of genes can render both copies necessary to execute the original function. Post duplication, there is a period of rapid gene-sequence evolution, where one gene copy can be freed to "explore" other functions and to change protein interaction partners (16), while selection acts to maintain the other (104). Gene duplication and subfunctionalization have occurred in many gene families involved in leaf development and morphogenesis, which may be why loss-of-function (LOF) mutations in some cases affect only specific developmental periods, such as juvenile or reproductive stages, or many mutations have to be stacked to see any major phenotypic affect. Gene duplication allows for a decrease in pleiotropic consequences of mutation and allows for evolutionary tinkering without risking catastrophic developmental consequences (34, 36).

## 2.5. Subfunctionalization: Neutrality to Novelty

Neutral mutations can allow sideways drift to phenotypic spaces not accessible from the initial genomic state and can allow evolution to conscript totally unrelated processes into developmental regulation. An illustrative example of this mechanism is the maize mutant *bladekiller1-R* (*blk1-R*), which encodes an enzyme in the thiamine biosynthesis pathway (THI2) (133) that produces adenosine diphospho-5-(β-ethyl)-4-methylthiazole-2-carboxylic acid (ADT). The two isoforms, *THI1* and *THI2*, result from gene duplication and have subfunctionalized spatiotemporal expression domains, such that the sum of both is needed to fulfill the needs of the organism. *THI1* is most active in mature leaves, and *THI2* is expressed in developing tissues, including very young primordia, but not in the meristem. The null mutant *blk1-R* produces leaves with normal sheath but decreasing amounts of leaf blade, and it decreases meristem size with each plastochron, as cell recruitment into primordia overtakes cell division.

The non-cell-autonomous nature of the requirement of ADT from the leaf primordia to the meristem to maintain indeterminacy allows loss of *BLK1/THI2* function to act as a choke on meristem proliferation, and the requirement for ADT produced by THI2 in developing leaf blade but not sheath tissues allows it to specifically limit the development of one leaf domain. One would presume that the THI1 gene product, produced in mature leaves, is developmentally too delayed to rescue these phenotypes. Had subfunctionalization partitioned expression domains differently, the effect of mutation to one or the other *THI* gene could be quite different. *blk1-R* provides a view of the evolutionary processes that lead to developmental

novelty via differential growth and mobile "signals" and shows how metabolic processes unrelated to canonical developmental regulatory processes could be co-opted into regulatory roles.

## 2.6. Reproductive Isolation

The relative insensitivity of plants to segmental and genomic duplications provides an opportunity for duplicated regulatory components such as TFs to adopt new roles in GRN regulation. Differential gene duplication and stochastic loss of duplicate homologs are two interconnected mechanisms that have led to hybrid incompatibility between ecotypes of *Arabidopsis* (10). DSD following such events could, in theory, contribute to reproductive barriers that lead to speciation. Differential shifts in GRN topology between two populations can create complex multilocus epistasis and misregulation of hybrid GRNs leading to regulatory incompatibility. Nonadditive hybrid misexpression of genes has been implicated in the generation of inviable or less-fit offspring (76, 101). DSD leading to regulatory incompatibility may in some systems contribute to the development of Bateson Dobzhansky Muller incompatibility (65), the modeling of which as a function of DSD-derived epistasis allows for transient reproductive isolation that enables populations to drift into and out of compatibility (91). Temporary reproductive isolation allows DSD operating independently between the two populations to sow seeds of epistatic interactions in hybrid organisms when reproductive barriers are lifted.

## 2.7. Epistasis

Transgressive epistasis is a powerful force of innovation in evolution (4): In flowering plants, the likelihood of transgressive traits increases with genetic distance (110). Hybridization between individuals with differing GRN topologies, either from selective processes or neutral drift, can be a source of evolutionary novelty. In the endemic Hawaiian genus *Lipochaeta*, two species with differing leaf morphologies, *L. tenuis* with simple leaves and *L. tenuifolia* with finely dissected leaves, have geographic ranges with only a small region of overlap (55). Within this region, hybrid individuals can be observed with a diversity of leaf morphologies, ranging from intermediate forms to forms that are more highly dissected than those of the compound leaf parent and that have an entirely different morphology. The observed hybrid individuals have varying genetic contributions from the parent species, but interestingly, the most compound hybrid shares more markers with the simple leaf parent. Hybrid misexpression of genes can be greater in backcrossed individuals than in the F1 hybrid (101). Transgressive epistasis is a well-documented phenomenon. DSD between populations could facilitate creation of epistatic GRN interactions that can instantly generate new variations that did not exist in the parental populations by leapfrogging to new morphological solutions that would otherwise require multiple mutational steps. Thus, the effects of DSD on developmental biology over evolutionary time is to promote speciation and provide new starting points for the evolution of morphological novelty, while preserving developmental and phenotypic outputs.

## 2.8. GRN Evolution as a Framework for Leaf Development

It is becoming ever clearer how a plant "threads the needle" through the vast space of all possible expression states through a coordinated developmental program to an end point with specific differentiated cell types in the proper spatial context. At many stages in the plant life cycle there is a need to initiate lateral organs such as leaves, lay down axes for developmental organization, and establish boundaries separating developmental domains. Just as understanding chicken development would be impossible without also studying the egg from which it hatched, the study of leaf development must remain conceptually linked

with the genes that regulate the patterning and development that comprise the developmental GRNs as well the evolution of their regulatory architectures. Clearly, more can be gained from understanding developmental genes in their network and evolutionary context than as isolated units of information.

## 3. GENES TO LEAVES

Leaves have proved an ideal system in which to identify the genes involved in various aspects of the developmental program and to infer their interactions within the network. In an evo-devo context, these networks can be compared between model species. It is, however, often difficult to extrapolate gene function from one species to another on the basis of homology alone. When comparing gene sequences between species, orthology is not always clear, such as when the processes of gene duplication and loss occur independently in each lineage. A single gene in one species may be represented by a gene family in another, or gene family expansions in both lineages may confuse inference of orthologous relationships (64). With work being done in multiple model species, it has been challenging to try to integrate disparate data to infer the network of genes directing development of the primordium, from initiation in the SAM through complete development of the organ.

The SAM of flowering plants is composed of a few cell types arranged into only three distinct domains. When a group of cells is specified to become a leaf primordium, the cells undergo a complex developmental program that culminates in a fully formed leaf consisting of dozens of cell types organized into numerous functional domains. After specification of the incipient primordium, cells from the SAM begin an intricate series of transcriptional changes that progress through states that determine to which leaf domains and cell types they will ultimately belong. There are an exceedingly large number of possible expressional states given the number of genes in a plant genome; however, there are a much smaller number of states that can result in a functional cell type. Cell-fate specification in plants is a plastic nondeterministic process in which, at many stages, the ultimate fate of a cell can be redefined on the basis of positional and environmental cues (23). The orderly development of the leaf depends not only on internal transcriptional regulation, but also on signaling and communication with other cells (64) that result in a progression of cells through a maze of GRN states that culminate in the correct cell types being placed in the correct locations and in the correct numbers.

### 3.1. Gazing at the Medusa

GRN states are more strongly controlled by a small number of "core" genes and less so by genes that are controlled by these core factors but do not directly feed back into the control mechanism (40). This "Medusa" structure of the GRN, with an interconnected hub at its center and effectors radiating away toward the periphery, allows a relatively small number of genes to coordinate the entire transcriptional profile. The role of miRNAs in gene networks is well studied in cancer. In human lung cancer cell lines, a set of only 538 TFs was better able to discriminate between various tumors than was the entire set of more than 9,000 genes or the same-sized sets of metabolic or random genes. However, clustering with only 195 miRNAs proved even better than using TFs (40). The power of such a small number of miRNAs to distinguish between tumor cell types results from their function in GRNs as "canalizing factors" that can override other regulatory components (48). Thus, miRNAs shape the GRN state and, consequently, the tumor type. In the evolutionary rewiring of GRN modules, miRNA-target modules change more slowly than protein-protein and TF-target modules (105). Of the 69 miRNA families conserved between *A. thaliana* and *A. lyrata*, 22 are also shared with maize and rice (31). Although the creation of new miRNA-target modules is a continuous process in

evolution (31), many of those utilized in plant developmental processes are ancient in origin (130). In animals, it has been proposed that the evolution of increasingly complex body plans is related to expansion of miRNA regulation, and conversely that evolutionary simplification corresponds to loss of miRNA families (30).

Plants have a greater diversity of TFs than do metazoans, suggesting a more important role for regulation at the level of transcription (81), possibly reflected in the totipotency of plant cells for which dedifferentiation and respecialization of cell fates is not uncommon. In *Arabidopsis*, there are >2,000 known TF genes (93) belonging to 81 gene families (93). Most plant miRNAs regulate their target genes by posttranscriptional methods (58, 71). This override of the target gene is highly effective because without intact mRNAs there can be no protein to affect the regulation of the GRN, and it allows miRNAs to shape very effectively the state of the GRN. Many miRNAs functioning in development target TFs, and several of these TFs play crucial roles in leaf development (see below).

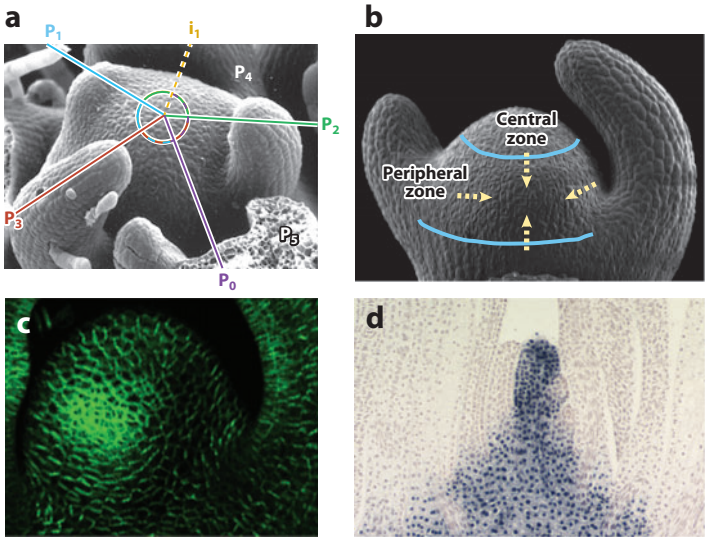


**Figure 2**

(*a*) Auxin regulates phyllotactic patterning of leaves in plants. (*b*) Local auxin flow (*yellow dashed arrows*) generates auxin maxima in the peripheral zone of the shoot apical meristem (SAM) to initiate leaf primordia. (*c*) PIN-GFP proteins concentrate auxin at location $i_1$ in tomato. (*d*) KNOXI proteins are present in the SAM but are downregulated after organ primordia, shown here in maize using Kn1 antibody staining.

## 3.2. On Your Mark: Initiation and Primary Recruitment

Auxin is the prime mover of much of plant development. Auxin is a small molecule that is a highly connected node in many developmental GRNs, and it serves as an output and an input in many GRN modules. The coordinated flow of auxin through the outermost layer of the SAM integrates positional information of other primordia to initiate the next primordium in the correct phyllotactic position (107) (**Figure 2*a***). PIN proteins direct auxin efflux from a cell by localizing to the cell membrane proximal to the highest local concentration. In this way, PIN proteins can organize an auxin flow toward the highest concentration (107) in what amounts to reverse diffusion to place the next primordium at the site of the local auxin maximum (**Figure 2*b*,*c***). The recruitment of founder cells from the meristem into this primordium must be tightly regulated both chronologically and in terms of the number of cells to ensure that the SAM is not depleted of its indeterminate cell population.

The chain of events connecting perception of the auxin signal to the specific changes in the GRN at the meristem is still largely unknown. Because only the peripheral zone is competent to respond to the auxin maxima to produce organ primordia, something unique to the expressional or epigenetic state of this region of the SAM allows an auxin signal to potentiate the leaf-primordium initiation program. An important component of the initiation process is the *as1/as2*-KNOXI module (53, 106). Class 1 KNOX (KNOX1) genes are TFs required for maintenance of the undifferentiated cell population in the SAM (7, 59, 72), and they are downregulated in the incipient primordium by *asymmetric leaves1* (*as1*) and *asymmetric leaves2* (*as2*) or their orthologs. Deactivation of KNOX1 activity in the $P_0$ may be necessary to conscript these cells into a determinate developmental fate (106) (**Figure 2*d***). This

module is very highly conserved in seed plants and may have originated independently in parallel in the lycophyte *Selaginella* (41).

### 3.3. Setting Boundaries

Boundaries are established during embryonic shoot patterning at the time of initiation of cotyledons to separate these organs from the meristem. Later, in vegetative growth, boundaries are established almost immediately after conscription of founder cells from the SAM to ensure separation of organs from one another and to separate lateral organs from the SAM (**Figure 3*a***). These boundaries can act as buffers between the differing hormonal environments in the primordium and the SAM; they also designate portions of the new organ that will not undergo growth to preserve the geometric compatibility of the organ to its attachment point. This is mediated in part by the CUC genes, NAC-domain TFs, which are required for organ-boundary determination (3).

The CUC and KNOX1 genes operate in a regulatory module involving mutual activation of the two classes of genes by each other (**Figures 3*d*** and **5**). In *Arabidopsis*, *CUC1* and *CUC2* act redundantly to activate the

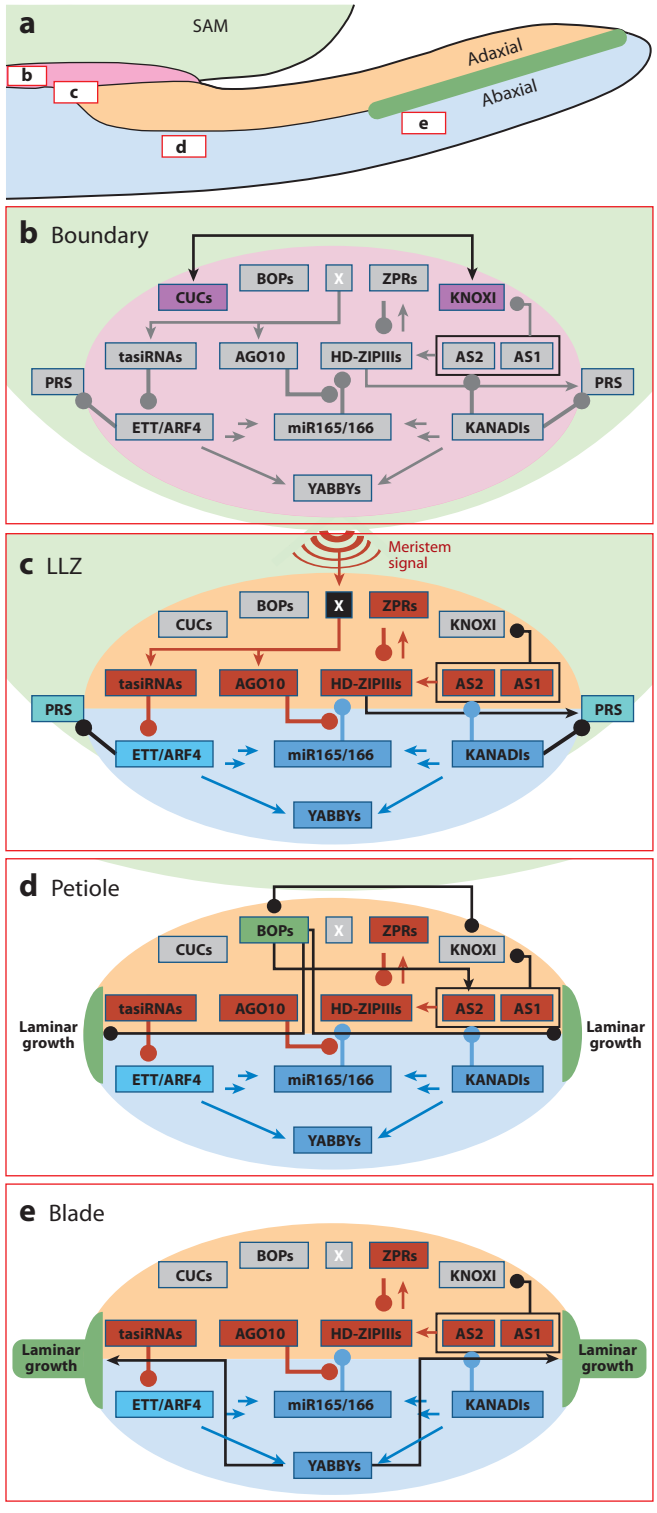


**Figure 3**

Fate decisions and patterning in the leaf primordium are governed by gene regulatory networks (GRNs) that differentially regulate development according to their position in the leaf primordium. (*a*) The leaf primordium adopts abaxial-adaxial polarity early in development. (*b*) Subtending the organ primordium CUC genes mutually upregulate KNOXI genes to create a boundary between the primordium and the shoot apical meristem (SAM). (*c*) The primary patterning fate decision involves abaxial-adaxial specification based on continuing signaling from the center of the meristem, where mutually suppressing states are generated by the opposing actions of the transcription factors: HD-ZIPIII in the adaxial domain and ETT/ARF4 genes in the abaxial domain. (*d–e*) The modules regulating other patterning programs interact with the abaxial-adaxial developmental GRN. Such interaction is also seen at the meristem (*c*).

expression of *STM* and *KNAT6*, and they require expression of those genes to delineate organ boundaries (8). *STM* and *KNAT6*, in turn, are required to activate *CUC3*. Interestingly, *KNAT2*, a closely related homolog of *KNAT6*, does not participate in this regulatory scheme (8), suggesting subfunctionalization of these recently duplicated KNOXI genes. The entire transcriptional cascade of *CUC1/CUC2* activation of *STM/KNAT6*-activating *CUC3* is required for proper boundary formation.

Identification of *CUC* genes from basal angiosperms including *Amborella* suggests that the gene family duplicated prior to the last common ancestor of the extant angiosperms and comprises two groups. One type, the *NAM-like CUC* genes, includes *Antirrhinum CUP*, *Petunia NAM*, and *Arabidopsis CUC1* and *CUC2* (120). The other type includes orthologs of *Arabidopsis CUC3*, which have lost regulation by miR164. The effects of loss of these *CUC* homologs in *Arabidopsis* and *Petunia* are limited to seedling and floral defects (2, 108, 121). In *Antirrhinum*, however, defects are also observed in vegetative growth: Mutant plants often have fused neighboring leaves and form fused spirals of bracts in continuous rings around the inflorescence stem (126).

It will be interesting to uncover what GRN topological changes cause *AmCUP* to be more integral to the vegetative phase than the *Petunia* or *Arabidopsis* orthologs. At this time, there is no published sequence of an *Antirrhinum CUC3*, so a possible loss of this gene in the course of evolution would reduce redundancy and mutational robustness and could explain the strong vegetative dependence on *AmCUP*. The distantly related species *Mimulus guttatus* has the only sequenced genome in the family, and it possesses a *CUC3* ortholog (mgv1a026208m.g). The severity of the *Antirrhinum cup* phenotype may be more likely due to shifts in the redundancy and overlap of *AmCUP* and the currently uncloned *AmCUC3* within the GRN. Given the lack of mutational robustness in boundary delimination, *Antirrhinum* could prove an insightful system in which to study the mechanisms of boundary determination.

### 3.4. Abaxial-Adaxial Polarity: Who's On Top?

The developmental regulation of abaxial-adaxial patterning is among the best worked out in terms of both the mapping of GRNs and the breadth of species studied. Abaxial-adaxial polarity is established before the primordium begins to grow out, with the adaxial (top of leaf) domain being established closest to the center of the SAM (**Figure 3*a***). In the evolution of flatted leaves from radial branched structures, developmental mechanisms had to evolve to restructure the inner-outer radial polarity into an upper (adaxial)-lower (abaxial) planar polarity (28). This restructuring involved creating an interface between these two domains, which, in turn, determines the location of laminar outgrowth. In this view, the abaxial domain could be thought of as the default, as it is developmentally equivalent to the outside of the radial shoot. Microsurgical experiments performed over the past 50 years, beginning with the insertion of mica slivers to separate the incipient primordium from the SAM and more recently using laser ablation to the same effect, show that, in the absence of positional information from the meristem, the leaf primordium will develop as a radial abaxialized structure (100, 111), that this positional signal is L1 specific (100), and that this signal must be sustained to maintain fate decision in polar patterning. The nature of the signal has yet to be determined. Both abaxial and adaxial fate decisions are governed by TFs unique to their domain, and these TFs are regulated by small RNAs (**Figure 3*c*,*d*,*e***). Many of the genes involved in the abaxial-adaxial patterning systems have been identified, and their roles in mutually regulating one another have allowed much of the core polarity determination GRN to be mapped out and compared between species.

**3.4.1. The pieces of the polarity GRN—from the bottom up.** HD-ZIPIIIs are TFs that promote adaxiality in lateral organs. Of the *HD-ZIPIII* genes, the group comprised of *REVOLUTA* (*REV*), *PHABULOSA* (*PHB*), and

*PHAVOLUTA* (*PHV*) is implicated in leaf development. These genes show a strictly adaxial expression in developing leaves in gymnosperms and basal angiosperms as well as in monocot and dicot species (138). *Arabidopsis REV* is orthologous to maize *RLD1* and *RLD2*, which together with *REV* are paralogous to *Arabidopsis PHV* and *PHB* (95).

*miR165/166* target *HD-ZIPIII* mRNAs and are expressed in the abaxial domain, preventing the target *HD-ZIPIII* transcripts from accumulating abaxially. Expression of *miR166* in the adaxial domain abaxializes the leaf (142), whereas *miR166*-resistant *HD-ZIPIII* transcripts cause adaxialization in both monocot and dicot systems (77, 86). In *Arabidopsis REV*, *PHB*, and *PHV* show a high level of functional redundancy, and triple mutants with strong LOF alleles result in seedling lethality. However, triple mutant plants heterozygous for *phb* produce trumpet-shaped leaves resulting from a severe reduction in adaxial domain to just the distal tip, with lamina outgrowth occurring only around this adaxial tissue boundary (96).

***ZPRs.*** Little zipper proteins (ZPRs) are small interfering peptides (siPEPs) that represent newfound members of the transcriptional control network involved in abaxial-adaxial polarity determination in *Arabidopsis*. HD-ZIPIII proteins function in the nucleus as dimers. ZPRs are small proteins with a leucine zipper domain similar to that of HD-ZIPIIIs, but they lack the other characteristic domains. They originated as truncated forms of ancestral HD-ZIPIII proteins (61, 127) and retain the ability to interact with other HD-ZIPIII proteins, but they prevent their action as TFs. The expression of ZPRs is activated by HD-ZIPIIIs, and the ZPRs repress HD-ZIPIII activity, attenuating the function of these genes within their own expression domain. Overexpression of ZPR genes diminishes HD-ZIPIII activity, resulting in abaxialized rod- and trumpet-shaped leaves.

The evolution of siPEPs from dimerizing TFs is a recurring regulatory theme in biology. The ease with which negative attenuators of TF activity can evolve from truncated forms of a gene suggests that siPEP regulation could be an important factor in the evolution of GRN architectures.

***KANADIs.*** KANADI genes are a class of MYB TFs that promote abaxial cell-fate differentiation (20, 135, 140), and are expressed in the abaxial domain throughout development. KANADIs feed back into the primary pattern GRN by directly repressing *AS2* in the leaf primordium (135). The *Arabidopsis* genes *KAN1* and *KAN2* act redundantly to repress *AS2* in embryonic patterning, but they have subfunctionalized roles in vegetative development where *KAN1* is required for *AS2* repression in the abaxial domain of the primordium (135). Mutants in monocot *KANADI* genes indicate that these genes also promote abaxial cell fate in this clade. The maize mutant *Milkweed Pod1* (*mwp1*) has sectors of adaxial tissue on the abaxial sheath (20), and rice *rolled9* (*rl9*) produces adaxialized leaves (139).

***ETT/ARF4.*** The abaxial patterning factors *ETT* (*ARF3*) and *ARF4* are class 1 auxin response factors, which act as transcriptional enhancers in the presence of auxin. Although a gene-duplication event prior to the radiation of the angiosperm crown group gave rise to the *ETT* and *ARF4* subtypes (33), they retain overlapping functions. Thus, either can substitute for the function of the other in *Arabidopsis* (92). *ett arf4* double mutants show a progressive loss of abaxiality in lateral organs after germination, suggesting some further redundancy with others among the 22 ARFs in *Arabidopsis* (92).

Rice and maize have lost the *ARF4* type but have proliferated the *ETT* type (33, 136). The domain architecture of the *ETT/ARF4*s has also shown a surprising amount of evolutionary plasticity. Canonical ARF proteins contain a pair of C-terminal interaction domains (III and IV) that facilitate PPI with the AUX/IAA proteins, which are negative regulators of ARF function (117). The ETT type in *Arabidopsis*, rice, and maize lack the interaction domains, whereas in the basal-most angiosperm

*Amborella trichopoda*, *AmETT* and *AmARF4* both retain this domain (33); all versions maintain DNA-binding domains. Loss of the interaction domain from one or the other members of this gene family is a recurring phenomenon in the course of angiosperm evolution, and interestingly, *AmARF4* produces two splice variants of the transcript, one lacking domains III and IV (33).

***TASI.*** *TAS3* *trans*-acting small interfering RNAs (ta-siRNAs) are siRNAs that direct cleavage of *ETT/ARF4* mRNAs to prevent translation and act cell nonautonomously to generate a diffusing suppression gradient in the adaxial domain. The *tasiR-ARF*s establish a morphogenic field in the adaxial domain by directing cleavage of abaxializing *ARF*s. *Arabidopsis ETT/ARF3* is transcribed throughout the leaf primordium; however, the transcript is restricted to the abaxial domain by the diffusing gradient of ta-siRNAs (22). These *tasiR-ARF*s are generated from directed cleavage of the noncoding *TAS3*-derived RNAs, which are targeted by miR390. The interaction of miR390 and the *TAS3* RNA is specifically mediated by the *Argonaute*-like gene *AGO7* in *Arabidopsis* and its maize ortholog *RAGGED SEEDLING2* (*RGD2*).

The *tasiR-ARF*s create the same pattern of *ETT/ARF4* mRNA suppression in *Arabidopsis*, maize, and rice; however, the various components of the system that generate these siRNAs conspire differently in these two species to produce the same pattern (17). As remarked in a recent review, "it is as if the same jigsaw puzzle pieces could be put together in different ways to produce the same final picture" (17). Despite the divergence of domains of expression of multiple interacting components, the regulation of *ETT*- and *ARF4*-like genes is the same, demonstrating the flexibility allowed in the mechanics of developmental systems while preserving the developmental output.

***YABBY.*** The juxtaposition of abaxial and adaxial domains is necessary for laminar outgrowth, and mutations abolishing this boundary fail to form a blade regardless of whether they are abaxialized or adaxialized (15). Mutations that produce sectors of reversed polarity also produce additional ectopic lamina at the junction of the abaxial and adaxial sectors (20). YABBYs are a group of TFs that until recently were believed to be unique to seed plants (134) that mediate laminar outgrowth at the abaxial-adaxial boundary. This may reflect the independent evolution of laminar development and megaphylls between different extant plant lineages.

The expression regulation of *YABBY* genes shows variability over evolutionary time. Whereas eudicot *YAB* genes are expressed in the abaxial domains of lateral organs, *Amborella YAB2* is expressed in the adaxial domain (137); within the grasses, the expression domains can vary both between individual *YAB* genes within a species and among orthologs between species. Rice *YAB2* orthologs show an abaxial expression domain as in *Arabidopsis* (6), rice and *triticum FIL* orthologs have expression in abaxial and adaxial domains (6), and maize *FIL* ortholog *ZYB14* is restricted to the adaxial domain (6).

**3.4.2. The puzzle: the polarity GRN from the top down.** Abaxial-adaxial fate determination is a binary developmental decision, where the cells in the primordium will adopt one of two cell fates, and is a fate decision superimposed atop the GRNs regulating specification of the various cell types within each domain. The identity of these two cell fates is dictated by which of the two mutually suppressive GRN expressional states is executed within those cells. This fate decision is influenced by positional signals from the meristem specifying the path individual cells will take. The primordium begins its existence with intrinsic polarity already in place. Polar expression of the YABBY gene *FIL* in *Arabidopsis* can be observed as soon as specification of the primordium is evident (43). This inherent polarity specification is not immutable and requires maintenance of expressional states in each of the domains before they become fixed in fate.

Cell-fate decisions require induction of the transcriptional profile for that cell type (5). Patterning of cell-fate decisions requires an outside input. This inductive signal has to be sustained and not transient for cells in the primordium proximal to the meristem to become adaxial in fate (100, 111). If the inductive signal is removed, the adaxial state of those cells is respecified as abaxial. Abaxial fate can be seen as a developmental default because, without outside signaling, the structure will be radial and abaxialized.

Stabilization of cell-fate decisions requires that the expressional state become self-reinforcing and ignore any further upstream inputs. This prevents change or loss of fate decision and is accomplished by multiple regulatory schemas acting concurrently, including auto-promotion of the regulatory state and active suppression of the alternate state.

In multicellular organisms, developmental GRNs are organized such that development is insulated from potentially detrimental variations in output arising from transcriptional noise (5). Noise, or stochastic fluctuation in transcript or protein abundance, is an intrinsic characteristic of transcription, particularly for genes expressed at low levels such as TFs. Noise in the expression of TFs transmits to their targets and can cause a switch between two stable states when positive feedback reinforces the new state (99). Gene duplication and polyploidy reduce intrinsic noise because this is inversely proportional to the square root of the copy number (99); indeed, many polarity-determination genes exist as multigene families such as the *KANADI*s in both maize and *Arabidopsis*. Extrinsic noise is another factor that is caused by differing cellular environments such as cell cycle, chromatin state, etc. miRNA regulation is one method GRNs use to buffer output against noise, and miRNA regulation is a key feature of developmental GRNs. miRNAs clear leaky transcripts, and in doing so, they ensure robustness of the developmental program (48). miRNAs canalize expression states, reducing perturbations from intrinsic and extrinsic noise. Canalization is an evolved buffering in a GRN that masks mutational and stochastic variance (48), ensuring robust and reproducible development and coherent tissue-level fate decisions.

A key feature of the development of abaxial-adaxial polarity specification is the boundary dividing cells that have adopted one fate from cells that have adopted the other. Each state is maintained by the key TFs it expresses, and each uses small RNAs to eliminate the key TFs of the other state. The adaxial state uses tasiR-ARF siRNAs to lock out *ETT ARF4* activity, by means of a diffusing gradient of tasiRNAs that decrease in concentration toward the abaxial-most tissues. Some point in the concentration gradient is no longer sufficient to suppress *ETT ARF4* transcripts, and a boundary is established by this non-cell-autonomous siRNA activity. The abaxial state uses miR166 to lock out the action of *HD-ZIPIII* genes, and these miRNAs act within the abaxial domain. This dichotomy of siRNA-mediated, mutually suppressive fate decisions is a common feature in developmental biology. It is similar to the fate decision of *Drosophila melanogaster* neuroectoderm to become nerve-cord primordium or epidermis, with each state using miR124 and miR9-A/miR279, respectively, to lock out the opposing cell fate (48). *ETT ARF4s* and *HD-ZIPIIIs* seem to be central regulators for their respective states, and they operate in local hubs, shaping the GRN expressional state.

"Community effect signaling" gives rise to uniformity of fate decisions among adjacent cells. Fate decisions are made when individual cells act together so that all the cells in proximity reinforce the decision made by their neighbors. This requires communication between cells to reinforce nascent states among neighbors until some threshold is reached (5). Although some TFs may exhibit non-cell-autonomous activity over short distances, the unique nature of plant cell cytoplasms as connected with their immediate neighbors allows the additional possibility that siRNAs can participate in quorum decisions, in addition to their role in establishment of patterning. siRNAs prevent opposing expressional states from

within, and they could reinforce suppression of opposing fates among neighboring cells, thereby generating a boundary between fates and uniformity within a domain. Both abaxial and adaxial GRN expressional states utilize small RNA suppression of the alternate expressional state. miRNAs and their targets often have mutually exclusive expression domains, are expressed in cells adjacent to one another, and lock out genes regulating alternate cell fates (109).

Mutations that reduce the robustness of the system to stochastic perturbances within individual cells allow one or a small group of cells to stochastically find an opposing expressional state that is then locked in. This would manifest as sectors of one domain that exist out of place among the opposite domain and are separated by a sharp boundary. This phenotype can be seen in numerous mutations in genes related to the polarity-determining GRNs. Strong *lbl1* alleles result in radial-abaxialized leaves, but milder *lbl1* shows ectopic sectors of abaxiality in the adaxial domain (115). Maize *mwp1* LOF is mild, but sectors of abaxiality develop on the sheath (20). Stochastic adoption of an inverted polarity expressional state in a small group of cells becomes locked in as a stable expression state inherited by daughter cells, resulting in sectors of inverted polarity.

Loss of mutational robustness by a portion of the GRN unmasks mutational phenotypes that would be otherwise phenotypically buffered (32, 37). This "cryptic variation" is a mutational load in a population of organisms that is masked, but loss of robustness of developmental GRNs is a tool employed by geneticists to discover additional components of a GRN. Mutations with relatively mild affects in one species, such as *as1* or *as2* in *Arabidopsis*, belong to portions of the GRN that, due to their particular properties, are able to buffer against that mutational insult. Reducing the buffering capacity of the GRN against further insults has allowed for additional mutations to be found that otherwise would have no phenotypic consequence owing to canalization of the GRN output. HSP90 has been found to be an important contributor to developmental canalization in both plants and animals (79), stabilizing client proteins and allowing cryptic variation to escape selection by buffering developmental outputs.

siRNAs are also important contributors to the robustness of developmental GRNs. siRNA function is dependent on the base-pairing interaction between the siRNA and the target, a process that is affected by temperature, as is the accumulation of siRNAs (45). Mutations affecting robustness of developmental GRNs differentially under various temperatures may potentially reveal the importance of siRNA regulation in plant-developmental GRNs. Loss of *as1* or *as2* in *Arabidopsis*, particularly in an *erecta* mutant background, shows a loss of adaxiality when grown under elevated temperatures (97). This suggests that where siRNA regulation will weaken, the ability of the GRN to canalize its output will also be weakened. Interestingly, *Antirrhinum phantastica* (*AmPHN*) mutants show an increasing degree of abaxialization when grown under cooler conditions at 17°C (122), which is also when base-pairing interactions would be strongest. The differences between *Antirrhinum* and *Arabidopsis* GRN architectures that lead to overcompensation under low-temperature conditions should prove interesting.

*ETT* and *ARF4*s are abaxial determination factors regulated by ta-siRNAs. The ta-siRNAs are produced in the adaxial domain to preclude *ETT* and *ARF4* function and to establish a diffusing suppression gradient of ETT and ARF4. Whereas *ETT/ARF3* is transcribed ubiquitously in the leaf primordium, the ta-siRNAs preclude its action in the adaxial domain (22, 88). tasiARF repression of *ETT* and *ARF4* is mediated by *SGS3* (129), although this mechanism is not yet understood. Loss of the *SGS3* ortholog in maize, *leaf bladeless* (*lbl1*), leads to abaxialization of the leaves. In *lbl1-rgd1* (strong allele), the *Revoluta* ortholog *rolled* (*rld*) expression is reduced and miR166 is increased and ubiquitous throughout the primordium (56, 88), indicating that unrestricted ARF expression is capable of overriding adaxial fate

determination in maize even with all other patterning components present. In *Arabidopsis*, mutations in tasiR-ARF components have a milder effect on polarity determination, demonstrating a GRN architecture more robust to insults to this subcircuit.

In the absence of siRNA regulation, state determination and stabilization of abaxial-adaxial state must rely on transcriptional regulatory activities, which, without the canalizing activities of siRNA regulation, could be a delicate balancing act. *AGO1* mediates both tasiRNA- and miRNA-mediated transcriptional silencing; thus, both domains are dependent on *AGO1* to mediate stabilization of state. *Arabidopsis ago1* mutants have strong polarity defects, with needle- or trumpet-shaped lateral organs that have been interpreted as adaxialized in leaves (60) and abaxialized in petals (73). *ago1* enhances *phb1-D* adaxialization, and, otherwise counterintuitively, also strongly enhances LOF of *rev* and *phb* (60). In a seemingly unnatural state of affairs, the radial leaf structures in *ago1* ubiquitously express *PHB* and miR165 simultaneously (60), a mixed expressional state that is not seen in normal leaf development. Allowing sets of abaxial and adaxial TFs to exist in the same domains, without one being able to direct siRNAs to mop up the other, forcing processes governed by each to be simultaneously coregulated, results in a mixture of expressed and repressed characteristics.

A hypomorphic allele *ago1-37* produces some trumpet-shaped leaves with abaxial epidermal cell types both within and outside the trumpet and with trichomes on both surfaces (69), a characteristic of adaxial fate in wild type. Radial needle-like petals have a mixture of abaxial and adaxial epidermal cell types (69), indicating lack of "community effect signaling" and stochastic acquisition of GRN states locally within individual cells (69). Within the early primordium, any cell can, regardless of current expression state, adopt either fate. This may be a function of factors such as *ETT/ARF3* being ubiquitously transcribed (22), thereby priming a spring-loaded fate switch if other factors shift in their direction.

Developmental complexity is the result of small highly stochastic, mutually reinforcing decisions to lead to a particular answer. Ultimately, the primary pattern still has to be established relative to external cues. Paradoxically, in radial-abaxialized *ago1* mutant primordia, the expression of *AGO10* [*PINHEAD* (*PNH*) or *ZWILLE* (*ZLL*)], although weak, is adaxial (60). The adaxial domain of expression of *AGO10* in the *ago1* mutant radial primordia suggests that its expression is not strictly determined by a particular domain, but that it is responding to the positional signal from the meristem. *AGO10* has evolved as a "decoy" for miR165/166; essentially, it interacts with miR165/166 to "soak it up." In a cellular environment with stochastic fluctuations in transcription, asymmetrically lifting siRNA repression of *HD-ZIPIII*s in one spatial domain would allow specification of "not default" adaxiality and act as a prepattern.

It remains to be seen how widely utilized dominant siRNA inhibition is in regulating the siRNAs involved in plant development, but orthologs of *AGO10* exist in monocots and dicots. There are three *AGO10*-like genes in maize and one in rice (98). Rice *PNH1* (*OsPNH1*) also plays a role in leaf patterning and is expressed in the most-adaxial cell layers in young primordia, but it shifts to a position adaxial to developing vascular bundles in older primordia (87). Strong antisense plants produce tendril-like leaves (87), and an adaxial early expression pattern is suggestive of a role in leaf patterning. Future works should reveal if use of *AGO10* as an miRNA decoy is ancient and conserved across plant species.

siRNAs may operate to override the default developmental pattern differently in both *Arabidopsis* and maize. In maize, the abaxialized radial *lbl1* leaf primordia show "adaxialized" expression of miR390 (89), suggesting that in this species the default override could operate by suppressing *ARF3*s, rather than lifting suppression of *HD-ZIPIII*s, and in maize, miR390 may be initially responding to the primary morphogenic signal. The evolution of GRN architectures to respond to override the default state differentially between maize and

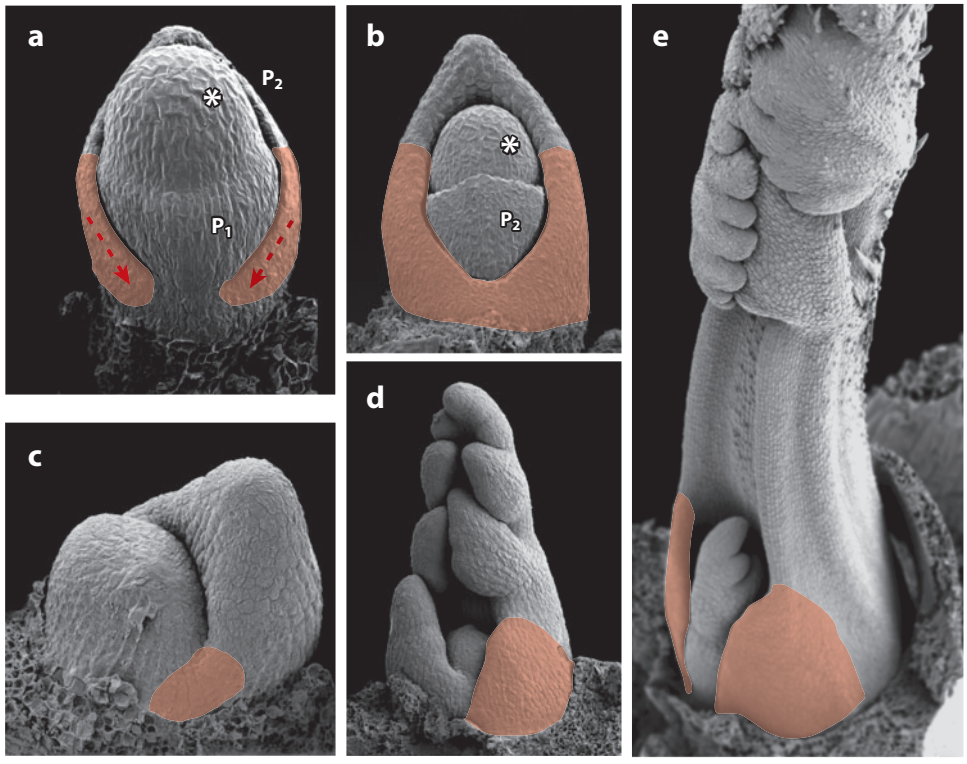


**Figure 4**

Lateral recruitment allows leaf primordia to continue to conscript cells from the shoot apical meristem (SAM) after the initial auxin-mediated cell recruitment: recruited cells (*red*), direction of recruitment (*red dashed arrows*). Cell recruitment fully encompasses the SAM in maize, resulting in the majority of mature leaf tissue (*a–b*), and leads to the formation of stipules in many dicots such as anise, also shown here in time series (*c–e*).

*Arabidopsis* could help explain the differential severity of mutations in the tasiR-ARF pathway between these two species. This evolution could also explain the increased severity in dicot species of mutations affecting *as1* and *as2*, which moderate the *HD-ZIPIII* subcircuit.

### 3.5. Stipules and Sheaths: Lateral Recruitment

The auxin maxima in the SAM specify only a discrete region for founder-cell recruitment, so an additional signal must propagate laterally to secondarily recruit stipule or sheath founder cells (102) (**Figure 4**). The primarily recruited founder cells then recruit additional cells into the primordium through a mechanism dependent on the expression of a *WOX* gene orthologous to *Pressed Flower* (*PRS*) in *Arabidopsis* (75) or the *Narrow Sheath* (*NS1* and *NS2*) genes in maize (102, 103). Double-mutant *ns1* and *ns2* plants show a deletion of the lateral portions of the sheath and leaf blade (103); in *Arabidopsis*, however, only the microscopic stipules are deleted (85). Cells that are recruited to become stipules or sheath are taken from the flanks of the primordium, suggesting this domain is positionally determined by early abaxial-adaxial juxtaposition in the primordium. Maize and *Arabidopsis* mutations affecting polarity determination also show defects in secondary recruitment and loss of stipule or sheath

from the lateral domains of the portion of the primordium most proximal to the SAM, otherwise known as the lower-leaf zone. This domain is elaborated into the major blade portion of the grass leaf (103). Dicot species tend to elaborate the distal portion of the leaf, i.e., the upper-leaf zone, and some species also elaborate prominent stipules. The maize leaf is more dependent on secondary recruitment of cells from the SAM, as it must form an ensheathing base that encircles the stem.

The systems governing patterning and development of the leaf primordium are highly interdependent, and the juxtaposition of abaxial-adaxial states may be necessary for secondary recruitment. However, abaxial factors appear to hinder this process. Mutations that abaxialize the leaf primordium also inhibit secondary recruitment. The maize *tasiARF* biogenesis mutants *lbl1* and *rgd2* have ectopic expression of *ARF3* homologs throughout the primordium, and they lack ensheathing leaf bases (44, 115). In *lbl1* and *Rgd2-R*, there is a reduction in the number of founder cells, as evidenced by the lack of downregulation of *KNOX* in those cells (44, 115). *Rgd2-R* also has reduced expression of *NS1* and *NS2* (44).

Disruption of the abaxial state appears to lift inhibition of secondary recruitment: *Arabidopsis* mutants that have adaxializing phenotypes develop ectopic stipules. Both *ett arf4* double mutants and *kan1 kan2* plants develop ectopic stipules on the abaxial leaf base, suggesting *ett arf4* and *KANADI* genes suppress secondary recruitment under normal developmental conditions.

Adaxializing mutants contribute to ectopic stipule recruitment. *Arabidopsis* heterozygous for *phb-1d* develop ectopic stipules, but strong homozygous plants do not, suggesting some abaxial factors are needed to promote or position secondary recruitment. Latent abaxiality may be less important for lateral recruitment in maize than in *Arabidopsis*. The adaxializing maize mutant *RLD1-O* has ensheathing leaf bases (56), unlike the abaxializing mutant phenotypes.

## 3.6. Going the Distance: Proximodistal Polarity

Proximodistal polarity becomes evident as the primordium begins to grow out and away from the SAM. Integral to the GRN regulation of the proximodistal patterning subnetwork are the *Blade on Petiole* (*BOP*) genes, *BOP1* and *BOP2*, which are BBT-POZ-type transcriptional activators that are required to pattern the proximal portion of the leaf in *Arabidopsis* (57) (**Figure 5**). Double-mutant *bop1 bop2* or dominant-negative *bop1-1* each lacks the proper distinction between leaf blade and petiole, and both show laminar development on what would be the petiole (57, 129). Both are expressed in the adaxial domain, where they act redundantly to suppress laminar outgrowth in the petiole region. *BOP2* binds to the promoter of *AS2* and activates its expression in the proximal domain (129). Regulation of *AS2*, a canonical abaxial-adaxial polarity patterning factor, by *BOP2* within the proximal region of the leaf primordium exemplifies the

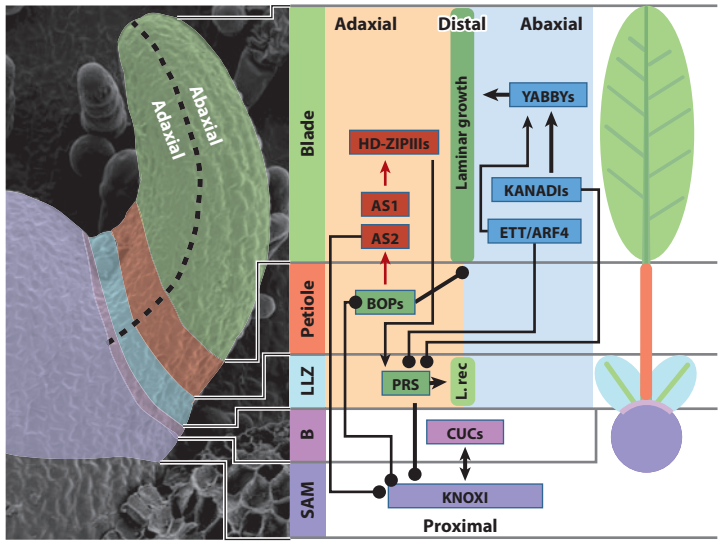


**Figure 5**

Regulation of proximal distal polarity allows the various parts of the leaf to differentiate into the correct domain of the leaf. The developmental zones are indicated here on a leaf primordium. Various genes involved in proximodistal patterning interact with the abaxial-adaxial developmental gene regulatory network (GRN). Abbreviations: B, boundary; LLZ, lower-leaf zone; SAM, shoot apical meristem. Also indicated are the petiole and the blade.

lack of complete distinction between GRNs directing differentiation of the various axes and developmental processes, because many components are simultaneously integral in multiple subnetworks (**Figures 3*d*** and **5**).

Developmental GRNs often have a hierarchical structure, with higher-level modules activating or precluding specific developmental GRNs (29). The hierarchical structure of developmental GRNs allows a relatively small mutational change to cause a wholesale redeployment of several genes. The unique anatomy of the grass leaf suggests specialized morphogenic patterning modules. At the boundary of the blade and sheath are the ligule and the auricle, specialized regions that facilitate blade bending and form a sharp transition zone between the sheath and the blade. The grass leaf is homologous to the dicot leaf despite superficial differences. However, the ligule and auricle have no clear homologous structure in dicot species, suggesting a distinct layer of patterning that sits atop the preexisting proximodistal patterning systems. A preexisting relationship between modules utilized together in one tissue or process may predispose a module to co-option where the other is in use, as the number of new GRN interconnections would be minimized. Some genes that regulate the developmental processes leading to the formation of the ligule have been co-opted from roles in patterning of floral parts, such as anthers, and in regulation of floral transition, a process that, in maize, appears to have been facilitated by gene duplication.

*Liguleless1* (*LG1*) belongs to the *SQUAMOSA*-binding protein-like (*SBP-like*) genes, which, in both monocots and dicots, chiefly function in floral induction, floral meristem identity, and floral organ patterning and differentiation (51). In grasses, orthologs of maize and rice *LG1* genes have a conserved function in differentiating ligule tissues. LOF in rice abolishes the ligule on most leaves and disrupts the blade-sheath boundary (68). In maize *lg1*, the disruption of the ligule is less severe, likely owing to the presence of a close homolog *ZmSBP14* (51). The closest homolog in *Arabidopsis* is *SPL8* (118, 141), which is involved in patterning and differentiation of anthers; *spl8* mutants do not have a leaf phenotype.

*Liguleless2* (*LG2*) may have been recently co-opted into leaf development in maize, where it restricts the region competent to form ligule and auricle. *LG2* orthologs do not appear to be involved in leaf patterning in other grasses (66). *lg2* mutants have additional pleiotropic phenotypes relating to the original function of the gene such as reduced tassel branching and extra vegetative leaves. *LG2* has a paralog in maize, *liguleless related sequence1* (*LRS1*), which resulted from gene duplication after maize and *Sorghum* diverged (66). Neither maize *LRS1* nor the rice ortholog are believed to be involved in leaf development (66). The *Arabidopsis* ortholog *TGA9* together with *TGA10* also act in anther patterning (84).

Over the past 80 years (113), changes in leaf architecture that allow for more efficient harvesting of light energy and higher planting densities have been selected for in agriculture. QTL analysis has identified both *LG1* and *LG2* as regions of strong effect for these changes, demonstrating the nexus between evolution, development, and application.

## 4. COMPOUND LEAF EVOLUTION: DO ALL ROADS LEAD TO KNOX?

Compound leaves have evolved independently numerous times from simple leaf forms (9), but despite the huge variation in form and degree of leaf complexity, the developmental mechanisms that lead to it are converged upon repeatedly (9). Numerous processes required for compound leaf development are already utilized in leaf initiation and primary development. Coordinated redeployment of these GRN subnetworks during leaf development allowed these processes to be reiterated in a temporally and spatially coordinated way, resulting in compound leaf morphogenesis.

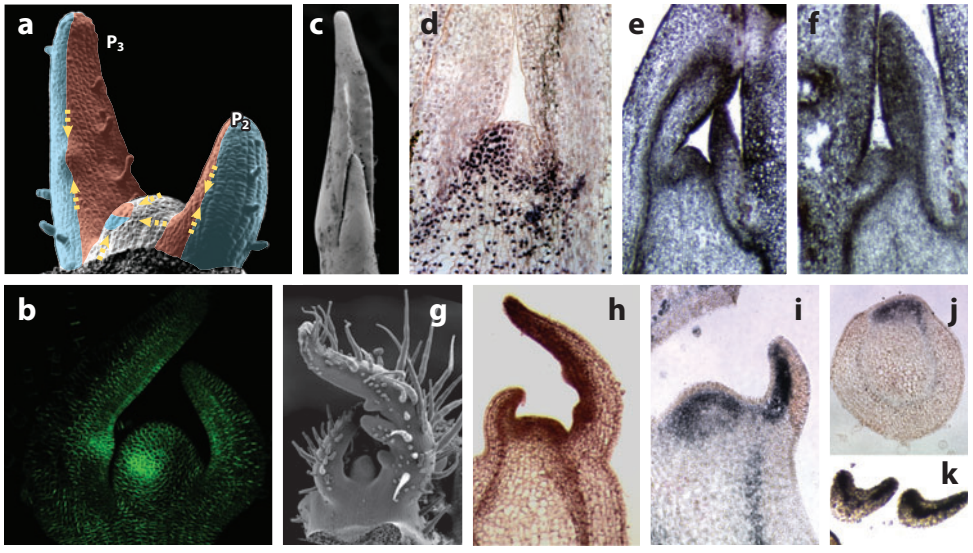


**Figure 6**

Compound leaf development requires elements of meristematic and leaf gene regulatory networks (GRNs) to act as part of a coordinated developmental program. (*a*) As with initiation of a leaf primordium in the shoot apical meristem by focusing an auxin maxima, leaflet primordia are also generated by the directed flow of auxin (*yellow dashed arrows*) along the abaxial-adaxial boundary in the leaflet. (*b*) Generation of the local auxin maxima in the leaf primordia is also directed by PIN proteins, shown here with PIN-GFP in the compound leaf species *Solanum lycopersicum*. Compound leaf development depends on derepression of *KNOXI* expression in the leaf primordium after *AS1/AS2*-mediated *KNOXI* repression occurs during founder-cell recruitment. (*c*) In most simple leaf species such as the basal-most angiosperm *Amborella trichopoda* shown in SEM, (*d*) *KNOXI* repression occurs in the $P_0$ and remains repressed in developing leaf primordia as shown with immunolocalization using antibodies to maize Kn1. *AS1-PHAN* orthologs have a conserved role in *KNOXI* suppression and abaxial-adaxial polarity in many species. (*e–f*) *Amborella* PHAN protein is localized to the adaxial domain of developing leaf primordia using Maize RS2 antibodies for immunolocalization. Developing compound leaf primordia such as (*g*) tomato derepress (*h*) *KNOX1* genes seen here using *LeT6* (*STM* ortholog) in situ hybridization. PHAN protein still accumulates in the adaxial domain of developing leaf (*i*) primordia, (*j*) petioles, and (*k*) leaflets. The presence of KNOXI and PHAN in the same expression domain illustrates the complexity afforded by non-Boolean regulatory interactions.

Leaflets are initiated by local auxin maxima in a manner similar to initiation of the leaf primordium, and PIN proteins focus auxin flow leading to formation of local maxima on the flanks of the primordium along the abaxial-adaxial boundary where leaflet primordia are initiated (12, 63) (**Figure 6*a*,*b***).

*KNOX1* genes are important components of the regulatory network involved in compound leaf development. *KNOX1* genes such as *STM* and *BP* or their orthologs become derepressed in the primordium (9) to promote the level of indeterminacy necessary for a prolonged period of development in compound leaf species (**Figure 6*c*,*d*,*g*,*b***). Consequently, overexpression of one of these *KNOX1* genes can result in an increased degree of complexity or lobing (54, 70). Mutations in genes that regulate *KNOX1* activity at the protein level have shown leaf complexity-related phenotypes in tomato and *Arabidopsis*. *BELL*-like genes are known *KNOX1* interaction partners. Although they form heterodimers with *KNOXI* proteins, they can act antagonistically in some

*KNOX1* regulatory roles (62, 74). LOF of the *BELL*-like genes *SAWTOOTH1* (*SAW1*) and *SAWTOOTH2* (*SAW2*) in *Arabidopsis* leads to serration of the leaf margin (22); similarly, LOF of the single tomato ortholog *BIPINNATE* (*BIP*) leads to an increased order of complexity, allowing development of tertiary leaflets on secondary leaflets (62).

The interaction between *BELL* and *KNOX1* genes is moderated by siPEPS evolutionarily derived from truncated *KNOX1* genes. These siPEPS contain only the KNOX domain, which mediates PPIs, and they physically interact with BIP/SAW proteins to prevent their entering the nucleus (62, 74). The siPEP gene *PETROSELENIUM* (*PTS*) was discovered as a natural variation with increased leaf complexity in a wild species of tomato from the Galapagos Islands (62). The variant with increased leaf complexity has a SNP in the promoter region that increases the expression level of the gene by twofold, thus reducing *BIP* transcriptional regulatory activity. *PTS* is an interesting siPEP because, unlike many others, its expression enhances the effect of the genes from which it was derived.

## 5. LEGUMINOUS LEAVES: A CASE IN POINT FOR DEVELOPMENTAL SYSTEMS DRIFT

Legumes are comprised of mostly compound leaved species, and most show the canonical KNOX1 expression mode. Within a subgroup of the legumes (ILRC), compound leaf architecture was maintained; however, the GRN responsible for this developmental process underwent a substantial shift in regulatory wiring away from *KNOX1*-mediated modules in a stepwise manner. Instead, the ILRC compound leaf development GRN architecture relies on *LFY/UFO*-mediated indeterminacy, and it maintains repression of *KNOX1* genes in the primordia.

Non-ILRC legumes have an intermediate reliance on *KNOX1* and *LFY* modules to regulate complexity in the developing leaf. These species express *KNOX1* genes in their leaf primordia, similar to other compound leaf angiosperms (21), but they have partial reliance on *LFY* for compound leaf formation. The intermediate *LFY* dependence was demonstrated with RNAi of the *LFY* ortholog in soybean (*Glycine max*) (*GmLFY*) and with an LOF mutation of the *LFY* ortholog in *Lotus*, *proliferating floral meristem* (*pfm*). The developmental consequences of these disruptions of *LFY* function are fusion of some leaflets in soybean (21) and reduction in leaflet number in *Lotus* from five to three or four (26).

Within the ILRC, *LFY* is absolutely required to develop the compound leaf architecture. The *Medicago LFY* ortholog *SINGLE-LEAFLET1* (*SGL1*) is required for compound leaf development, and *sgl1* plants have only a single leaflet (123). The pea *LFY* ortholog *UNIFOLIATA* (*UNI*) is expressed in leaf primordia early in development, and it is downregulated at the time of leaflet initiation (38, 46); *uni* mutant leaves consist of only a single leaflet (46). Mutations that release the downregulation of *UNI* in the leaf primordia of pea result in an increased degree of compoundness in the leaf (80), indicating that *LFY* expression in these species prolongs the window of morphogenic plasticity in leaf development, reminiscent of *KNOX1* function outside of the ILRC. *LFY* was first co-opted into a supporting role in the compound leaf development program in legumes, thus allowing the *KNOX1* function to be attenuated in leaf development until it could be lost without developmental consequence.

*LFY* also has a role in leaf development outside of the legumes. In tomato, the *LFY* ortholog *FALSIFLORA* (*FA*) is expressed in leaf primordia and in vegetative meristems (82). *fa* mutants have only a mild leaf phenotype, with no reduction in primary leaflets but fewer minor leaflets (82) that originate later in development. In tomato, this suggests that *LFY* can also extend the period of developmental plasticity in leaf primordia, allowing more time to initiate leaflets. Overexpression of *LFY* in

*Arabidopsis* does not lead to a leaf complexity–related phenotype, but instead causes lateral shoots to emerge as individual flowers (125). Overexpressing the *LFY* coregulator *UFO*, however, leads to a lobing phenotype, but only in an *LFY* background (67), indicating that a specific subset of *LFY*-regulated processes is responsible for promoting some indeterminacy in leaf development.

*LFY* target genes are regulated in a context-dependent manner: *Arabidopsis LFY* binds promoters of different sets of genes in seedlings and inflorescences, and it transcriptionally regulates different sets of genes in these two environments (131). Motif prediction based on these two sets of genes shows consistent differences in the secondary regulatory sequences (131). In a floral primordium, *LFY* must function to maintain a sufficient degree of indeterminacy to produce multiple floral whorls: Each whorl has multiple organs, although all share homology with the leaf. Thus, it is easy to envision modules from that developmental program being grafted onto the GRNs responsible for promotion of leaf indeterminacy.

Despite a radical transfer of power away from *KNOX1* regulation in compound leaf development in the ILRC, a core *KNOX1* regulatory module remains intact in pea. In diverse angiosperm species, *AS1* and *PHAN* are utilized to suppress *KNOX1* (19, 39, 78, 112, 114) and directly repress the *BP* orthologs (39) in the incipient primordium (42). In simple leaf species, repression is maintained within the developing primordium. In pea in the mutant *crispa* (*cri*), the *BP* ortholog *PSKN2* is ectopically expressed in leaf primordia (112), just as in *Arabidopsis as1* (19). Maintaining *KNOX1* repression in leaf primordia is typical of simple leaf species, so this state may be easily mutationally accessible. In addition, recruitment of *LFY* into the compound leaf-developmental program of IRLC legumes may be what allowed this reversion to occur without gross morphological consequences. It is noteworthy that the GRN-regulating leaf development in the ILRC is still somewhat competent to respond to the expression of a *KNOX1* gene. *Medicago* transformed with 35s:*LeT6* (tomato *STM*) shows increased complexity, averaging less than one additional leaflet per leaf (21).

## 6. NATURE'S WHIMSY: NOVELTY IN EVOLUTION OF LEAF DEVELOPMENT

Leaf development can be modified by regulatory alteration to find "out of the box" solutions to evolutionary pressures. Alterations to the GRNs involved in leaf development can produce surprisingly novel adaptations. Within the genus *Kalanchoe*, some species are capable of asexually producing plantlets directly from the leaf margins. In the basal-most groups with this characteristic, the plantlets are formed by growing an ectopic meristem from a pool of undifferentiated cells in the sinus between leaf serrations (35). The underlying mechanism by which these cells are set aside and later triggered to undergo development is not well understood, but it is *KNOX1* dependent (35). The process is superficially similar to what occurs in *Arabidopsis* when *MIF1* or *MIF3* is overexpressed (49), i.e., when plants form serrated leaves with ectopic meristems on the leaf margin in the sinus between serrations. MIFs are MINI ZINC FINGER proteins that possess a zinc-finger domain and lack a DNA-binding domain (50). They are another example of siPEPs in plant GRN regulation and are an ancient group of genes originating prior to the gymnosperm-angiosperm divergence. Overexpression of either *MIF1* or *MIF3* results in sustained expression of *STM* along the leaf margin, which may maintain cells in an undifferentiated state or cause dedifferentiation to occur. In *Kalanchoe*, mutations in MIFs are unlikely to be the direct cause of the reproductive syndrome in the genus, but these mutations illustrate how modifying a single element in the GRN can create a drastic phenotypic consequence on which selection can act. The vegetative mode of reproduction in *Kalanchoe* species has been implicated by ecological modeling as the largest factor contributing to their invasiveness (45),

demonstrating that alterations to GRNs regulating leaf development can have very favorable fitness consequences.

## 7. CONCLUSIONS

The evolution of leaf development has involved both conservation of core modules and cooption of familiar GRNs into new functions. The modular nature of GRNs involved in development has provided an extensive evolutionary tool kit that can be assembled in various combinations to provide a final outcome—a flattened organ that is optimized to capture photosynthetic light. Leaves come in a variety of shapes and sizes and have been studied extensively in a select group of model organisms. The current approach of utilizing a handful of candidate genes to understand leaf development in a nonmodel species provides only incomplete answers. We propose that adopting a GRN-based approach will allow us to understand more completely how innovation was achieved in the evolution of these organs.



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