

Megaphylls, microphylls and the evolution of leaf development

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Originally coined to emphasize morphological differences, ‘microphyll’ and ‘megaphyll’ became synonymous with the idea that vascular plant leaves are not homologous. Although it is now accepted that leaves evolved independently in several euphyllophyte lineages, ‘megaphyll’ has grown to reflect another type of homology, that of euphyllophyte leaf precursor structures. However, evidence from the fossil record and developmental pathways fails to indicate homology and suggests homoplasy of precursor structures. Thus, as I discuss here, ‘megaphyll’ should be abandoned because it perpetuates an unsupported idea of homology, leading to misconceptions that pervade plant biology thinking and can bias hypothesis and inference in developmental and phylogenetic studies. Alternative definitions are needed that are based on development and phylogeny for different independently evolved leaf types.

The microphyll–megaphyll dichotomy

In vascular plant sporophytes, leaves are lateral appendages that share four defining features: vascularization, determinate growth, bilateral symmetry (adaxial–abaxial polarity; hereafter referred to as ad/abaxial polarity) and definite arrangement (phyllotaxis). Despite these shared characteristics, leaves are not homologous across all vascular plants. An early step toward the recognition of this situation [1] was the defining of a major dichotomy between microphylls, small and simple leaves, and megaphylls, larger and more complex leaves. The dichotomy, based on morphological criteria, was reinforced as subsequent progress in paleobotany led to the realization that vascular plant phylogeny was itself divided by a major dichotomy [2], dating back ~415 million years to the Late Silurian–Early Devonian, that paralleled, to some extent, the taxonomic distribution of the two leaf types. The two major lineages of that phylogenetic divide originated from among two distinct grades of early vascular plants, the zosterophylls and the trimerophytes. The descendants of those two lineages form the two clades that comprise most vascular plant phylogeny, the lycophytes and the euphyllophytes, and which were regarded as having evolved microphylls and megaphylls, respectively. These ideas provided the impetus and framework for an early wave of thinking that would later become known as evo-devo, which led to the formulation of seminal hypotheses of leaf evolution.

However, the microphyll–megaphyll divide is not as clear cut, and morphological definitions that contrast microphylls and megaphylls as mutually exclusive concepts of leaves are inconsistent. Moreover, current understanding of plant phylogeny and leaf development fails to shed light on the origin of microphylls, and supports several independent origins of megaphylls. Here, I review plant phylogeny and developmental data from fossil and extant tracheophytes, as well as the current understanding of genetic pathways controlling leaf development, to argue that the megaphyll concept should be abandoned because it perpetuates misconceptions and confusion based on unsupported homology.

Morphological inconsistencies and overlap in the microphyll–megaphyll dichotomy

Microphylls are defined as leaves of small size, with simple venation (one vein) and associated with steles that lack leaf gaps (protosteles). By contrast, megaphylls are defined as leaves of generally larger size, with complex venation and associated with leaf gaps in the stele [3]. However, each of these criteria has inconsistencies [4] that highlight the disconnection between phylogeny and current morphological definitions at the level of the microphyll–megaphyll divide.

First, the presence or absence of leaf gaps does not provide a basic distinction between the two types. In many plants nested among the megaphyll-bearing euphyllophytes, leaf trace divergence is not associated with a leaf gap (e.g. the non-protostelic *Equisetum*, cladoxylalean pteridophytes and all extant seed plants [5]). Moreover, several groups of euphyllophytes are similar to microphyll-bearing lycophytes in that they have protosteles (e.g. the extant filicalean ferns *Lygodium* and *Gleichenia*, fossil *Kaplanopteris* and *Botryopteris*, some extinct coenopteridalean ferns and sphenophyllales, the aneurophytalean progymnosperms, and even early seed plants such as *Elkinsia*).

Second, leaf size and venation complexity are also inconsistent as distinguishing criteria between microphylls and megaphylls. *Equisetum* and some fossil sphenophyllales, as well as several extant and fossil gymnosperms, all of which are megaphyllous euphyllophytes, have highly reduced leaves supplied by one vein. Conversely, some lycophyte microphylls are large (up to 1 m in some of the extinct lepidodendrales or up to 0.5 m in extant *Isoetes* species), whereas others have complex venation patterns (some *Selaginella* species [6]) or morphologies (e.g. the dissected leaves of extinct protolepidodendrales such as

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Colpodexylon, *Protolepidodendron*, *Leclerqia* and *Estinnohyton* [7]).

Phylogenetic and developmental perspectives

Definitions based on morphology thus seem to create more confusion and overlap between the microphyll and megaphyll concepts instead of clarifying them. The validity of the two concepts needs to be verified against comparative developmental data, genetic developmental pathways controlling leaf development and plant phylogenies that illuminate the relationships between extant taxa and their closest leafless relatives in the fossil record. Leaf evolution was the earliest major plant biology question to be addressed using a comprehensive evo-devo approach. Work on leaf evolution integrated all the data types available (morphology, anatomy, development, phylogenetic hypotheses and the fossil record) to produce consistent theories. Such pre-molecular era evo-devo approaches led to several hypotheses for the evolution of leaves. Of those, two prevailing hypotheses explained the origin of microphylls from enations (small unvascularized flaps of tissue that characterized early vascular plants), and that of megaphylls from three-dimensional (3D) branching systems of undifferentiated photosynthetic axes [7].

Most modern phylogenies support the lycophyte–euphyllophyte divide [8–12], but phylogenetic studies have so far failed to provide unequivocal answers on the nature of the ancestral structures and evolutionary processes that generated the lycophyte leaf and have produced conflicting hypotheses of euphyllophyte phylogeny [9,10,12]. A developmental feature potentially congruent with the lycophyte–euphyllophyte dichotomy could be the mode of origination of leaf primordia on the flanks of the shoot apical meristem (SAM). A study of *Selaginella* [13] led to the idea that lycophyte leaves originate from a few initials specified exclusively in the outermost cell layer of the SAM; this is in contrast with euphyllophyte leaves, which originate from larger populations of initials to which both the outermost cell layer, as well as subjacent layers, contribute. The mode of origination of *Selaginella* leaves is similar to the development of bryophytic gametophyte leaves and fern scales, and could be the cause for a different mode of ad/abaxial polarity determination that is uncoupled from the layering of the leaf primordium [13]. Data available on other extant lycophytes seem to support this proposed developmental dichotomy, but an exhaustive survey of extant lycophytes, as well as data from fossils, are needed to confirm the hypothesis.

Another direction of investigation is suggested by the results of a study that found limited interrelationships between phyllotaxis and cauline vascular architecture in two *Lycopodium* species [14]. This uncoupling of two major shoot morphological features contrasts the situation seen in euphyllophytes, where phyllotaxis and cauline vasculature are well correlated. If confirmed by studies in other lycophytes, this could represent another fundamental developmental characteristic separating lycophyte leaves from those of euphyllophytes.

Phylogenetic analyses could provide another set of criteria for defining leaves in lycophytes and in the different euphyllophyte groups by identifying the type of

precursor structure at the origin of each, as reflected by their respective leafless sister groups. That requires inclusion of as many fossil taxa as possible in morphology-based phylogenies. In lycophytes, three competing hypotheses propose evolution of the leaf ('microphyll'): (i) by vascularization of enations; (ii) from sporangia by sterilization; or (iii) from telome trusses by reduction [7,8]. However, although monophyly of the group is generally accepted, neither the scarce data on genetic pathways controlling leaf development [15,16] nor phylogenetic studies [8] lend unequivocal support to any of the three hypotheses.

Our understanding of euphyllophyte phylogeny is hampered by major disparities between the results of studies based exclusively on extant taxa and those of studies that include fossils [8–12]. 'Extant-only' studies support monophyly of extant seed-free euphyllophytes (the 'monilophyte' clade), whereas studies including fossils support a sequence of paraphyletic grades comprised of extant and fossil seed-free euphyllophytes that led to the seed plants. Although they have been gaining wide acceptance, the results of extant-only studies are equivocal to the question of euphyllophyte leaf origin because they lack fossil taxa that: (i) could represent transitional stages bearing precursor structures in transformational series; and (ii) generate the amount of phylogenetic resolution needed to answer such a question. Nevertheless, the fossil record shows unequivocally that the common ancestor of 'megaphyllous' euphyllophytes was leafless. This indicates that, among euphyllophytes, leaves are not homologous because they evolved independently in at least two lineages or, as indicated by phylogenies that include fossils, in as many as nine different lineages. A close look at leaf development and the genetic pathways that control it across euphyllophytes provides strong support for homoplasy of the leaf across euphyllophytes.

How many different 'megaphylls' are there?

Multiple lines of evidence indicate that leaves that have been categorized as megaphylls are fundamentally different from each other. Some are associated with eustelic stems, whereas others are borne on siphonostelic or protostelic stems. In seed plants the adaxial domain of leaves has a developmentally associated axillary meristem; such structures are missing from seed-free plants. Cell differentiation and tissue maturation progress exclusively acropetally in fern leaves, but in seed-plant leaves, development is a more complex process in which final tissue maturation progresses largely basipetally. All of these indicate that there are several different types of 'megaphylls' that have probably evolved independently. Current understanding of plant phylogeny supports several independent origins of leaves in different euphyllophyte lineages.

The studies that used the most complete datasets pertinent to euphyllophyte phylogeny in general, and leaf evolution among euphyllophytes in particular [9,12], support two contrasting topologies of the euphyllophyte tree. One of these topologies, not emphasized here, proposes an unorthodox placement of the zosterophylls–lycophyte clade as the sister group to the lignophytes (*sensu* Ref. [17]) and,

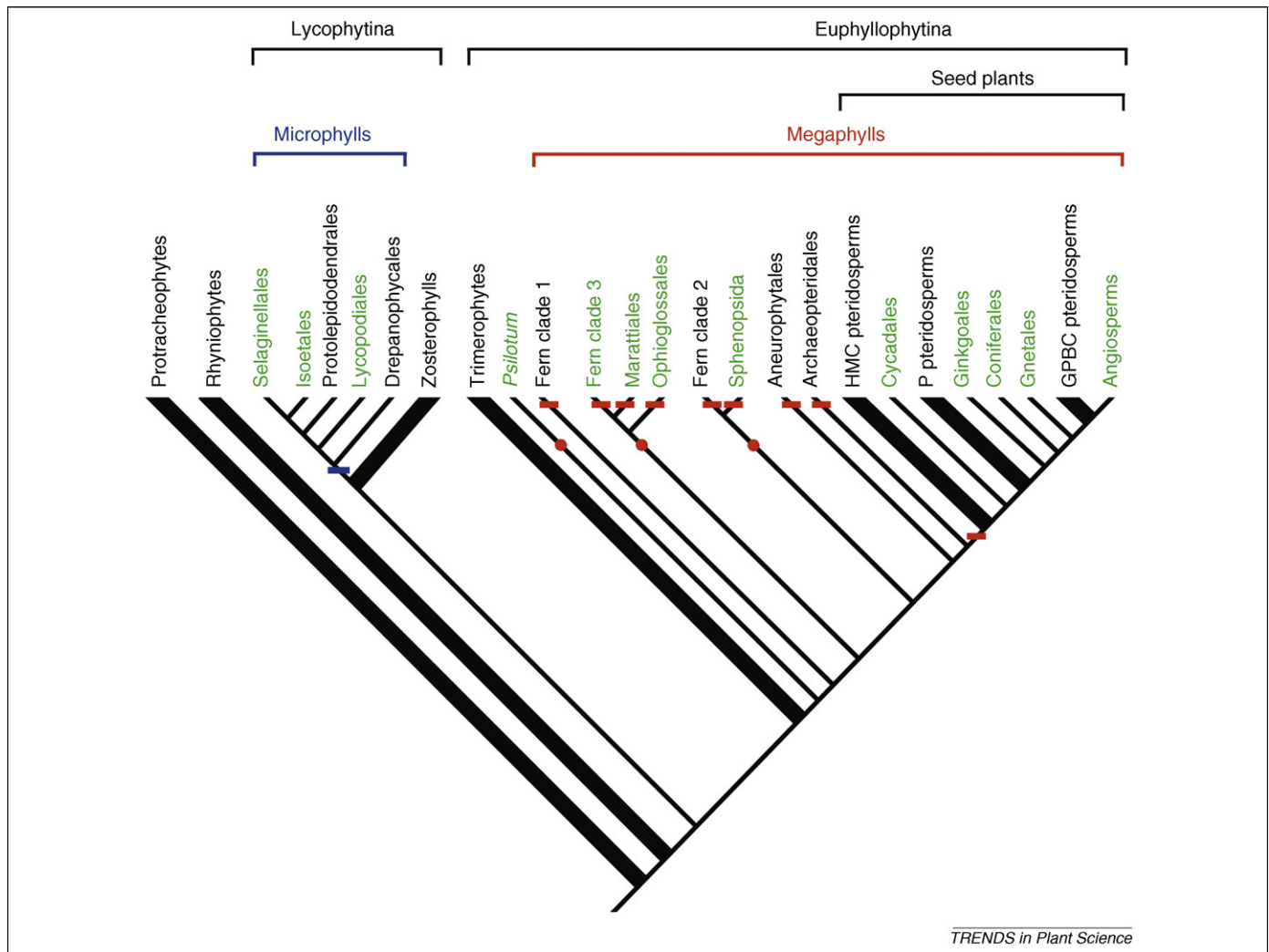


Figure 1. Microphyll and megaphyll origins. 'Extant + extinct' polysporangiophyte phylogeny (based on Refs [9,33]) supports a single origin of microphylls (blue line) and several independent origins of megaphylls (red lines); red dots represent alternative megaphyll origins. Paraphyletic grades within which relationships are not detailed are collapsed and represented by the broader branches of the tree; names of lineages with extant representatives are in green; names of extinct lineages are in black. Fern clades 1 and 2 are as defined in Ref [9]: fern clade 1 – Stauropteridales; fern clade 2 – Zygoteridales + Cladoxylales; fern clade 3 includes living and extinct Filicales and Hydropteridales. Aneurophytales and Archaeopteridales are progymnosperms. The pteridosperms are seed ferns: HMC (hydrasperman, medullosan and callistophytalean seed ferns); P (peltaspermalean and corystospermalean seed ferns); and GPBC (glossopteridalean, pentoxylalean, bennettitalean and caytonialean seed ferns).

hence, paraphyly of the euphyllophytes. The other topology (Figure 1) supports the more generally accepted hypothesis of euphyllophyte monophyly [8]. However, independent of euphyllophyte monophyly, because of the inclusion of a high number of fossil representatives of all lineages (some of which are entirely extinct), both of the proposed topologies bring considerable resolution to the question of euphyllophyte leaf evolution – both support up to nine independent origins of euphyllophyte leaves (Figure 1). The morphology of fossil taxa that occupy basal positions in the different lineages that have evolved leaves independently indicates that in all cases the precursor structures that evolved into leaves were systems of 3D-branching undifferentiated axes.

'Megaphyll' precursor structures

The origin of euphyllophyte leaves from systems of 3D-branching axes, long advocated by students of the fossil record, received support from cladistic studies that proposed that synapomorphies of the euphyllophyte clade

include: (i) monopodial or pseudomonopodial branching; and (ii) small, pinnulelike vegetative branches [8]. The corollaries of these synapomorphies are the presence of a main axis with subordinated lateral branching systems and determinate growth of the lateral branching systems. These proposed synapomorphies led to a hypothesis of partial homology, namely that megaphylls are homologous at the level of precursor structures ('megaphyll precursors' [8]): 3D dichotomous lateral branches that shared a fundamental leaf characteristic, determinacy [8].

However, currently there is no support for homology of lateral appendage determinacy across euphyllophytes. Structural homology means sameness as a result of inheritance from a common ancestor that had the structure in discussion, yet evidence that the common ancestor of euphyllophytes had determinate lateral branching systems is equivocal. In fact, a recent study of 380–340-million-year-old fossils (Heather L. Sanders, PhD thesis, Ohio University, 2007) shows that ferns and seed plants

acquired determinacy and ad/abaxial symmetry of lateral branching systems in a different sequence: whereas evolution of ad/abaxial symmetry in ferns preceded that of determinacy, ad/abaxial symmetry in seed plants is a more-derived character, preceded by the evolution of determinacy. This finding has significant implications because it demonstrates that neither determinacy nor ad/abaxial symmetry were present in the common ancestor of the lineages from which ferns and seed plants evolved. Therefore, homology of ‘megaphyll’ precursor structures could be invoked only at the most basic level of body plan organization, that of 3D branching systems of undifferentiated axes. However, at that level everything would be homologous to everything else in the organography of polysporangiophyte sporophytes, because it all evolved from the same fundamental precursor structure, the branched system of undifferentiated axes.

Furthermore, if megaphyll precursor structures were homologous as determinate lateral branching systems across all basal euphyllophytes, then we should be able to find evidence of process homology [18] for leaf determinacy across all extant ‘megaphyllous’ lineages. In other words, we would expect the gene pathways that control determinacy in ‘megaphyllous’ leaves to be shared among all euphyllophytes. However, although they are still short of addressing the whole breadth and depth of polysporangiophyte phylogeny, genetic studies of leaf development indicate that this is not the case. Interactions between class 1 *KNOX* (*KNOTTED-LIKE HOMEODOMAIN*) genes and *ARP* (*ASYMMETRIC LEAVES*, *ROUGH SHEATH* and *PHANTASTICA*) genes, a main candidate regulator of determinacy in leaf development, show a breadth of diversity that eliminates the possibility of process homology, and so do the genetic pathways involved in another major leaf feature, ad/abaxial polarity.

Process homology and homoplasy

Process homology refers to common inheritance of developmental genetic pathways [18]. In terms of gene pathways, three major determinants of leaf development have been commonly considered in discussions of the evolution of shoot systems: (i) interactions between class 1 *KNOX* genes and *ARP* genes; (ii) class III *homeodomain-leucine zipper* (*HD-Zip*) genes and their interactions with *KANADI* genes and microRNAs 165 and 166; and (iii) *YABBY* genes and their interactions with class 1 *KNOX* genes and leaf ad/abaxial polarity pathways.

KNOX-ARP interactions

Class 1 *KNOX* genes (hereafter referred to as *KNOX*) are thought to be responsible for maintaining the meristematic status of tissues characterized by cell totipotency and thus for indeterminacy of growth. Consistent with this role, they are expressed at the shoot apex in all extant lineages of tracheophytes (Box 1). Conversely, genes of the *ARP* group are expressed in the leaf primordia of all living tracheophytes (Box 1), where they are thought to induce determinacy of growth by promoting cell fate determination, starting with the specification of leaf primordium founder cells at the periphery of the apical meristem. Thus, expression of *KNOX* genes at the shoot apex and of *ARP* genes in

leaf primordia seem to be common denominators of leaf development in all extant tracheophytes. Therefore, it is tempting to suggest that some form of *KNOX-ARP* antagonistic interaction is the fundamental shared mechanism responsible for determinacy of lateral appendages, which would then represent a ‘process synapomorphy’ that characterized all leaf precursors in tracheophytes. However, a closer look at *KNOX* phylogeny and *KNOX-ARP* patterns of expression in the different tracheophyte lineages indicates complex evolutionary patterns. These demonstrate that *KNOX-ARP* interactions are modulated differently in the different lineages (Box 1) and weaken the case for *KNOX-ARP* interactions as a major regulator of determinacy.

Box 1. Genetic pathways in leaf development

Broadening of the taxonomic spectrum for which genetic developmental data are available, along with innovative developmental approaches to the plant fossil record, will help answer specific questions that will lead to a deeper understanding of the evolution of leaf development in all tracheophyte lineages.

- Is *KNOX* expression in the shoot apex (Figure 1a, dark green) and *ARP* expression in leaf primordia (Figure 1a, red) shared by all vascular plants, living and extinct? With respect to extinct lineages, this might seem a strictly rhetorical question. However, several studies [25,35,36] have already used morpho-anatomical fingerprints to demonstrate developmental processes and regulatory mechanisms in plant fossils, showing that documentation of such characteristics in extinct lineages has become reality. Discovery of morpho-anatomical fingerprints for regulatory mechanisms of leaf development ([37], Heather L. Sanders, PhD thesis, Ohio University, 2007) could enable the inclusion of many extinct lineages in studies addressing the evolution of leaf development.
- Is *KNOX* expression in complex leaf primordia (Figure 1b, light green) exclusively characteristic of euphyllophytes, or were *KNOX* genes also expressed in the leaf primordia of lycophytes with complex leaf morphology (now extinct), such as *Leclerqia*?
- *KNOX* downregulation in leaf primordium initials (Figure 1b, yellow) has been documented in *Selaginella* and seed plants but is absent in filicalean ferns and some angiosperms (*Medicago*). Is this pattern due to loss of a shared characteristic of lycophytes and euphyllophytes or to parallel evolution in ferns and *Medicago*?
- Was *ARP* expression in the shoot apical meristem (SAM; Figure 1b, pink) present in the common ancestor of lycophytes and euphyllophytes and subsequently lost in seed plants, only to evolve independently in some angiosperms (tomato)? Or was it acquired independently in lycophytes, ferns and flowering plants?
- What are the functions of *HD-ZipIII* genes in ferns? Are they responsible only for SAM functioning and vascular tissue patterning, as they are in all other extant vascular plants (Figure 1c, purple), or are they also involved in leaf ad/abaxial symmetry patterning, as is the case in seed plants (Figure 1c, light blue)? Is the latter function a process synapomorphy of seed plants or of all euphyllophytes?
- Are *HD-ZipIII-KANADI* interactions conserved across all embryophytes, as suggested by the presence of *KANADI* homologs in *Selaginella* and *Physcomitrella*, and is their canalization into regulation of (i) vascular tissue radial patterning and (ii) leaf ad/abaxial polarity regulation homologous or homoplastic in different vascular plant lineages? Are any of the other regulatory pathways of leaf ad/abaxial polarity documented in angiosperms active and interacting in other lineages?
- What gene pathways control leaf ad/abaxial polarity patterning in lycophytes? Are any of the pathways that are involved in leaf ad/abaxial polarity patterning in seed plants also active in lycophytes?

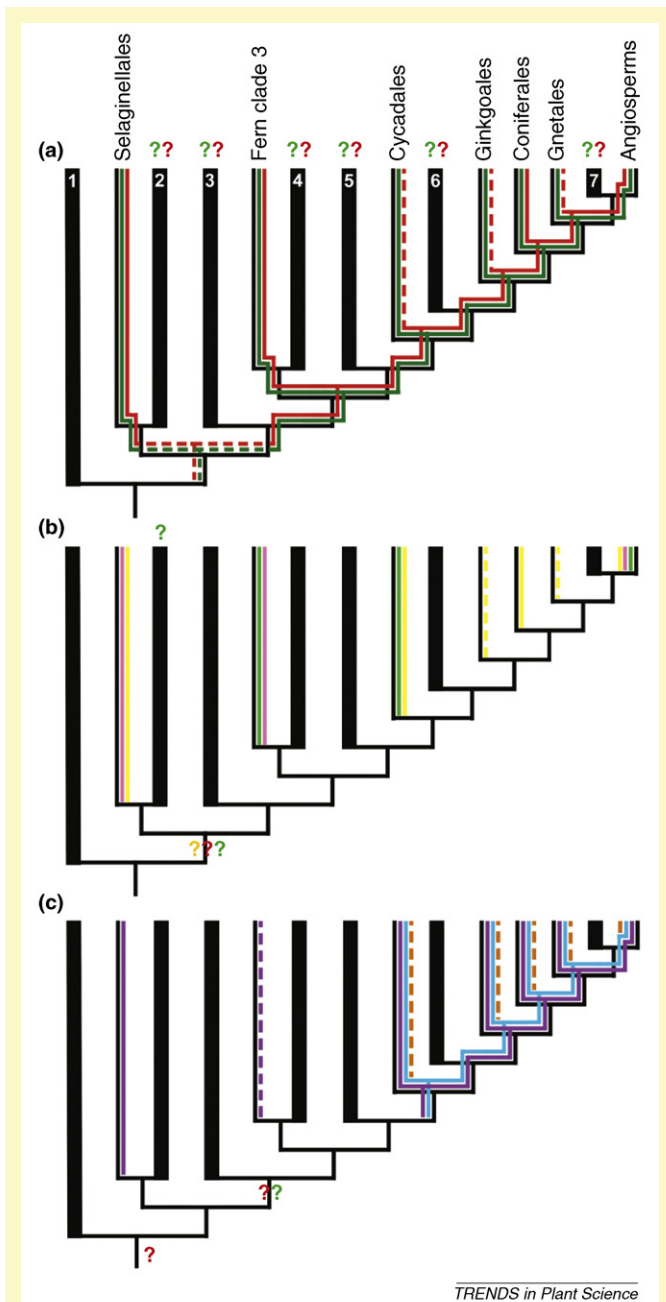


Figure 1. Expression patterns of genes associated with leaf development. Polysporangiophyte phylogeny is simplified based on the tree in Figure 1 (main text) and shows only extant lineages for which gene expression data are available (data from Refs [15,16,18,21–24,26,29,34]); thicker branches are placeholders for paraphyletic grades within which relationships are not detailed: 1, protracheophytes and rhyniophytes; 2, Isoetales, Protolpidodendrales, Lycopodiales, Drepanophycales and zosterophylls; 3, trimerophytes, *Psilotum* and Stauropteridales; 4, Marattiales and Ophioglossales (fern clade 3 comprises extinct and living Filicales + Hydropteridales); 5, Zygopteridales, Cladoxylales, sphenopsids, progymnosperms plus the hydrasperman, medullosan and callistophytalean seed ferns; 6, peltaspermalean and corystospermalean seed ferns; 7, glossopteridalean, pentoxylalean, bennettitalean and caytonialean seed ferns. (a) *KNOX* and *ARP* expression patterns potentially shared by all living tracheophytes: *KNOX* genes expressed in the SAM (dark green) and *ARP* genes expressed in leaf primordia (red). (b) *KNOX* and *ARP* expression patterns that demonstrate widely divergent types of interaction: *KNOX* genes expressed in leaf primordia (plants with complex leaf primordia) (light green); *KNOX* genes downregulated, even if only transiently, in leaf primordia (*Medicago* shows no downregulation, in contrast to the main angiosperm pattern) (yellow); *ARP* genes expressed in the SAM (*Lycopersicon* is the only angiosperm known with this pattern) (pink). (c) *HD-ZipIII* and *YABBY* functions: *HD-ZipIII*s involved in SAM functioning and vascular tissue patterning that are potentially repressed by microRNAs 165 and 166 (purple); *HD-ZipIII*s involved in leaf ad/abaxial polarity patterning (light blue); *YABBY*s involved in leaf ad/abaxial polarity patterning (brown). Question marks refer to questions detailed in the box text.

KNOX phylogenies show that members of the gene family expressed in the SAMs of different living lycophte and euphyllophyte lineages are not orthologs of each other [15,19] because some of them are more closely related to *KNOX* genes with different expression patterns of expression than to those with similar expression patterns. This has been taken to indicate independent evolution of the SAM expression pattern (and presumably function) of *KNOX* genes in the different lineages [15]. Alternatively, it has been proposed [19] that the different patterns of expression could be the result of subfunctionalization after duplication of a single *KNOX* gene inherited from the tracheophyte common ancestor, where it covered all of the expression domains of the different derived members of the family, including the SAM. This seems plausible in light of the finding that in mosses (*Physcomitrella*) *KNOX* genes promote production of sporogenous tissue by preventing premature sporogenesis [20], a function similar to the maintenance of meristematic status, and thus indeterminacy, at the shoot apices of higher embryophytes.

KNOX and *ARP* genes have been identified in all the tracheophytes studied to date. It has been proposed that *KNOX-ARP* interactions have evolved independently in lycophtes and euphyllophytes [15], but an alternative hypothesis is that they are shared across all vascular plants and, in lycophtes, they could have contributed to the acquisition of determinacy of sterilized-sporangia appendages [19]. Irrespective of that discussion, *KNOX-ARP* interactions are modulated differently between extant plant lineages. A reflection of this situation is that *KNOX* expression is not limited to the SAM; neither is *ARP* expression limited to leaf primordia (Box 1). Additionally, the degree of antagonism in *KNOX-ARP* interactions varies between lineages:

- *KNOX* genes are downregulated in the leaf primordia of lycophtes and seed plants (cycads, conifers, *Welwitschia* and angiosperms; no information available for *Ginkgo*). Although this pattern has been attributed to *ARP* gene activity, unequivocal evidence is not available for all lineages that exhibit *KNOX* downregulation. In fact, in some angiosperms, *ARP* genes are not needed for downregulation of *KNOX* genes (which are still downregulated in the leaf primordia of some *Arabidopsis* and maize *ARP* loss-of-function mutants [21]), and they do not repress all *KNOX* genes in the leaf primordia (a feat achieved only by *YABBY* genes [16]).
- *KNOX* genes are expressed in the leaf primordia of ferns and seed plants that have complex leaves that start off as complex primordia [22]. In most of those seed plants, *KNOX* expression characterizes later stages of leaf primordium development and follows a transient stage of *KNOX* downregulation that is thought to be linked to leaf primordium specification. By contrast, ferns do not exhibit *KNOX* downregulation associated with any stage of leaf primordium development; additionally, *KNOX* proteins do not seem to have the same role in leaf development in *Ceratopteris* as they do in angiosperms (Heather L. Sanders, PhD thesis, Ohio University, 2007). Recently, the angiosperm *Medicago* has been shown to lack *KNOX* downregulation during leaf primordium development [23].

- Ferns are also peculiar in that both *KNOX* and *ARP* genes are expressed in both the SAM and leaf primordia, suggesting that interactions between the two gene groups are anything but antagonistic.
- *ARP* expression in the SAM has been documented not only in ferns but also in *Selaginella* and has been hypothesized to be responsible for apical branching in these plants [15]. However, the same pattern of expression has been documented in an angiosperm (*Lycopersicon* [24]) that is characterized by axillary and not apical branching.
- Genes of the *ASYMMETRIC LEAVES2/LATERAL ORGAN BOUNDARIES (AS2/LOB)* family act in concert with *AS1* to downregulate *KNOX* genes in angiosperm leaf primordia. Putative *AS2/LOB* homologs have been identified in *Selaginella* and *Physcomitrella* [19], but their patterns of expression and phylogeny at the level of all living tracheophytes are not resolved.

In conclusion, across the lycophyte–euphyllophyte divide, gene expression patterns do not indicate unequivocally whether particular types of *KNOX–ARP* interactions are homologous or homoplastic; neither do they differentiate unequivocally lycophyte leaves from those of euphyllophytes. Within euphyllophytes, the existence of a shared fundamental mechanism underpinning leaf development has been proposed based on studies of the evolution of leaf morphology [25], and *KNOX–ARP* interactions have been suggested as a candidate for that role [15]. However, the multitude of interaction types suggested by the diversity of *KNOX* and *ARP* expression patterns indicates that this is not the case and does not translate into any clear patterns of process homology or homoplasy in leaf development, even just across euphyllophytes.

Class III *HD-Zip* genes

Class III *HD-Zip* genes (hereafter referred to as *HD-Zip*) have been identified in representatives of most extant streptophytes [26]. Based on *HD-Zip* expression patterns and inferred functionality, as well as on gene phylogeny, it has been proposed [26] that the common ancestor of vascular plants had a single *HD-Zip* gene that was involved in regulation of apical meristem functioning and radial patterning of vascular tissues (Box 1). Evolutionary duplication of the ancestral *HD-Zip* and subsequent lineage-specific functional diversification led to the acquisition of new roles by the resulting paralogs in lycophyte and euphyllophyte lineages. Consequently, *HD-Zip* functions mark a fundamental difference in developmental regulation between lycophyte and seed-plant leaves [16]. Whereas *HD-Zips* are involved in procambium specification and vascular tissue patterning in lycophyte leaves (*Selaginella*), in seed-plant leaves, they are involved in procambium specification and patterning, as well as in primordium specification and ad/abaxial polarity (Box 1). Particularly, in seed-plant leaves, *HD-Zips* determine adaxial polarity as a result of antagonistic interactions with *KANADI* genes (promoters of abaxial identity in leaf tissues) and microRNAs 165 and 166 [27,28]. A conserved target sequence for microRNAs 165 and 166 suggests that

negative regulation of *HD-Zip* by those microRNAs might be shared across all embryophytes [29]. By contrast, absence of *HD-Zip* expression data for seed-free euphyllophytes (in which *HD-Zips* have been identified [16]) and scarcity of data on non-angiosperm *KANADI* homologs (which were identified, nevertheless, in *Selaginella* and *Physcomitrella* [19]) preclude understanding of the role of *HD-Zip–KANADI* interactions in leaf evolution.

In conclusion, phylogenetic patterns of *HD-Zip* functions parallel the lycophyte–euphyllophyte divide and concur with homoplasy of leaves between the two clades. However, the functions and interactions of these genes are insufficiently documented to bring resolution to questions of homology and homoplasy in leaf evolution among euphyllophytes.

YABBY genes

The *YABBY* gene family is believed to be specific to seed plants because members have been identified in both angiosperms and gymnosperms but none were detected in seed-free plants [19] (Box 1). To date, *YABBY* functions have been investigated only in angiosperms. There, they are responsible for repression of all *KNOX* genes in the leaf primordium [16] and regulation of leaf ad/abaxial polarity and laminar growth, and they might also influence phyllotaxis [30]. However, in terms of leaf ad/abaxial polarity regulation, *YABBY* genes act downstream of all other known polarity determinants. These include three pathways (*ETTIN–AUXIN RESPONSE FACTOR4*, *AS1–AS2* and *HD-ZipIII–KANADI*) with complex interactions that differ significantly among studied angiosperm species, and none of these pathways are concerned exclusively with leaf ad/abaxial polarity [28].

Genetic pathways – overview

Studies of genetic pathways of leaf development that address gene phylogeny, expression patterns and functionality are opening new perspectives in the way we think about leaf evolution. *KNOX–ARP* interactions have been proposed as a fundamental mechanism responsible for determinacy of leaf precursor structures. However, patterns of *KNOX* and *ARP* expression at the shoot apex and in leaf primordia exhibit a multitude of combinations that indicate widely divergent types of *KNOX–ARP* interaction in different major lineages and raise the question whether that is an important interaction for leaf determinacy. If the latter is true, no independent evidence is available to indicate whether it represents homology or homoplasy, in terms of process, between lycophytes and euphyllophytes. The diversity of *KNOX* and *ARP* expression patterns rather supports many independent origins of determinacy of leaf precursor structures (even among euphyllophytes), possibly by different modulation of *KNOX–ARP* interactions, but it is unclear whether those interactions existed before the evolution of determinacy or evolved initially as a determinacy mechanism.

The expression patterns and known or inferred functions of *HD-Zip* genes mark a fundamental difference in developmental regulation between the seed-plant clade, where *HD-Zips* participate in leaf ad/abaxial polarity regulation in interaction with *KANADI* genes and microRNAs

165 and 166, and lycophytes with no *HD-Zip* involvement in leaf ad/abaxial polarity. Data on *HD-Zips* from seed-free euphyllophytes are needed to understand whether that fundamental difference corroborates the lycophyte–euphyllophyte divide as a ‘process synapomorphy’ of euphyllophytes or reflects a seed plant synapomorphy. The latter is probably the case for *YABBY* genes, which are involved in seed plant leaf ad/abaxial polarity regulation (among other functions, such as laminar growth and phyllotaxis) and which seem to be absent in seed-free plants.

Conclusions and future perspectives

The classic concepts of microphyll and megaphyll pervade thinking on the evolution of leaf development. As such, they influence significantly the process of science in plant biology by contributing to the shaping of evolutionary hypotheses and to inferences of developmental studies. However, the two concepts, as currently defined, are equivocal, partially overlapping and inconsistent with current understanding of plant phylogeny. Most workers are aware of this situation when they agree that ‘microphylls’ (as referring to lycophyte leaves) probably have a single common origin and that ‘megaphylls’ (as referring to euphyllophyte leaves) evolved independently in several lineages. Under this scenario, the ‘megaphyll’ (and its more recent version, the ‘euphyll’) has been retained to account for a hypothesis of partial homology whereby the leaves of all euphyllophytes can be traced back to a common ancestor that had determinate lateral branching systems. However, the fossil record and genetic pathways controlling leaf development (as documented in different vascular plant lineages) indicate that euphyllophyte leaves are neither homologous at the level of their precursor structures nor at the level of the genetic pathways that control their development. Thus, by grouping together non-homologous structures, the megaphyll concept perpetuates an unsupported evolutionary scenario. The centrality of the concept to many hypotheses on the evolution of leaf development makes this issue more than just a matter of naming things, and I argue that the ‘megaphyll’ should be abandoned before it introduces more bias in plant science. ‘Leaf’, accompanied by a specifier (e.g. ‘filicalean leaf’) works just as well, and it is neutral in terms of any implication of homology. Ultimately, we need unequivocal definitions of the different leaf types based on development (including genetic pathways) and phylogeny.

Phylogeny and the fossil record show that the ancestors of the two crown clades of vascular plants, lycophytes and euphyllophytes, were leafless, which is consistent with homoplasy of leaves between the two clades. Within the lycophyte clade, homology of leaves is widely accepted, thus the microphyll concept could be retained if it is redefined to designate lycophyte leaves. Within euphyllophytes, fossil-based phylogenies support as many as nine independent origins of leaves. Justification for the use of ‘megaphyll’ to designate euphyllophyte leaves stemmed from the idea that they all evolved from precursor structures that were homologous as branching systems with determinate growth. However, genetic pathways suggest homoplasy between euphyllophyte lineages in terms of leaf

developmental processes, and studies of fossil ferns and seed plants indicate that the branching axes of the common ancestor of the two lineages had not evolved determinacy nor ad/abaxial symmetry. Thus, use of a specialized term has no logical justification because it would group together structures that are not homologous.

Despite spotty taxonomic coverage, data on developmental genetic pathways add another layer of complexity to the understanding of leaf evolution. The diversity and phylogenies of genes in major gene families involved in leaf development are well documented in several angiosperm taxa. Putative homologs of some of those genes have been identified in gymnosperms, filicalean ferns, *Psilotum*, the lycophyte *Selaginella*, the moss *Physcomitrella*, liverworts and hornworts, and phylogenies have been constructed for some of those genes. By contrast, while attempts are being made to study developmental genes in non-flowering plants, the difficulties have thus far prevented much from being learned. Studies of expression patterns have been performed only on a limited subset of genes and taxa, and studies of gene functions are rare outside the angiosperm clade. Mechanisms controlling ad/abaxial leaf polarity, characterized in significant detail in flowering plants, are undocumented in seed-free plants. Nevertheless, available data indicate that *KNOX-ARP* interactions are too diverse among (and sometimes within) major lineages to be unequivocally regarded as the fundamental mechanism of leaf determinacy in tracheophytes. It is unclear which aspects of the *KNOX-ARP* interactions are homologous and which are homoplastic between lycophytes and euphyllophytes.

Two major unanswered questions are – what were the precursor structures of lycophyte leaves, and exactly how many independent origins of euphyllophyte leaves there were? Answers to these questions and understanding of leaf evolution could come from addressing several more specific questions (Box 1). These will require broadening of the taxonomic spectrum for which genetic developmental data are available [31]. Whether or not the monilophyte clade stands the test of time [10,12,32], the seed-free euphyllophytes include a considerable amount of diversity. Of these, only one lineage, the filicalean ferns, has been sampled to date. Thus, even considering extant euphyllophytes only, several lineages that are highly divergent morphologically require detailed study: eusporangiate ferns (Marattiales and Ophioglossales), psilotophytes, sphenopsids and heterosporous leptosporangiate ferns (Hydropteridales). Getting the full picture of leaf development will also require data on lycophytes other than *Selaginella* (the only taxon of the clade studied to date), such as homosporous lycophytes and the more highly derived, heterosporous Isoetales. Inclusion of data from all of these taxa, ideally supplemented with data on fossil lineages, will illuminate patterns of gene evolution, expression and functionality that could ultimately be used as characters in phylogenetic studies.

Finally, one could also wonder what good is the microphyll if the megaphyll is to be abandoned, because the two concepts were defined as mutually exclusive. However, irrespective of whether we decide to call lycophyte leaves microphylls, defining their synapomorphies represents a

valid task with significant implications. That task is rendered difficult by the lack of understanding of the evolutionary history of lycophyte leaves. Although some fossil evidence points to origin by vascularization of enations, two other proposed evolutionary mechanisms (sporangial sterilization and reduction of branching systems) cannot be rejected, and neither phylogeny nor genetic developmental data provide unequivocal resolution to the enigma of the precursor structures of the lycophyte leaf. One potential lycophyte synapomorphy and microphyll-defining character that needs confirmation is the origination from only a few initials, all of which are specified in the outermost cell layer of the shoot apex. Another potential shared feature of lycophyte leaves is a partial uncoupling of phyllotaxis and cauline vascular architecture. These emphasize the need for basic studies of leaf developmental anatomy and morphology, a need which is by no means limited to the lycophytes – it is humbling to realize, in the post-genomics era, how much we do not know about development and morphology in many plant species.

Acknowledgements

I thank Gar Rothwell, Heather Sanders and two anonymous reviewers for their constructive critique, helpful comments and suggestions.

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