Advanced Review

Morphogenesis of simple leaves: regulation of leaf size and shape

Ramiro E. Rodriguez*, Juan M. Debernardi and Javier F. Palatnik*

Plants produce new organs throughout their life span. Leaves first initiate as rod-like structures protruding from the shoot apical meristem, while they need to pass through different developmental stages to become the flat organ specialized in photosynthesis. Leaf morphogenesis is an active process regulated by many genes and pathways that can generate organs with a wide variety of sizes and shapes. Important differences in leaf architecture can be seen among different species, but also in single individuals. A key aspect of leaf morphogenesis is the precise control of cell proliferation. Modification or manipulation of this process may lead to leaves with different sizes and shapes, and changes in the organ margins and curvature. Many genes required for leaf development have been identified in *Arabidopsis thaliana*, and the mechanisms underlying leaf morphogenesis are starting to be unraveled at the molecular level.

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INTRODUCTION

Oxygen, food, fibers, fuels, and other products essential to life and human activity ultimately rely on photosynthesis. Most of the photosynthetic processes carried out by land plants occur in leaves, which are nearly flat organs designed to efficiently capture light and perform photosynthesis. These functions are carried out by specialized tissues disposed in layers with the top (adaxial) side focusing on light capture, and the bottom (abaxial) side on gas exchange. From an anatomical perspective, the leaf is formed by two epidermal layers surrounding the mesophyll, which consists of a tight array of columnar cells (palisade parenchyma) and a layer of irregular shaped cells with extensive intercellular air spaces (spongy parenchyma) (Figure 1(b)).

There is a wide variety of leaf shapes and sizes in nature. Leaves can be simple or compound organs, depending on the leaf blade, which can be undivided or dissected into leaflets (Figure 1(c)). Leaves are usually described according to their general shape (rounded, ovate, or lanceolate), margins (smooth, lobed, or serrated), and curvature (planar or not) (Figure 1). Interestingly, even leaves from a single plant might have different morphologies. For example, the first and juvenile leaves of *Arabidopsis thaliana* are small and rounded with smooth margins, while the later and adult leaves are bigger ovate-shaped organs with partially serrated margins (Figure 1(a)). Here, we describe the genetic mechanisms that control the shape and size of simple leaves, with an emphasis on the regulatory modules characterized in the model plant *A. thaliana*.

EARLY EVENTS IN LEAF DEVELOPMENT

Establishing the Leaf Primordia: Hormone Signaling and Transcription Factor Networks

In contrast to animals, plants produce new organs throughout their life cycle. The above-ground parts of the plant, such as the leaves, stem, and flowers derive from a small collection of stem cells located at the shoot apical meristem (Figure 2). Two classes of homeodomain transcription factors have essential roles in meristem formation and

*Correspondence to: rrodriguez@ibr-conicet.gov.ar; palatnik@ibr-conicet.gov.ar

IBR (Instituto de Biología Molecular y Celular de Rosario) - CONICET/UNR, Rosario, Argentina

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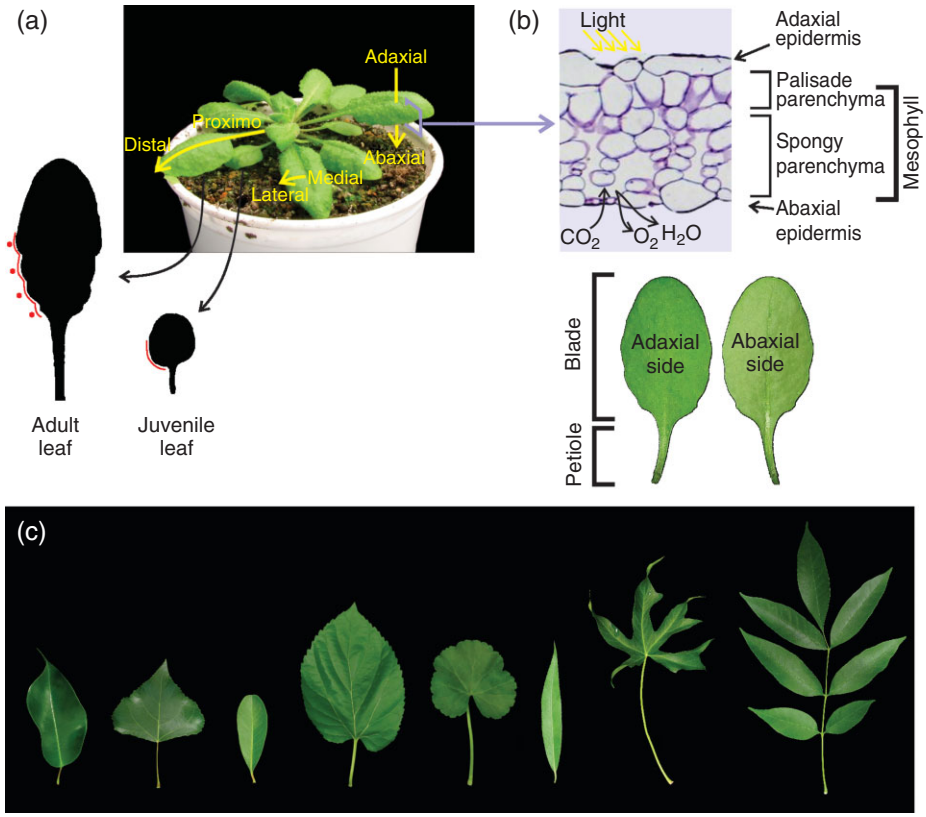

FIGURE 1 | Dicotyledonous leaves and their variations in size and shape. (a) *Arabidopsis thaliana* plant showing the three different axes of asymmetries. Bottom left, adult and juvenile leaves. (b) Cross sections of an *Arabidopsis* leaf blade showing the organization of the different tissues. Two single-celled layers of epidermis enclose the mesophyll, organized in palisade and spongy parenchyma. (c) Samples for leaf shape and size variations found in nature. The rightmost leaf is a compound or dissected leaf with seven leaflets, while all the others are simple leaves with different shapes, sizes, and margins. From left to right, leaves of *Ficus benjamina, Populus nigra, Pyracantha coccinea, Morus nigra, Pelargonium hortorum, Salix babylonica, Hedera helix*, and *Fraxinus excelsior*.

maintenance. A group of cells in the meristem central zone express *WUSCHEL* (*WUS*), which defines the stem cell niche in the over-laying cells.[1] The class I KNOTTED1-like homeobox (KNOXI) gene *SHOOT MERISTEMLESS* (*STM*) is expressed throughout the meristem, preventing an early cell differentiation. Together with *STM*, other KNOXI transcription factors such as *BREVIPEDICELLUS*/*KNAT1*, *KNAT2*, and *KNAT6* are also expressed in the meristem (recently reviewed in Refs 2–4).

Leaf primordia are initiated at the peripheral zone of the shoot apical meristem (Figure 2(a) and (b)). Newly developing organs are positioned in a specific pattern called phyllotaxy. *A. thaliana* has a spiral phyllotaxy and leaves are arranged in angles of approximately 137°. The location of the leaf primordia founder cells at the meristem periphery is defined by a local auxin maximum (Figure 2(c)). This high concentration of auxin is generated by the polarized transport mediated by PIN-FORMED1 (PIN1) through the epidermis.[5–7]

Specific transcription factor networks distinguish the leaf primordia from the rest of the meristem, such as the MYB transcription factor *ASYMMETRIC LEAVES1* (*AS1*)[8,9] (Figure 2(c)). AS1 acts in concert with AS2, a LOB domain protein, to repress *STM* and other *KNOXI* genes in the leaf primordia.[8,10,11] This repression is important for the developmental program of the leaf and is achieved through several different pathways.

The boundaries between the meristem and the growing leaf primordia are delimited by transcription factors of the http://planttfdb.cbi.edu.cn/family.php?fam=NAC family termed *CUP-SHAPED COTYLEDON1* (*CUC1*), *CUC2*, and *CUC3* (Figure 2(c)). These genes are specifically expressed in a small group of cells separating the domains of the meristem and the incipient leaf primordia.[12–14] Mutations in two of them cause the fusion of the cotyledons and loss of the apical meristem.[15,16] Expression of *STM* and *CUC* genes reinforce each other, while STM directly activates *CUC1* expression.[17–19]

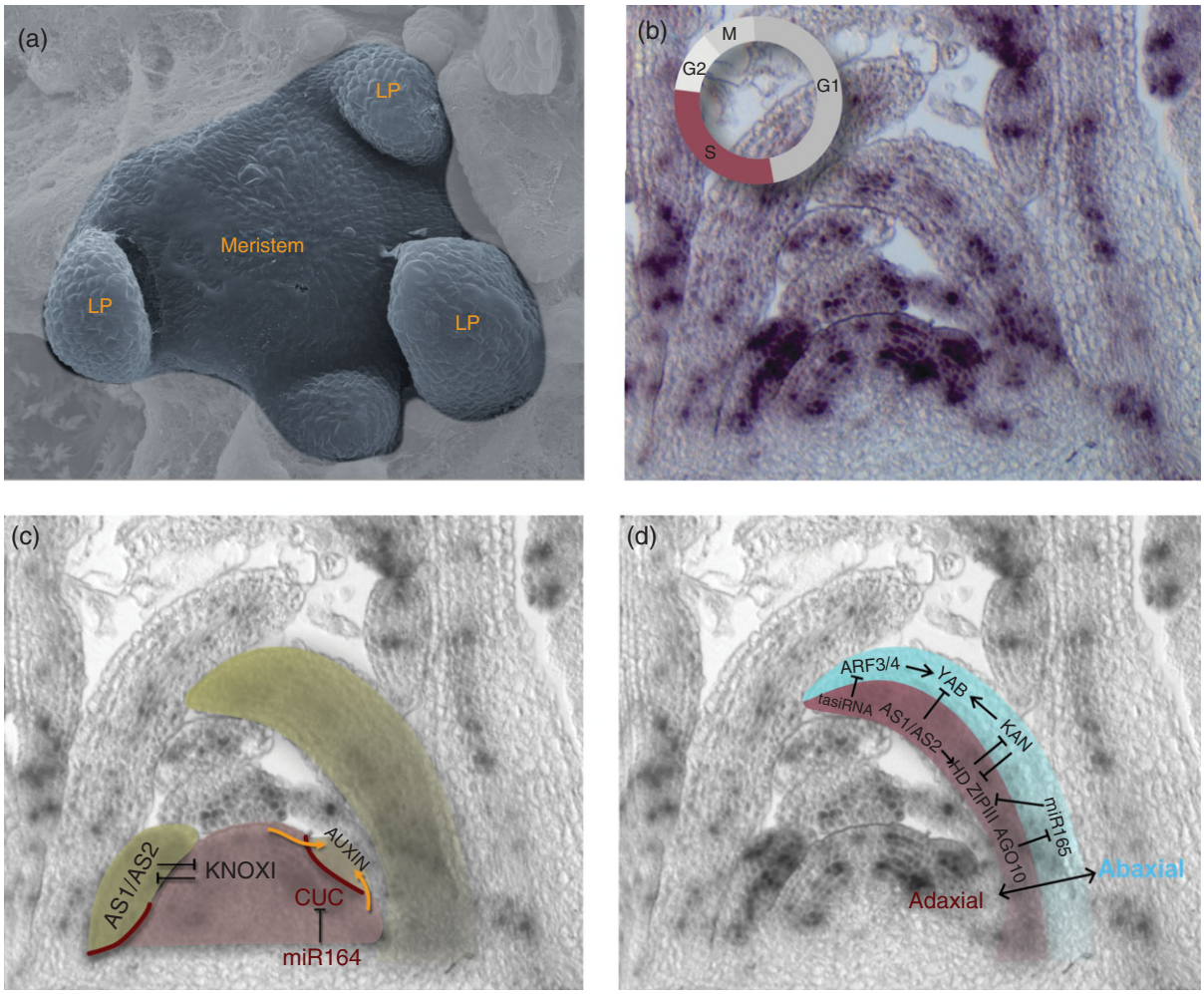

FIGURE 2 | Early events in leaf development. (a) Scanning electron microscopy of a vegetative shoot apical meristem. Note the spiral phyllotaxy of leaf primordia (LP) around the meristem. (b) Architecture of the vegetative shoot apical meristem. Cells in the S-phase of the cell cycle are revealed by the expression of *HISTONE H4* detected by *in situ* hybridization in medial-longitudinal sections. (c) Genes and hormones involved in meristem maintenance and leaf initiation. (d) Expression and activity patterns of genes and small-RNAs essential for the establishment of leaf polarity.

Definition of the Dorso-Ventral Axis of the Leaf

Initially, the leaf primordia extending out of the meristem has a rod-like structure (Figure 2(a)) and will soon expand to form a flat lamina consisting of two anatomically distinct surfaces: the adaxial side (top), that will be prepared to capture light, and the abaxial side (bottom), that will specialize in gas exchange (reviewed in Refs 20 and 21). An early step in the development of the leaf primordia is, therefore, the establishment of this dorso-ventral axis, which will finally generate the organized structure of tissues layers that compose the leaf (see Figure 1). This process relies on a complex network of transcription factors that have synergistic interactions and mutual exclusions (Figure 2). Furthermore, as primordia develop on the flank of the meristem, the adaxial side is in closer proximity to the meristem (Figure 2), leading to the model suggesting that this inherent asymmetry directs the leaf patterning (reviewed in Refs 20 and 21).

The adaxial side is specified by the activity of members of the class III HOMEODOMAIN-LEUCINE ZIPPER (HD-ZIP) family of transcription factors, such as *PHABULOSA* and related genes, and loss of their functions causes abaxialized organs.[22–24] A second pathway promoting the adaxial fate involves *AS1* and *AS2*,[25] which are also required for the repression of *KNOXI* genes.[11] In turn, the abaxial side is specified by the activity of transcription factors of the KANADI,[26,27] YABBY,[28,29] and AUXIN RESPONSE FACTOR http://planttfdb.cbi.edu.cn/family.php?fam=ARF(ARF)[30] class. Remarkably, transcription factors that promote the definition

of one side tend to repress the expression of those of the other one, therefore, contributing to the establishment of sharp complementary domains.

POSTTRANSCRIPTIONAL CONTROL DURING LEAF DEVELOPMENT: THE ROLE OF SMALL RNAs

Small RNAs are key regulators of gene expression in animals and plants (reviewed in Refs 31 and 32) and play a major role in leaf development. MicroRNAs (miRNAs) are one class of small RNAs defined by their unique biogenesis pathway, which requires the cleavage of a noncoding RNA harboring a fold-back precursor by a ribonuclease type III called DICER-LIKE1 (DCL1).[33] MiRNAs are around 21 nt and function in the context of a complex containing an ARGONAUTE (AGO) protein, generally AGO1.[34] MiRNAs guide these complexes to target RNAs that have base complementarity to the miRNA. Target mRNAs in plants are usually cleaved, although they can also be translationally repressed in some cases. *A. thaliana* contains more than *200 MIRNA genes* dispersed in its genome. Although many of them have appeared recently in evolution, there are 21 families of miRNAs deeply conserved in angiosperms.[32] Many of the conserved miRNAs regulate transcription factors involved in plant development and, more specifically, leaf development.

CUC1, *CUC2*, and other *NAC* transcription factors are posttranscriptionally regulated by miRNA miR164.[35,36] Gene expression studies revealed that *CUC1* and *CUC2* expression occurs in a broader domain and that miR164 limits them to the cells in the organ boundary[37] (Figure 2(c)). Overexpression of the miR164 compromises organ separation causing fusion between cotyledons, leaves, and also between the inflorescence stem and cauline leaves.[35,36,38] Furthermore, plants harboring mutations in the miR164-binding site of *CUC* genes have altered leaf morphology and phyllotaxis.[37,39,40] MiR164, together with other miRNAs, such as miR319 and miR396, also controls later stages of leaf development (see below).

Two different small RNA pathways have been implicated in the precise expression pattern of polarity genes. The first one involves the miR165/166 miRNAs, which regulates the class III HD-ZIP genes, such as *PHABULOSA* and *PHAVOLUTA*.[24,41–44] MiR165/166 act in the abaxial side, limiting the expression of the HD-ZIP transcription factors to the adaxial side (Figure 2(d)). Several dominant gain-of-function mutations in these transcription factors have been identified that cause an expanded expression domain, promoting adaxialization and radialization of leaves. These mutations are located in the miR165/166 target site and diminish the complementarity to the miRNA.[22,24] In turn, it has been shown that AGO10 represses the activity of miR165/166.[45–47] Specifically, AGO10, which is expressed in defined domains, binds and sequesters miR165/166, generating a catalytically inactive complex in *A. thaliana*. Therefore, AGO10 functions as a decoy reducing the miR165/166 levels available for AGO1, which constitutes the active complex *in vivo*[43,46] (Figure 2(d)).

A particularly interesting miRNA is miR390, which functions in the context of AGO7 instead of the common AGO1.[48] MiR390 guides AGO7 to two partially complementary target sites in *TAS3*, a noncoding RNA widely conserved in plants.[48–50] Targeting of *TAS3* by miR390 causes its cleavage in one of its target sites, which then serves as proxy for the activity of RDR6 that converts the TAS3 fragment into double stranded RNA. Afterwards, the ribonuclease DCL4 cleaves this dsRNA TAS3 fragment to generate small RNAs termed ta-siRNAs (*t*rans-*a*cting *s*mall *i*nterfering RNAs). Ta-siRNA formation triggered by the activity of miR390 occurs in the adaxial side of the leaf generating a gradient of ta-siRNAs that in turn inhibit the expression of *ARF3/4*.[50,51]

GROWTH OF THE LEAF BLADE: COORDINATION OF CELL PROLIFERATION AND CELL EXPANSION DURING LEAF DEVELOPMENT

Once the different domains of a leaf primordium are established, the leaf lamina expands to acquire its final size and shape. First, cell division occurs through-out the small leaf primordium, which can be visualized using reporters of mitotic cyclins such as *CYCLINB1;1-GUS*[52] (Figure 3). Dividing cells also grow, and the term proliferation is used to refer to this joint activity.[53] During this phase, cell growth and cell division are tightly regulated, so that the average size of the proliferating cells is maintained fairly constant.[54]

As the organ grows, the region containing proliferative cells becomes restricted to the base of the organ (Figure 3(a)). The cells located in the distal part of the developing leaf leave the mitotic cell cycle and begin their expansion, which is defined as cell growth without division. The proliferating cells in the leaf have just a fraction of their final size of the fully expanded cells[52–54] (Figure 3(b)). As a consequence of the ontological pathway of the leaf, a growing organ

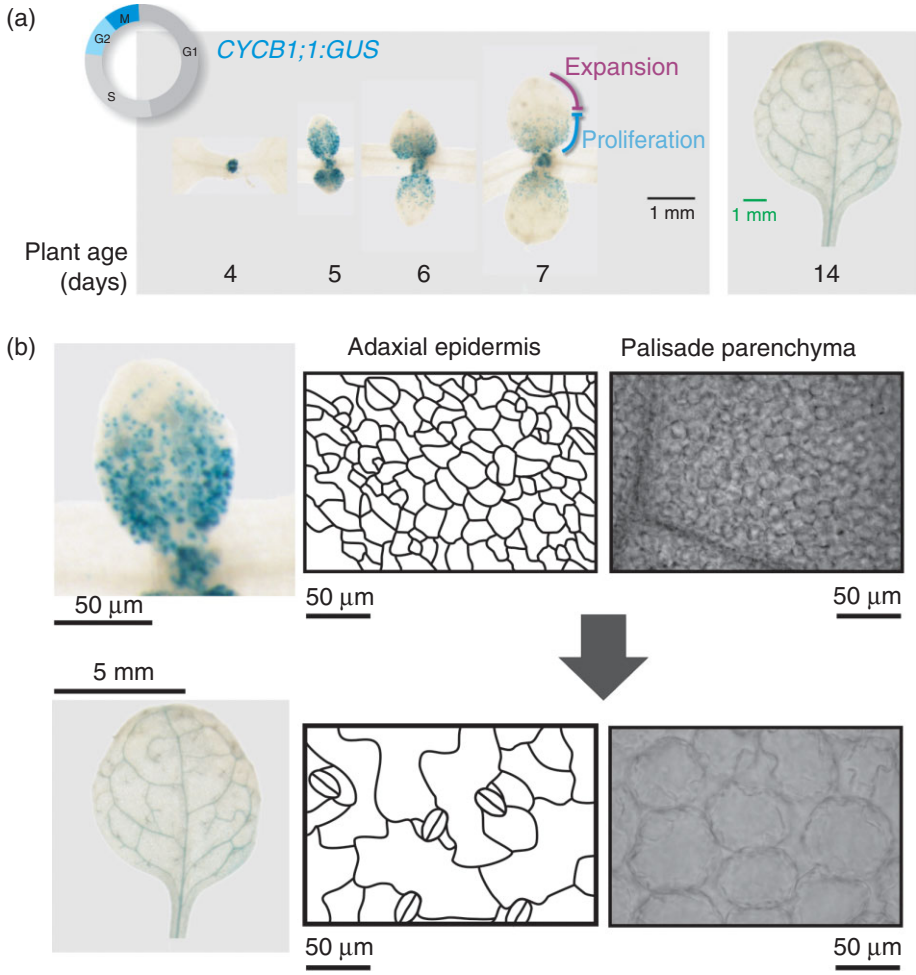

FIGURE 3 | Cell proliferation and expansion during leaf development. (a) Distribution of the cell proliferation and expansion phases in developing leaves of increasing ages. A *CYCLINB1;1*, (*CYCB1;1:GUS*) reporter labels cells in the G2-M phase of the cell cycle. Note that cell proliferation is restricted to the proximal part of the leaf, while cell expansion occurs in the distal part. (b) Magnitude of the cell expansion process during leaf maturation. Outline of adaxial epidermal pavement cells and paradermal view of palisade parenchyma cells in proliferating and mature leaves (Reprinted with permission from Ref 74. Copyright 2010 The Company of Biologists Ltd).

may contain small proliferative cells at the base, a gradient of expanding cells of different size along the proximo-distal axis and fully expanded differentiated cells at the tip (Figure 3).

Then, cell proliferation in the organ finally ceases rather abruptly and the leaf lamina continues to grow only by cell expansion. As proliferation first stops at the distal part of the leaf, it has been proposed that a mitotic arrest front moves from the tip to the bottom of the organ. The timing of the arrest front is critical to determine the final size of the organ and many factors are integrated to decide its onset and progression.

Although the concept of the arrest front might be interpreted as a steady decrease in size of the cell proliferation zone, current evidence indicates that the length of this region remains rather constant during a certain period of time until it rapidly disappears.[53,55] Therefore, cell proliferation in dicot leaves might be more similar to monocots than previously thought.[56] The final shape of the cells is acquired after complete expansion. For example, the jigsaw-shaped epidermal cells of a mature leaf are initially small and polygonal during proliferation, while the photosynthetic capacity of the leaf organ is only acquired after the arrest of mitosis.[52,53]

The arrest front can be divided in two temporally distinct components depending on the responding cells.[52,57] A primary arrest front affects the majority of cell types including the abaxial and adaxial epidermis, and the palisade and spongy parenchyma.[52,53] A secondary arrest front is temporally delayed and genetically distinct from the primary arrest front and is specific for meristemoids and procambial cells, which are the precursors of stomata and the vasculature,

respectively.[57] The contribution of the proliferation of meristemoid cells to the leaf area is not negligible, as many pavement cells derive from them in addition to the guard cells.[53,58]

In the small developing leaf primordium, cell proliferation is distributed rather homogeneously throughout the growing lamina leading to the enlargement of the whole blade. These divisions are mainly anticlinal (perpendicular to the leaf surface), generating, in this way, a flat lamina. It has been observed that the adaxial side of the *Arabidopsis* leaf requires more cell proliferation than the abaxial side, so that mutants that affect cell proliferation might in turn affect the polarity of the organ compromising the adaxial domain.[52,59–62]

GENETIC CONTROL OF CELL PROLIFERATION AND LEAF DEVELOPMENT

Leaf Development and the Cell Cycle

The progression through the different phases of the mitotic cell cycle (G1, S, G2, M) is the driving force for increasing cell number.[63] The core machinery controlling the cell cycle relies on the activity of cyclin-dependent kinases.[64] Many aspects and components of this core machinery are highly conserved among eukaryotes. In contrast, the enormous phenotypic variations are caused by the integration of these basic cell cycle mechanisms with the developmental program of each organism.[64] In this context, the final size and shape of a leaf might be related to the extension of the cell proliferation phase, the localization of the dividing cells in the growing organ and the orientation of the plane of division. Studies performed on *A. thaliana* identified many different genes and pathways that affect leaf development, including several transcription factor networks. However, the molecular mechanisms that link the different pathways with each other or to the core machinery of the cell cycle are still poorly understood.

The progression of the cell cycle through mitosis is controlled by the anaphase-promoting complex/cyclosome (APC/C), a largely conserved protein complex found in eukaryotes. This complex triggers the destruction of A- and B-type cyclins allowing the progression of the cell cycle.[64–66] Overexpression of *APC10*, encoding a subunit of the APC/C complex, causes a faster degradation of CYCLIN B1;1 (CYCB1;1) and an increase in the cell cycle rate, which is then translated into bigger leaves with more cells.[67] On the contrary, overexpression of inhibitors of cyclin-dependent kinases (ICK/KRP) causes a reduction in the numbers of cells.[68,69]

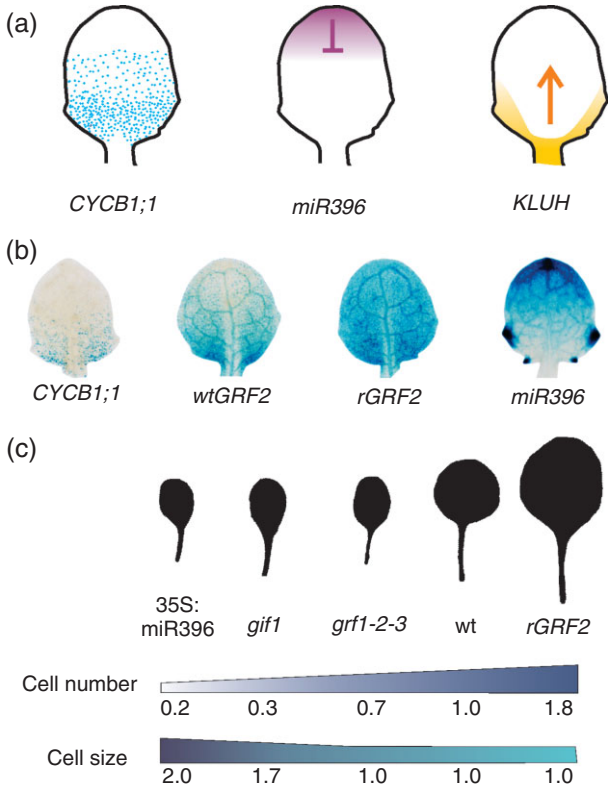

FIGURE 4 | Role of the miR396-*GRF-GIF* regulatory network in leaf growth. (a) Scheme showing the expression pattern of *CYCLINB1;1* (*CYCB1;1*), miR396, and *KLUH*. *CYCB1;1* indicates dividing cells; miR396 negatively regulates *GRF* transcription factors and therefore cell proliferation; and KLUH generates an unknown mobile signal that stimulates cell proliferation. (b) GUS staining in developing leaves #5 of transgenic plants harboring *CYCB1;1*, *wtGRF2*, *rGRF2*, and miR396, reporters. The *GRF2* reporter is sensitive to the posttranscriptional regulation by miR396, while *rGRF2* has mutations in the miRNA-binding site, so that it is resistant to miR396 action. (c) *GRFs* and *GIFs* promote cell proliferation. Silhouette of first leaves of plants overexpressing miR396 (35S:miR396) or *GRF2* (*rGRF2*) and knock-outs in *GRFs* or *GIF1* (also known as *AN3*). Palisade parenchyma cell number and size normalized to wild-type are indicated below each leaf. (Reprinted with permission from Ref 80. Copyright 2012 PLOS).

Control of Cell Proliferation by miR396 and the *GRF-GIF* Pathway

The GROWTH-REGULATING FACTORs are a family of plant-specific transcription factors defined by the presence of the conserved WRC and QLQ protein domains.[70–72] Mutations in *GRF5*[73] and a triple *GRF1-3* mutant[74] cause a reduction in leaf size (Figure 4). In contrast, overexpression of *GRF1*, *GRF2*, and *GRF5* results in bigger leaves with more cells.[71] The GRFs form complexes with GRF-INTERACTING FACTORs (GIFs), which are small proteins with homology to the human SYT transcriptional co-activator.[73,74] Mutations in

GIF1,[74] also known as *ANGUSTIFOLIA3* (*AN3*),[73] cause smaller leaves as a result of a reduction in the cell proliferation phase and a slowdown of the cell cycle rate.[75] Triple mutants of *GIF1-3* in *Arabidopsis* severely compromise the development of the whole plant and the shoot apical meristem[75] (Figure 4). In addition of being smaller than wild-type, leaves of *gif1 mutants* are also proportionally narrower. Interestingly, a specific decrease of division planes parallel to the proximo-distal axis is observed in *gif1* mutant leaves, which might subsequently cause the change in leaf shape.[61]

Seven out of the nine *Arabidopsis GRFs* have a binding site for miRNA miR396,[77] a target site present in the *GRFs* of many plant species.[77,78] Overexpression of miR396 causes a significant reduction of *GRF* expression and smaller leaves with reduced cell number,[60,76,79] while plants harboring a *GRF2* gene with mutations in the miR396 binding site have a higher level of the *GRF* transcript and an increased number of leaf cells[76] (Figure 4(c)). In young developing leaves, miR396 is expressed in a gradient along the longitudinal axis of the organ, with higher expression at the distal part. In turn, miR396 represses *GRF2*, generating an opposing gradient of expression with higher levels in the proximal part of the organ (Figure 4(b)). The posttranscriptional regulation of *GRF2* by miR396 results in the co-expression of the transcription factors with the cells undergoing mitosis,[76] while the presence of mismatches in the miRNA-target interaction ensure the quantitative regulation by miR396.[80] At later developing stages, miR396 is expressed in whole organs suggesting that it is a marker of cell differentiation.[76]

The *ARGOS-AINTEGUMENTA* Pathway

Factors controlling leaf development might also have a general role in the promotion of organ growth in plants. The AP2-domain transcription factor *AINTEGUMENTA* (*ANT*) was initially identified by its role in ovule development and floral organ growth,[81,82] while it was later shown to control leaf size too.[83] *ANT* and *ANT-like* transcription factors prolong the meristematic capacity of the cells during leaf growth. Mutations in *ANT* cause the cells to exit the mitotic cell cycle prematurely reducing the number of cells in the leaf, while an increase of *ANT* extends the proliferation phase.[83,84] *ANT* modifies the leaf size without altering the morphogenesis of the leaf.[83]

There is evidence linking the *ANT* pathway with auxin signaling. *AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE* (*ARGOS*), an auxin-inducible gene activates *ANT* by an unknown mechanism, which in turn activates *CYCD3* expression.[85] Plants overexpressing *ARGOS* or the homologous protein ORGAN SIZE RELATED PROTEIN1 have bigger leaves due to an extended proliferative phase, while mutations in these genes reduce leaf size.[86,87] In turn, the increase in leaf size caused by *ARGOS* requires *ANT*.[85] On the other hand, mutations in *AUXIN RESPONSE FACTOR2* (*ARF2*) cause pleiotropic phenotypic effects including bigger leaves due to an excess in cell proliferation.[88–90] Mature leaves and stems of *arf2* mutants ectopically express *ANT* and *CYCD3;1*, indicating that *ARF2* normally represses *ANT*.[90]

KLUH and a Potentially New Mobile Signal

Mutations in *KLUH* cause smaller leaves, sepals, and petals, while plants overexpressing *KLUH* have larger organs due to an increase in cell proliferation.[91,92] *KLUH* is expressed at the base of the developing organs and overlaps only partially with the proliferative zone of the leaf.[91,93] It encodes a putative microsomal cytochrome P450 monooxygenase and is one of the six members of the CYP78A family in *Arabidopsis* (CYP78A5). These proteins comprise a large superfamily of heme-dependent enzymes, known to be involved in the biosynthesis and modification of many different molecules, including gibberellins and brassinosteroids. That *KLUH* requires its enzymatic activity for its function and acts non-cell autonomously has led to the hypothesis that *KLUH* generates a mobile signal promoting cell proliferation.[91,93] The chemical nature of this mobile signal remains to be identified, although it has been suggested that is different from other known hormones.[91]

The Translation Machinery and Leaf Development

Many mutations in ribosomal proteins result in problems during leaf development, including defects in leaf polarity, cell proliferation and shape,[94–99] (recently reviewed in Refs 100 and 101). These data might indicate that early events in leaf development, such as the proliferative phase, demand an efficient translational machinery due to the constant need for proteins to sustain cell growth. However, the scenario is not so simple, as these mutations can affect one specific developmental process. For example, mutations in *RPS28* compromise leaf polarity, without affecting cell proliferation, while inactivation of *RPL4* causes opposite effects.[95] Therefore, a link between the ribosomal proteins and

the translation of specific sets of mRNAs cannot be ruled out.

CONTROL OF LEAF CURVATURE

The generation of a leaf as a flat organ is not a trivial process and the available data indicates that it is under genetic control.[102] Snapdragon leaves are normally flat but they become crinkled in *CINCINNATA* mutants.[102] *CINCINNATA* is a member of the TCP family, a plant-specific class of transcription factors known to participate in many developmental pathways (reviewed in Ref 103). In plants lacking *CINCINNATA*, the mitotic arrest front is delayed leading to increased cell proliferation especially at the leaf margins. The coordination of growth in the center and the periphery of the growing lamina is essential to generate a flat organ. The excess of cells in the periphery of the organ with respect to the center has been shown to be the cause of the crinkled leaves in *CINCINNATA* mutants.[102]

The *Arabidopsis* activation-tagged mutant *jaw-D* also has crinkled leaves[104] (Figure 5). The phenotype is caused by the insertion of viral enhancers that activate the transcription of the *MIRNA* gene *MIR319a*.[105] MiR319 guides the cleavage of the transcripts of five *TCP* genes that have homology to *CINCINNATA* of snapdragon. Overexpression of miR319 or multiple knock outs in the miR319-regulated *TCP* genes generate crinkled *Arabidopsis* leaves.[105–109] It has been suggested that strong down-regulation of these *TCP* genes also affects cell differentiation.[107] MiR319 is widely conserved and many species contain *TCP* genes with miR319-binding sites, including *CINCINNATA*.[78,110] High levels of the miR319-regulated TCP4, which is the possible ortholog of *CINCINNATA* cause smaller leaves due to a reduction in cell number.[76,106,107,111] Interestingly, plants with high TCP4 levels have an increase of miR396 and a decrease in the *GRF* transcripts, thereby, linking two miRNA regulatory networks in the control of cell proliferation and leaf morphogenesis[76] (Figure 5).

It has been predicted that an excess of cell proliferation in the center of the lamina with respect to the margins should generate dome-like structures.[102] Experimental manipulation of *ICK1*/*KRP1*, has shown that specific cell proliferation repression in the leaf margins altered the leaf curvature generating 3D structures.[112] On the other hand, mutations in *PEAPOD1* and *2* result in dome-shaped leaves.[57] It has been suggested that *PEAPOD* regulates the secondary mitotic arrest front causing an extended proliferation of stomata and vasculature precursors.[57]

CONTROL OF LEAF GROWTH TROUGH CELL EXPANSION

The final leaf size is the result of a combination of both cell proliferation and cell expansion, and there are interactions between these two processes. Many mutants with reduced cell numbers show a compensating increase of cell size (reviewed in Ref 113). For example, the decrease in cell number observed in mutants defective in *GRFs*/*GIFs* or plants with an increase in KRP2 is associated with an increase in cell size that partially compensates the organ size.[54,68,73,76]

A key aspect during cell expansion is the transition from the mitotic cycle to the endoreduplication cycle. In the mitotic cycle, cells pass through G1, S, and G2 before dividing in M phase and producing two daughter cells. In the endoreduplication cycle, the M phase is omitted and cells double their nuclear DNA content giving rise to cells with higher ploidy levels.[66] The transition from the mitotic to the endoreduplication cycle coincides with the onset of the period where cells only expands.[114] Usually, but not always,[68] there is a correlation between final cell size and nuclear ploidy level.[115] For example, mutations in *CYCD3;1* cause a premature exit from cell proliferation phase and reduced cell number, which is compensated by an increase in the endoreduplication and larger cell sizes. Conversely, leaves overexpressing *CYCD3;1* have smaller cells with reduced ploidy levels.[116]

Cell expansion is driven by turgor-pressure and its magnitude and direction is regulated by the biomechanical properties of the cell wall.[117] Expansins are plant cell-wall loosening proteins involved in cell expansion during various developmental processes.[118] Cell size is reduced in transgenic plants expressing an *EXP10* antisense transcript and increased when the gene is overexpressed.[119]

Hormone signaling also influences cell expansion during leaf development. For example, mutants of brassinosteroid biosynthesis and signaling develop small leaves mainly due to defects in cell expansion.[87,120] *ARGOS-like*, a protein with sequence homology to *ARGOS*, is induced by brassinosteroids and promotes cell expansion during leaf growth.[87] This is in contrast to ARGOS-mediated growth, which mainly affects cell proliferation. In turn, *CYP90C1*/*ROTUNDIFOLIA3* encodes a cytochrome P450 involved in brassinoesteroid biosynthesis that is required for longitudinal cell elongation.[121]

SCULPTING THE LEAF MARGINS

Leaf margins can be smooth (entire leaves), have small tooth-like indentations (serrated leaves), or large

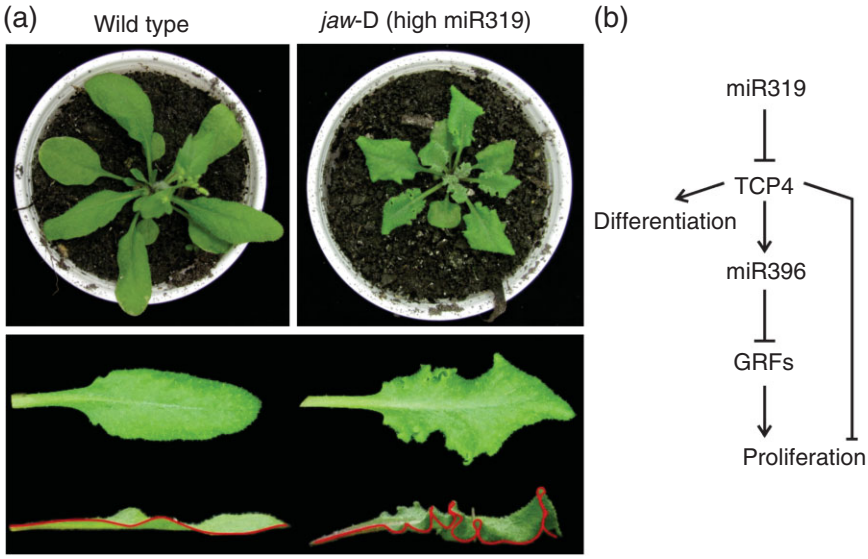

FIGURE 5 | Regulation of leaf curvature. (a) Overexpression of miRNA miR319, which negatively regulates TCP transcription factors as seen in the *jaw-D* mutant produces an excess of cells in the margins that causes crinkled leaves. (b) Integration of the miR319/TCP and miR396/GRF regulatory nodes in cell proliferation and differentiation.

outgrowths (lobed leaves) (Figure 1(c)). In particular, serrations are characterized by their number and depth, measured as the distance between the tip of the tooth and the sinus. The margins might even differ from between leaves of the same plant; as it is in the case for *Arabidopsis* (see below). The current evidence indicates that *KNOXI* and *CUC* transcription factors, which are required for meristem function, are recruited again during leaf development to control organ morphogenesis. *Arabidopsis* mutants that fail to repress *STM* and/or other *KNOXI* genes in the leaf primordia develop organs with outgrowths and lobes. Furthermore, both *KNOXI* and *CUC* transcription factors act in concert during the formation of complex leaves and lobed organs in many species.[17,122]

In *Arabidopsis*, *mir164a* mutants have deeper serrations than wild-type leaves and *cuc2* mutants produce leaves with smooth margins, indicating that the quantitative balance between miR164 and the *CUCs* is important to determine the presence and depth of the serrations.[39] Actually, many different transgenic plants and mutants with altered leaf shape recruit *CUC2* to generate serrations.[123]

PIN1-mediated polar auxin transport, which defines the position of leaf primordia initiation, also shapes leaf margins. This transport concentrates auxin at discrete places in the margins of developing leaves to promotes the outgrowth of the tooth of serrations.[124–126] Mutants in *PIN1* and treatments with chemical inhibitors of auxin transport produce leaves with smooth margins.[125] *PIN1* and *CUC2* expression alternates in the margins, with *PIN1*

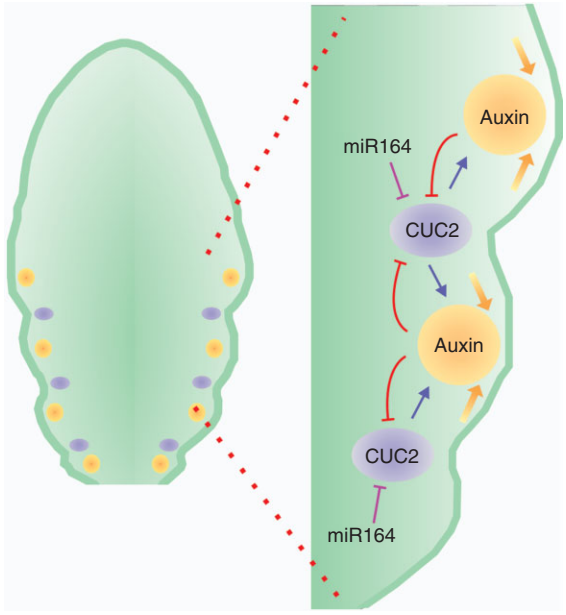

FIGURE 6 | Generation of leaf serrations. Scheme showing the interplay between *CUC2* and auxin in the formation of leaf serrations in *Arabidopsis* leaves. CUC2 promotes the establishment of PIN1 convergence points which in turn generate auxin maxima at the tip of serrations. The auxin maximum represses CUC2 at the serration tip and promotes tooth growth. Note that *CUC2* is regulated by miR164.

expression and auxin maxima at the tip and *CUC2* at the sinus of serrations[124] (Figure 6). This pattern is generated by a feedback loop that is critical for serration development. In this loop, *CUC2* promotes the establishment of *PIN1* convergence points to

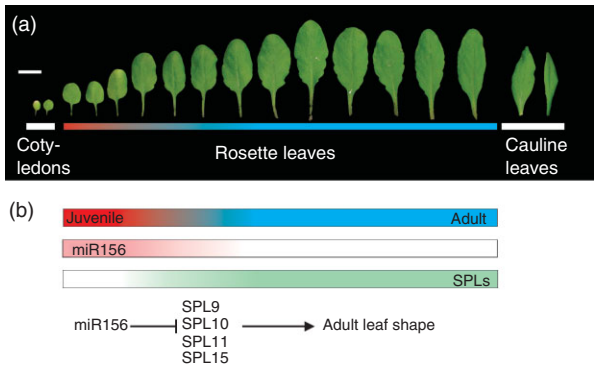

FIGURE 7 | Heteroblasty. (a) Cotyledons, successive true rosette, and cauline leaves in *Arabidopsis thaliana*. (b) Scheme showing the regulation of vegetative phase change by the miR156-mediated repression of *SPL* genes.

generate auxin maxima at the tip of serrations that promote tooth growth. In turn, the auxin maximum represses *CUC2* and induces miR164 expression at the serration tip[39,124] (Figure 6).

Interestingly, infections by Turnip mosaic virus and other viruses alter leaf development, and sometimes produce an increase in leaf serration. It seems that miss-regulation of the miR167-regulated *ARF8* during the viral infection is responsible of these serrations,[127] further confirming the role of auxin in the generation of different leaf shapes. In principle, sculpting the leaf margins might be achieved by the localized control of the cell proliferation machinery in specific regions of the leaf. In this context, expression of *ICK1/KRP1* trough different promoters generated leaves with different shapes.[112]

HETEROBLASTY

The developmental stage of the plant affects the size and morphology of the leaves produced by the shoot, a phenomenon called heteroblasty.[128–130] During vegetative development, *Arabidopsis* plants go through a juvenile-to-adult phase transition, and only plants in the adult phase have the competence to flower. Phase transition is manifested in changes in leaf morphology, as adult organs have trichomes also on their abaxial side, their shapes are more elongated and their margins contain deeper serrations than juvenile leaves[129,131–133] (Figures 1 and 7).

Both, intrinsic and environmental conditions, such as day length[134,135] can affect heteroblastic features, suggesting that there are multiple pathways involved in the process. A major regulator of the developmental timing in plants involved in the juvenile to adult transition is the miRNA miR156 network.[132,133,136–140] This miRNA regulates *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) transcription factors.[38,132] As the plant ages, miR156 levels decrease steadily, allowing for the concomitant accumulation of SPL proteins, which promote the adult traits.[132,139]

The identity of the *SPL* genes that mediate the effect of miR156 on vegetative phase change is difficult to establish because of the high degree of functional redundancy within this family. However, recent studies have shown that *SPL9* and *SPL10*, and their paralogues *SPL15* and *SPL11* respectively, promote most of the traits associated with the adult phase.[131,133,141]

VARIATIONS IN LEAF SIZE AND SHAPE AMONG *ARABIDOPSIS* NATURAL ACCESSIONS

Most of the genes identified as part of the developmental program of the leaf were isolated through genetic screens for plants with defects in organ growth. Nevertheless, variants in the architecture of leaves can be found in natural accessions of the same species. Allelic variants of certain genes involved in leaf development could explain this variation, which, in some cases, might have a role in the adaptation to the local environment.[142,143]

Previous work has characterized the natural variation in the morphogenesis of *Arabidopsis* leaves in 188 accessions.[142] Leaves were classified into 14 different groups according to their margins, lamina shape, and petiole (Box 1), demonstrating that there is a substantial variability in leaf architecture among *Arabidopsis* natural accessions, although the characterized changes were less extreme than those obtained in genetic screens. Studies performing genetic approaches, such as QTLs, have shown that mechanisms underlying natural variation in leaf morphogenesis have a multifactorial nature.[142,144–146]

While it is known that miR164 control leaf serrations in *Arabidopsis*, it has recently been found that differences in the sequences of the gene-encoding miR164a can underlie differences in leaf shape in *Arabidopsis* accessions.[148] Plants of the Col-0 accession with an introgression of the C24 *MIR164A* allele have leaves with deeper serrations due to a decrease in the levels of miR164 caused by a point mutation in the precursor of miR164a in C24.[148]

In some cases, natural variation in leaf size could reflect alternative ecological strategies. *ACCELERATED CELL DEATH 6* (*ACD6*) is a gene associated with differences in both, resistance to a broad range of pathogens and degree of vegetative growth. Some *A. thaliana* natural accessions have a hyperactive allele

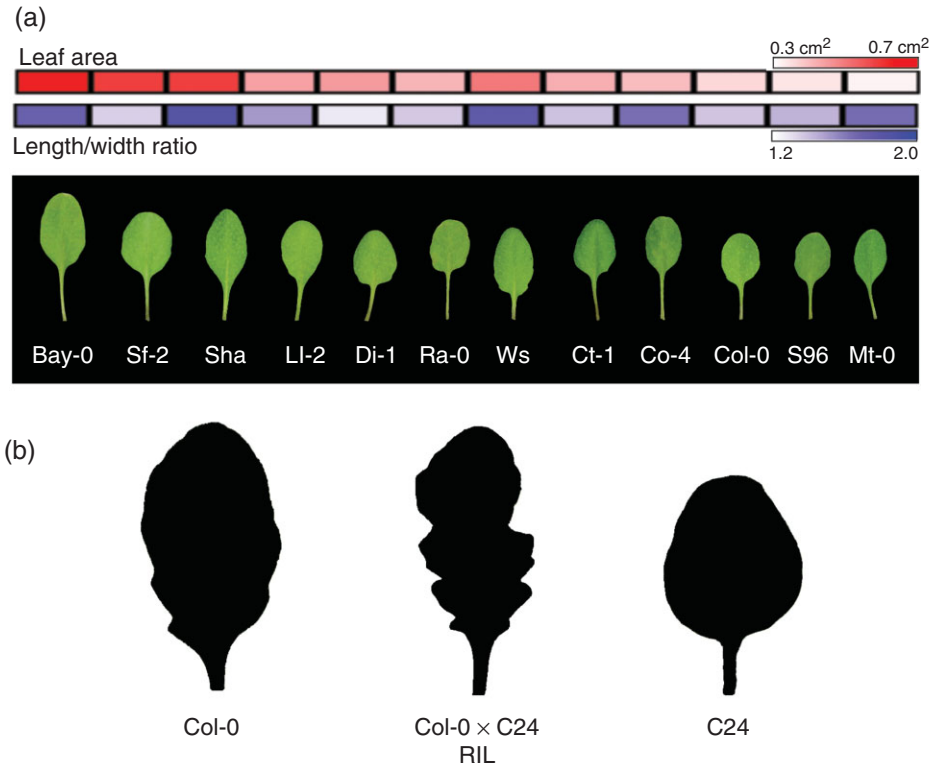

FIGURE 8 | Variation of leaf shape and size in *Arabidopsis* natural accessions. (a) Leaf #1 shape and size of 12 *Arabidopsis thaliana* accessions. The heatmaps represent the leaf area (top) or the length-to-width ratio of each accession. (b) A naturally occurring miR164 allele defective in the biogenesis of the miRNA found in the C24 *Arabidopsis* accession produces deeper serrations when introgressed in Col-0 plants (Col-0 × C24 RIL).

BOX 1

THE PETIOLE

In addition to the leaf lamina, most dicotyledonous leaves plants have a petiole, which is a stem-like structure that supports the leaf blade and attaches them to the stem (Figure 1(b)). There is variation in the presence and size of the petiole between and also within species (Figures 1, 7, and 8). In *Arabidopsis*, juvenile and adult rosette leaves have petioles of different length, while cauline leaves lack them.[129]

During the early steps of leaf primordia development there is no clear distinction of the proximo-distal axis. The petiole becomes first visible as a basal constricted region after primordia initiation. Recently, detailed studies using clonal analysis revealed that petiole cells originate in files from a proliferative region located in the junction between the blade and petiole.[147] The proliferative zone of leaf primordia then supplies leaf-blade cells acropetally (toward the tip) and leaf-petiole cells basipetally (toward the base).[147]

of ACD6 with enhanced resistance to pathogens, but the presence of this allele causes a penalty in organ development greatly reducing the biomass of mature leaves.[149] In this way, natural *Arabidopsis* accessions might choose between two alternative strategies: bearing small leaves well protected against pathogens or have larger organs that might be potentially more susceptible to pathogens.[149]

CONCLUSION

A large collection of genes that participate in leaf development and morphogenesis have been identified during the past years; however, the molecular mechanisms and relationships between them are only starting to be unraveled. The great advance of genomic approaches, especially those meant to identify genes directly regulated by transcription factors will certainly aid to the dissection of the different pathways. The impressive set of genomic tools and approaches to study natural variation will surely complement classic genetic approaches and will help to bring insights into mechanisms responsible for differences in leaf architecture.[143]

A key event during leaf development is the transition from cell proliferation to cell expansion and differentiation. The process seems to integrate many different pathways. Noteworthy, mobile signals might be involved in the process, such as that generated by *KLUH*.[55,91,150] The identification of these signals will certainly improve the current knowledge about plant development.

Most of the studies trying to understand the molecular mechanisms underlying leaf development have been performed in *A. thaliana*. Recent studies have, however, developed detailed strategies to study the *Zea mays* leaf combining genomic approaches with the dissection of the organ domains.[151,152] In this context, the maize leaf offers the advantage of a being a larger organ and different domains can be separated and further used for additional studies. Therefore, these studies might provide results at single organ resolution, which are difficult to achieve with the small *Arabidopsis* leaves. It might be possible that future breakthroughs in the field will come from additional model systems other than *A. thaliana*.

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