Chapter 4

LEAF DEVELOPMENT IN VASCULAR PLANTS

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ABSTRACT

The most versatile organs of extant vascular plants are leaves, along with their modifications. Because leaves vary in form, function, and development, several hypotheses have arisen to explain how leaves have developed throughout evolution. Therefore, to consolidate current thought, this chapter will unite classic morphological and anatomical data with the most recent advances in molecular biology to understand the different aspects of leaf ontogeny during the evolution of lycophytes, ferns, and seed plants. Accordingly, we will discuss the concept of leaf and its inseparability from the shoot system, as well as their overlapping regions and developmental mechanisms. We further address the initiation of primordia, leaf base and blade delimitations and concepts, leaf base modifications, including those involving petiole, stipules, ligules, and sheaths, growth regions during leaf ontogenesis and, finally, abaxial-adaxial polarity and determinacy. Finally, in our concluding remarks, we

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will indicate perspectives, including possible future directions toward filling the gaps in our knowledge about this important plant organ.

Keywords: indeterminate growth, leaf base, leaf morphogenesis, simple and compound leaves

INTRODUCTION

Flattened lateral structures are widespread in photosynthesizing organisms, indicating that they may be a very efficient way of receiving light by allowing rays to penetrate the cells (Fahn 1982; Plackett, Di Stilio, and Langdale 2015). Although they are present in many different clades of macroalgae (brown, red, and green algae) and bryophytes (liverworts and mosses), we frequently remember only of leaves present in the vascular plant's sporophytes. While such arcane terminology is still used mainly by tracheophyte botanists, many phycologists and bryologists now use terms like leaves, fronds or laminae for different organs of nonvascular plants (Shaw and Renzaglia 2004; Plackett, Di Stilio, and Langdale 2015), even though tracheophytes are the largest group in number of species and have unique leaves that deserve special attention.

The following may be considered general features present in the leaves of tracheophytes: (a) vascularization, (b) determined growth, (c) adaxial-abaxial flattening, and (d) definite arrangement around the axis (phyllotaxy) (Dengler and Tsukaya 2001; Tomescu 2009). However, the presence of these features in such groups of vascular plants as lycophytes, ferns, gymnosperms, and angiosperms is not final evidence that these leaves are completely homologous. In addition, a cautious look at the diversity of vascular plants will quickly reveal many exceptions to these features, e.g., avascular, indeterminate, or cylindrical leaves.

Leaves of vascular plants are diverse in form, function and evolutionary history; therefore, it is reasonable to expect substantial variation in their developmental program. Still, basic patterns are already known for many model plants, like *Arabidopsis*, tomato, and maize.

Indeed, an understanding of the basic aspects of leaf development is a good starting point to grasp the impressive variation within tracheophytes. In this chapter, we will focus on the evolution and developmental aspects available for this group.

EVOLUTION IN VASCULAR PLANTS

Given their variation in form, development and function, a frequent question that arises about tracheophytes involves homology. Different hypotheses support one, two, three or more origins of leaves in vascular plants (as reviewed by Vasco, Moran, and Ambrose 2013). Each is supported by different structural, ontogenetic, or paleontological datasets. Most researchers concede that leaves have evolved at least twice, once in the ancestors of lycophytes (microphylls) and at least one more time in the common ancestor of ferns and seed plants (megaphylls) (Gifford and Foster 1989; Tomescu 2009; Tsukaya 2014).

According to classical definitions, the microphyll is a small structure with a single nonramified vein, and its vasculature is connected to a stele that lacks parenchyma leaf gaps. Megaphylls, on the other hand, are big with ramified venation, and their leaf traces leave parenchyma gaps in the stele. Many exceptions to these definitions have emerged, and the concepts seem equivocal, partially overlapping and phylogenetically inconsistent (Gifford and Foster 1989; Tomescu 2009). Even though ferns and seed plants are still a well-supported monophyletic group with leaves that share complex and unique mechanisms of development (Vasco, Moran, and Ambrose 2013; Ambrose and Vasco 2016; Vasco et al. 2016).

The enation theory is most often used to explain the origin of microphylls. This holds that leaves evolved as new structures after the vascularization of sterile emergences on the surface of a leafless type of axis (Gifford and Foster 1989; Crane and Kenrick 1997). Another competing, albeit less popular, model of microphyll evolution supports the sterilization hypothesis. In this case, microphylls are not *de novo* structures, but rather, sterilized sporangia (Crane and Kenrick 1997).

Recent molecular data demonstrated that leaves in lycophytes may have co-opted both branching and sporangial molecular mechanisms of development, reinforcing their origination from pre-existing structures, possibly sterilized sporangia (Vasco et al. 2016).

The telome theory, as proposed by Walter Zimmerman, is often used to explain the origin of megaphylls (Zimmermann 1952; Claßen-Bockhoff 2001; Beerling and Fleming 2007). The ancestors of vascular plants possibly looked like the fossil species *Rhynia*, a single-species genus of Devonian vascular plants, which had leafless and dichotomously branched axes. The distal protostelic branches able to produce sporangia are the telomes. The telome theory basically proposes a process of modification of these structures, ultimately transforming them into megaphylls. The steps necessary to make this possible may be summarized as follows. First, some telomes take a lateral position after being overtopped by others that are more dominant and grow farther. Second, planation occurs in which the lateralized, overtopped telomes are disposed horizontally in a bidimensional way. Third, fusion, or webbing, allows tissue to grow between the telomes, thus creating the leaf blade with a branched vascular system. Such steps and the leaf patterns thereof can be detected in several extinct species (Corvez, Barriel, and Dubuisson 2012). Molecular data have demonstrated mechanisms that explain the processes of overtopping and planation. However, only limited evidence is available for the webbing process that can possibly be substituted by lateral outgrowth during the formation of the blade (Beerling and Fleming 2007).

Ontogeny

A new leaf primordium is developed from a specific site in the periphery of the shoot apical meristem (SAM) (Figure 1) that receives the highest concentration of auxin hormone (auxin-maximum area). In most vascular plants, Class I KNOTTED HOMEOBOX (Class I KNOX) genes responsible for indeterminacy maintenance are usually expressed in the SAM. These genes are downregulated at the site of a new primordium in

order to allow the emergence of a determined organ (Uchida et al. 2010). As the new primordium develops, a new SAM site will become the next auxin-maximum area, and the process repeats. Each such cycle has come to be known as a plastochron, i.e., the emergence of leaf primordia at any two successive nodes in a stem apex. During this period, we see different arrangements of leaves around a shoot (phyllotaxis) that may be classified as opposite, distichous, decussate, alternate, verticillate, or spiral.

For didactic purposes, leaf morphogenesis can be better described in three phases: initiation, primary morphogenesis and secondary morphogenesis (Dengler and Tsukaya 2001). The growth of leaf primordium also involves different regions with meristematic activity, like apical and intercalary meristems, responsible for proximodistal growth in the length of leaf axis, as well as marginal and plate meristems responsible for the production of the leaf blade. The axis also increases in thickness, often through the activity of a strip of cells beneath the adaxial protoderm, the adaxial meristem (Figure 2).
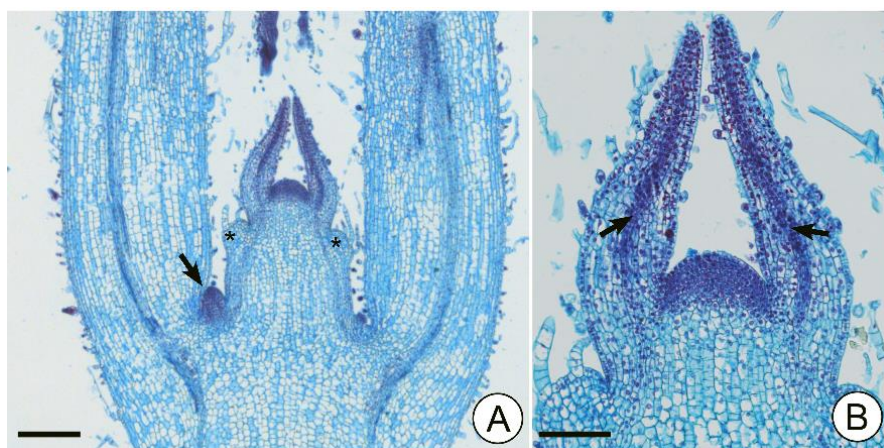

Figure 1. Longitudinal sections of the shoot apex of *Plectranthus scutellarioides* (L.) R. Br. (Lamiaceae). A. Axis with decussate phyllotaxis (successive opposite pairs that are 90 degrees apart). Notice the leaf bases of the second pair of young leaves (asterisks) and the axillary bud of one leaf of the third node (arrow). B. Detail showing the SAM and two developing leaves with procambial strands (arrows) establishing the vascular connection with the stem. Bars: 250µm (A), 100µm (B).
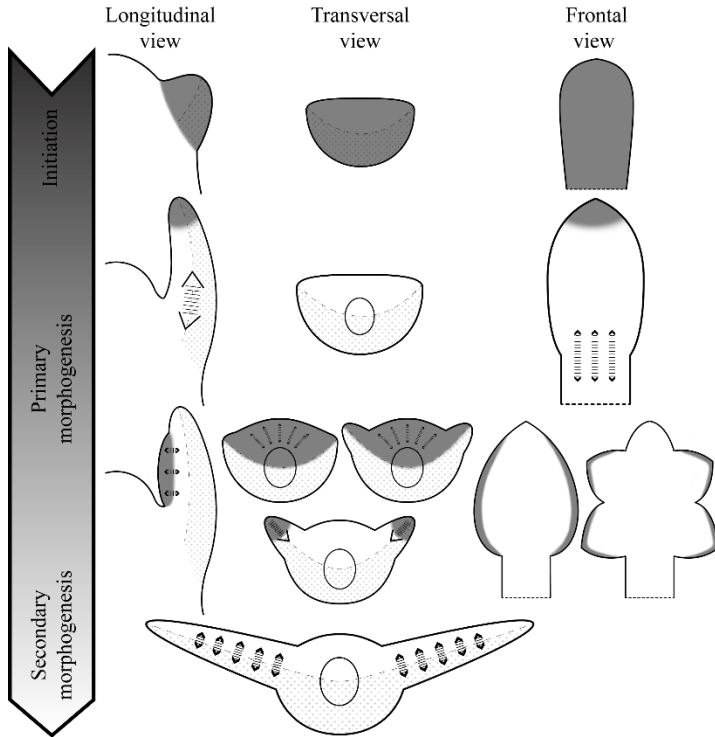
Figure 2. Phases of leaf development showing longitudinal, transverse and frontal views of the primordium. Dashed lines indicate the boundary between adaxial and abaxial domains. Grey regions represent the different growth regions, beginning with apical meristematic activity, followed by intercalary meristem activity, which promotes proximodistal growth. Growth in the adaxial-abaxial axis occurs from the adaxial meristematic region. After this phase, the meristematic region is restricted at the juxtaposition of adaxial and abaxial domains, resulting in activity of the marginal meristem, which, together with the plate meristem (arrows), will form the leaf blade. Illustration by Yasmin V. Hirao.

The new leaf primordium grows along proximodistal, mediolateral, and adaxial-abaxial axes (Hibara et al. 2009). Genetic and molecular studies have demonstrated that the development of each axis is regulated by different mechanisms (Bowman, Eshed, and Baum 2002; Chitwood et al. 2007; Ishikawa et al. 2009; Nakayama, Yamaguchi, and Tsukaya 2010).

Initiation begins with an initial lateral protrusion of the axis. The development of this new leaf primordium commonly results from

periclinal divisions in the flank of the SAM. These divisions occur in one or more of the layers near the surface, or in the superficial layer, as in certain grasses (Esau 1953). In this phase, apical growth of the primordium occurs, and it is typically short in duration. The remaining proximodistal growth results from intercalary meristematic activity. The resulting peg-like structure has a well-delimited adaxial-abaxial polarity that is defined during the earliest stages of leaf development (Waites and Hudson 1995; Reinhardt et al. 2005), while the leaf primordium acquires its dorsiventral symmetry even earlier. The side that is closest to the SAM is, by definition, the adaxial side, and the opposite side is the abaxial side. Abaxial and adaxial domains are structurally distinct in that the adaxial face is usually flattened, while the abaxial face is convex. In this phase, the upper and the lower zones are established in the primordium from which the free region of the leaf and the constitutive base will be respectively formed. Foliage leaf is united to the shoot at the constitutive leaf base (Figure 3). In other words, it is the part of the primordium which, individually, or with bases of other leaves, forms the mantle of the shoot axis (Mitra and Majumdar 1952; Majumdar 1955).
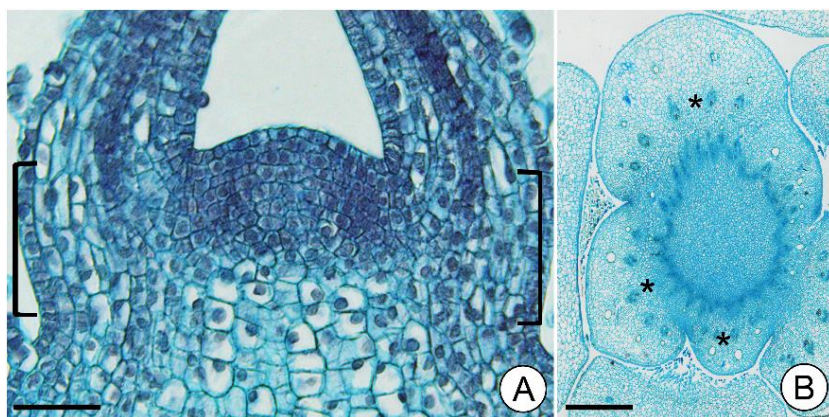

Figure 3. A. Longitudinal section of shoot apex of *Plectranthus scutellarioides* (L.) R. Br. (Lamiaceae) with two young leaves and their constitutive bases. B. Transverse section of the shoot apex of *Senecio sakamaliensis* (Humbert) Humbert (Asteraceae), showing the shoot axis formed by the constitutive base of different leaves (asterisk). Bars: 50μm (A), 500μm (B).

During primary morphogenesis, differential activity of the adaxial, marginal, and plate meristematic regions in the upper region of the primordium establishes two important regions of the foliage leaf: the blade (or leaf lamina) and the free base (i.e., petiole and other basal modifications).

Under the stereomicroscope, most leaves present distinct and visible modifications of the base. The most recognizable feature of a leaf base is the petiole since most megaphylls are not sessile, i.e., the blade directly fixed in one place. In angiosperms, some of the cells that make up the leaf blade come from the basal region located at the actual base of a monocot leaf (Tsukaya 2014) or at a junction between the blade and the petiole, as in *Arabidopsis* (Ichihashi et al. 2011). In most non-monocotyledon species, the proximodistal growth of the primordium that began during the initiation phase keeps occurring during primary morphogenesis, mostly by intercalary cell divisions and expansion since apical meristematic activity had already stopped earlier in most leaves. This growth generates a region that will become both the petiole and the midrib. Although the midrib region will experience marginal growth at a later time, most petioles limit their growth to the proximodistal axis and some radial increase (Foster 1936). Most petioles are cylindrical or subcylindrical, with some exceptions (e.g., winged petioles). Usually, the region of petiole differentiation experiences intense adaxial meristematic activity that promotes an increase in the leaf's basal diameter, becoming more cylindrical. Apart from adaxial growth, some species with winged petiole show mediolateral growth through the activity of the marginal meristematic region. The petiole is attached to the shoot axis through the constitutive base, and its growth occurs later by intercalation between the blade and the base.

During secondary morphogenesis, the growth of the leaf blade, which was initiated in the earlier stages by elongation of the leaf axis (Foster 1936), is followed by marginal meristem activity positioned at the juxtaposition of adaxial and abaxial domains (Steeves and Sussex 1989; Donnelly et al. 1999; Rolland-Lagan 2008; Tsukaya 2014; Fukushima and Hasebe 2014). Expansion and differentiation occur during this phase and

last longer than primary morphogenesis. During this process, the basic form of the primordium at the end of primary morphogenesis can be retained (isometric growth) or may result in differences in its proportions (allometric growth) (Dengler and Tsukaya 2001).

The morphological diversity of the leaf blade, e.g., lobate and non-lobate leaves, as well as different degrees of lobation, is a reflection of the complexity, duration, and mode of marginal growth activity. Some authors have stated that marginal growth is short in duration (Avery 1933; Denne 1966), while other authors have argued that such growth takes place over a longer period of time in the meristematic region (Maksymowych and Erickson 1960; Maksymowych and Wochok 1969; Poethig and Sussex 1985). Thus, it appears that an understanding of the cellular mechanisms underlying marginal growth remains controversial. This marginal meristematic activity occurs in two steps. First, we see periclinal division of submarginal cells and anticlinal divisions of marginal cells, mostly without cell expansion. Thereafter, we see a prolonged growth phase that arises via plate meristem in which cell divisions are predominantly anticlinal with cell expansion (Foster 1936). However, according to Maksymowych and Wochok (1969), in *Xanthium*, "the marginal meristem can be defined as a group of cells which initiates the leaf blade by forming five or six basic cell layers, thus giving rise to the plate meristem". Periclinal, anticlinal and oblique cell divisions of the plate meristem form epidermal, mesophyll and vascular cell layers, thus allowing the lamina to grow in thickness (Maksymowych and Wochok 1969). Analysis of cytological chimeras revealed that the epidermal layers of the leaf are clonally related, whereas the mesophyll and vascular bundles are derived from subepidermal layers (Baur 1908; Avery 1933).

Molecular studies in *Arabidopsis thaliana* (L.) Heynh leaves show that two encoding WUSCHEL-RELATED HOMEOBOX (WOX) transcription factors, PRESSED FLOWER (PRS)/WOX3 and WOX1, act in leaf blade outgrowth and that their expression defines a middle domain, including two middle mesophyll layers and the margin (Nakata et al. 2012). In a recent study, Alvarez et al. (2016) demonstrated that NGATHA (NGA) and CINCINNATA-class-TCP (CIN-TCP) transcription factors act

redundantly in *Arabidopsis thaliana*. This occurs shortly after leaf initiation to gradually restrict activity in the marginal and basal domains of the primordium. The absence of these transcription factors confers persistent marginal growth to leaves.

Marginal meristematic activity is a key feature to understanding leaf development. To repeat, lamina outgrowth through marginal activity occurs at the juxtaposition of adaxial and abaxial domains (Steeves and Sussex 1989; Donnelly et al. 1999; Rolland-Lagan 2008; Tsukaya 2014; Fukushima and Hasebe 2014), and its mitotic activity can be long-lasting, short or absent, resulting in wide flattened lamina or cylindrical leaves, respectively (Esau 1960; Yamaguchi, Yano, and Tsukaya 2010; Yamaguchi, Nukazuka, and Tsukaya 2012; Tsukaya 2014).

In *Antirrhinum majus* L. PHANTASTICA (PHAN) loss-of-function mutants, leaves develop such that adaxial-abaxial polarity is entirely lost without the development of the lamina, and "abaxialized" leaves are radially symmetric (Waites and Hudson 1995, Figure 4). However, some unifacial leaves develop flattened lamina, and this suggests that the regulation of lamina outgrowth is not completely identical to that of the adaxial-abaxial juxtaposition system in bifacial leaves (Yamaguchi and Tsukaya 2010).
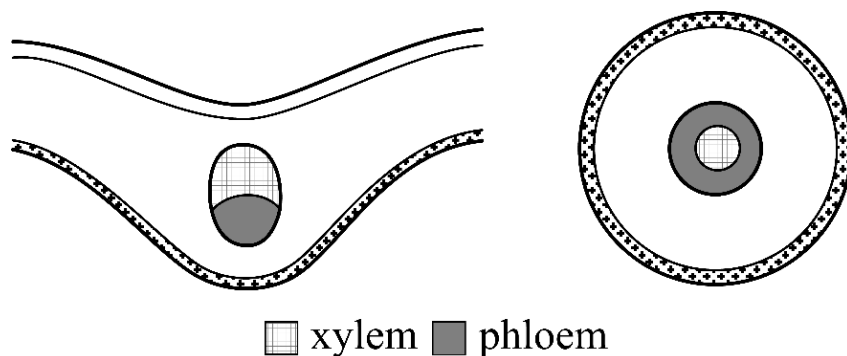

Figure 4. Schematic illustration of leaves (transversal sections) in wild-type (A) and PHAN mutant (B) of *Antirrhinum majus*. Dashed lines indicate abaxial side. Note the collateral vascular bundle in A and amphicribal bundle in B. Based on the results of Waites and Hudson (1995). Illustration by Yasmin V. Hirao.

The terms unifacial and bifacial are used to describe how many faces (sides) are in the blade. Unifacial blades have only one face (adaxial or abaxial domains) in contrast to bifacial blades that have two faces, each with its own specific organization (Kaplan 1970, 2001; Tsukaya 2014). However, studies have shown that leaf primordia, including those described as unifacial leaves, still retain adaxial-abaxial polarity at their bases (Troll and Meyer 1955; Hagemann 1970; Ozerova and Timonin 2009; Melo-de-Pinna et al. 2016; Ogura, Hernandes-Lopes, and Melo-de-Pinna 2018). Since this adaxial-abaxial polarity occurs at the earlier stage of leaf development, irrespective of unifacial or bifacial lamina, it is possible that the molecular mechanism involved in the establishment of the adaxial-abaxial domains is conserved in leaves and that this polarity can be lost during lamina development. In some succulents with unifacial leaves and cylindrical leaf blades, a peripheral growth region was named as "rounding meristem" by Troll and Meyer (1955), while it was called "peripheral meristem" and "peripheral blastozone" by Boke (1944) and Hernandes-Lopes, Oliveira-Neto, and Melo-de-Pinna (2016), respectively. These leaves show a peripheral vascular system with collateral bundles that can be endoscopic wherein xylem cells are oriented toward the center or exoscopic wherein xylem cells are oriented toward the surface. In leaves with peripheral endoscopic vascular bundles, peripheral growth begins at the abaxial side toward the adaxial side, while in leaves with exoscopic bundles, growth begins at the adaxial side toward the abaxial side, resulting in lamina with abaxialized and adaxialized feature, respectively. This peripheral growth must be a variation of the activity of the marginal and plate meristematic region, and this may be related to a possible loss of adaxial-abaxial polarity during lamina growth.

STRUCTURAL MODIFICATIONS OF THE LEAF BASE

The anatomy of a petiole resembles stem anatomy in most tracheophyte species, although bilateral features are evident in relation to the well-defined polarity of the primordium during leaf development. The

stelar anatomy of a megaphyll petiole is very diverse, but mostly exhibits siphonostelic organization formed by bundles, rings or arcs of vascular tissue with parenchymatic pith (Radford et al. 1974). This organization allows the petiole of woody plants to have a vascular cambium installed, and a petiole may present a limited amount of secondary xylem and phloem. The presence of a phellogen, or secondary meristem, is also frequent in these species, and their older petioles may have cork substituting for the epidermis. Fern petioles, also known as stipes, may even have a more diverse organization of the vascular system that is directly related to a diversity of steles in the group. This anatomical diversity in fern petioles is taxonomically important for the group and may help with field identification since bundles and meristeles are often visible to the naked eye (Ogura 1972).

Other important outgrowths of the leaf base may be described as stipules, sheaths, and ligules (Figure 5), even though further modifications may be present, such as ocreas in Polygonaceae and pulvini in Fabaceae.
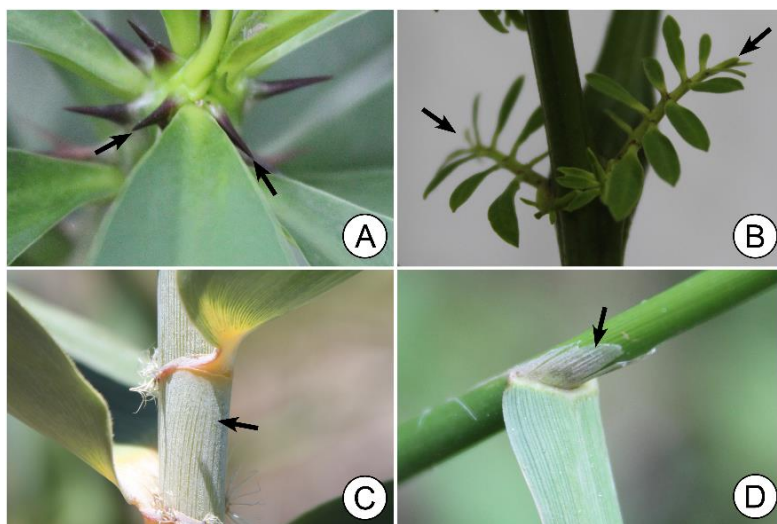

Figure 5. Structural modifications of the leaf base. A. Pair of spinescent stipules (arrows) in *Euphorbia milii* Des Moul. (Euphorbiaceae). B. Compound stipules (arrows) in *Delonix regia* (Bojer ex Hook.) Raf. (Fabaceae). C. Sheathing base (arrow) in Poaceae. D. Ligule in Poaceae.

Groups of cells are recruited from the primordium flanks to become stipules or sheaths, possibly after the determination of abaxial-adaxial juxtaposition (Townsley and Sinha 2012). However, this recruitment seems to be more associated with adaxial identity. Mutations that abaxialize the leaf can lead to the inhibition of sheath formation, and adaxialized mutants of *Arabidopsis* can bear ectopic stipules (Townsley and Sinha 2012).

Stipules are very frequent in rosids, but not restricted to them (Stevens 2001 onwards). Particularly in this group, they may seem to be directly attached to the stem, but they are actually outgrowths of the constitutive leaf base. Stipules, which are usually developed from paired primordia at the leaf base, may either remain paired or undergo alterations during development by becoming fused and thus conferring protection to the buds (Cruz et al. 2015). The resulting morphology may be leafy and green, or even divided (e.g., the Leguminosae *Delonix regia* (Bojer ex Hook.) Raf.), and in this case, they reiterate part of the development of the plant's compound leaves. They also may be modified into secondary defensive structures like spines, as in some *Euphorbia* species, or bear extrafloral nectaries that attract defensive ants, such as some *Passiflora* species.

Sheaths are one of the most distinguishable features of monocotyledons. A sheathing base is a more or less tubular structure that surrounds part of the stem. Since most monocot leaves show basipetal development, blade differentiation precedes sheath elaboration in a crescent-shaped primordium, even though the sheath already seems present in a very young primordium (Conklin et al. 2019). Meristematic activity becomes restricted to the base, and the sheath is elaborated, surrounding the shoot axis (Fahn 1982). It is interesting that some fused stipules may form a sheath-like structure, and as a result, some of these structures may be difficult to define, requiring a developmental study for correct classification (Cruz et al. 2015).

Ligules are usually small membranous flaps, but may be reduced and hair-bearing (ciliated ligules). They are usually described for monocotyledon groups, e.g., Arecaceae, Cyperaceae, Poaceae, and Zingiberaceae, and they are always associated with the upper and abaxial part of the sheath. They are also described for the reproductive microphylls

of *Selaginella* and *Isoetes*. Because they are present in such different groups, it is clear that these structures are not homologous for lycophytes and monocotyledons. In Poaceae, ligules are developed from a band of small cells transversal to the proximodistal axis of the developing leaf (Johnston et al. 2014). A surprising number of genes related to leaf initiation and lateral branching have been shown to express during ligule development, suggesting the co-option of these developmental mechanisms (Johnston et al. 2014).

SIMPLE AND COMPOUND LEAVES

An important theme concerning determinacy control in leaves is the presence of simple and compound (also known as complex or divided) forms. A mainstream view holds that compound leaves derived from a deeply lobed morphology. Such view is mainly based on a very restrictive and frequent interpretation of the leaf as a structure totally independent from the stem (Claßen-Bockhoff 2001).

However, it is notable that a leaflet is similar to a simple leaf, usually bearing a midvein and a flattened blade, or even modifications at the base, e.g., petiolules and stipels. Therefore, an alternative interpretation of this structure would describe it as a reduced shoot with an axial rachis equivalent to a stem and lateral flattened leaflets equivalent to a simple leaf. This concept is called "identity in parallel," and it was proposed by the British scientist Agnes Arber (Arber 1950; Sattler 1996; Rutishauser and Isler 2001).

Strong morphological, anatomical and molecular evidence suggests that compound leaves are actually reduced shoots. Some plants, like the garden rose and *Murraya exotica* (L.) Jack., bear pinnately compound leaves. However, just before their branches start to produce flowers, as determined structures, the number of leaflets decreases, and these plants eventually start to produce simple leaves (Efroni, Eshed, and Lifschitz 2010). In ferns, this similarity is even more evident since they present long-lasting apical meristems, very similar to shoot apical meristems, in

their developing leaves, that are usually pinnate (Vasco, Moran, and Ambrose 2013). Arber consolidated this evidence to then consider a compound leaf as a partial shoot since it probably uses developmental mechanisms that are present in both stems and leaves (Arber 1950).
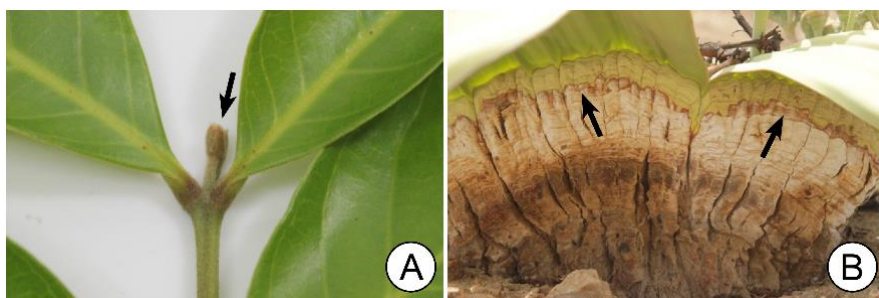

Figure 6. Leaves with indeterminate growth. A. Terminal bud (arrow) in a pinnate compound leaf of *Guarea macrophylla* Vahl. that continuously produces new pinnae. B. Basal generating regions (arrows) of *Welwitschia mirabilis* Hook. f. leaves that continuously provide new cells. These two simple leaves are the only ones that this plant will have during its entire lifetime that can last for centuries.

Genes from Class I KNOX, as previously mentioned, are known to maintain meristematic identity in angiosperm meristems. One example is SHOOT MERISTEMLESS (STM) of *Arabidopsis thaliana*. STM is downregulated in meristem regions where a simple leaf primordium will arise, delimiting a group of cells with a determined fate. However, in many plants with compound leaves, Class I KNOX expresses in the leaf primordium, thereby allowing it to reduce its determinacy and maintain prolonged meristematic activity in order to create lateral leaflets. Some examples of plants that have this system are ferns, tomato and *Cardamine hirsuta* L. (Bharathan et al. 2002; Hay and Tsiantis 2010; Bar and Ori 2015; Ambrose and Vasco 2016).

The genera *Chisocheton* and *Guarea* (Meliaceae, Figure 6A) and ferns of the genera *Lygodium* (Lygodiaceae) and *Salpichlaena* (Blechnaceae) bear long compound leaves of indeterminate growth and persistent apical meristems, indicating SAM-like activity of their apices (Steingraeber and Fisher 1986; Fisher 2002; Vasco, Moran, and Ambrose 2013; Tsukaya

2014). Indeterminate leaves, however, are not restricted to compound leaves. The gymnosperm *Welwitschia mirabilis* Hook.f. (Welwitschiaceae) has only two indeterminate and persistent leaves that are simple and grow for years using cells provided by a meristematic region of their bases (Figure 6B) that keeps expressing Class I KNOX genes (Pham and Sinha 2003).

CONCLUSION

The abundant variety of leaf forms is a consequence of the complexity of interconnected developmental pathways after millennia of evolutionary selection in vascular plants. From both molecular and morphological perspectives, model plants have proven very useful for identifying the basic mechanisms of leaf ontogeny. While much is known about leaf development in vascular plants, much remains to be explored, perhaps arguing for an integrative approach to morphoanatomy, molecular biology, and evolutionary biology, as well as the recruitment of a new generation of botanists equipped to accomplish that mission within the plant sciences.

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