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Chapter 6
The Sporophytes of Seed-Free Vascular Plants – Major Vegetative Developmental Features and Molecular Genetic Pathways

Alexandru M.F. Tomescu

6.1 Introduction

Seed-free vascular plants, collectively and informally referred to as pteridophytes, span most of the taxonomic breadth of vascular plant diversity above the order rank, yet all seed-free lineages taken together are considerably less speciose than the seed plants (spermatophytes). The latter are sharply distinct from the seed-free plants in sharing a unique fundamental sporophyte feature, a body plan dictated by two major developmental characteristics: bipolar growth that originates in a cotyledonary embryo, and axillary branching of the shoots. In contrast, seed-free plants encompass a much broader spectrum of sporophytic body plans and they are much more diverse from a developmental standpoint, a situation reflected in the breadth of their taxonomic span.

Numerous formal taxonomic schemes have been proposed in plant systematics (e.g., Judd et al. 2007; Cantino et al. 2007; Chase and Reveal 2009). They represent differing views and encompass a considerable range of differences in terms of the underlying systematic principles and nomenclatural rules they employ. This broad range of options can generate confusion regarding the legitimate formal name and rank of taxonomic entities above the genus rank. Since taxonomy is beyond the scope of the present volume, I opted for simplicity and equidistance, using a system of informal names, which do not carry any taxonomic rank significance, to designate the different groups of seed-free vascular plants, which are defined as follows. Lycopsids include the extant lycopodiales (club mosses and *Phylloglossum*), selaginellales (spike mosses) and *Isoetes* (quillworts), as well as several extinct lineages (protolepidodendrales, lepidodendrales, pleuromeiales); *Isoetes* and its closest fossil relatives, the lepidodendrales and pleuromeiales are grouped in the isoetalean clade (Stewart and Rothwell 1993). Psilotopsids comprise only two extant genera, *Psilotum* (whisk fern) and *Tmesipteris*. The pteropsids ("ferns") consist likely of three major lineages, of which two are extinct and one includes extant representatives.

A.M.F. Tomescu (✉)
Department of Biological Sciences, Humboldt State University, Arcata, CA, 95521, USA
e-mail: mihai@humboldt.edu

Extant pteropsids comprise the eusporangiate ophioglossales (*Ophioglossum,* *Botrychium, Helminthostachys, Mankyua*) and marattiales (*Marattia, Angiopteris,* *Archangiopteris, Christensenia, Danaea*), and the leptosporangiate filicales (with numerous genera) and hydropteridales (*Marsilea, Regnellidium, Pilularia, Salvinia,* *Azolla*). Sphenopsids are represented by only one living genus, *Equisetum* (horse-tail), but have a rich fossil record including the sphenophyllales and calamitales.

A major divide in the phylogeny of vascular plants, recognized very early (Haeckel 1866), is generally well agreed-upon (Rothwell and Nixon 2006). It separates the lycopsids from a clade (the euphyllophytes) comprising all other pteridophytes and the seed plants; depending on whether their organography is considered highly simplified or plesiomophic (see discussion below), the psilotopsids are either included or excluded from the euphyllophyte clade, respectively. On the lycopsid side, monophyly of the group, as well as its close relationship with the zosterophylls (an extinct, probably paraphyletic group of vascular plants with simple organography), are strongly supported (Kenrick and Crane 1997). On the euphyllophyte side, the positions of deep internal nodes are disputed between two competing phylogenetic hypotheses. An apparently robust phylogeny based exclusively on extant taxa (Pryer et al. 2001) proposes that all extant seed-free euphyllophytes form a clade (the moniliformopses) that is sister to the spermatophyte clade. This widely popular hypothesis is apparently supported by the exclusively fossil-based phylogeny of Kenrick and Crane (1997).

However, on closer look, the analysis of Kenrick and Crane, which was the study that proposed the moniliformopses clade, is based on a very different taxon sampling compared to Pryer et al.'s (2001) study: the taxonomic affinities of the three fossils used by Kenrick and Crane as placeholders for pteropsids and sphenopsids are equivocal at best (Rothwell and Nixon 2006); and no seed plant is included in their analysis. Consequently, the apparent overlap between Kenrick and Crane's (1997) and Pryer et al.'s (2001) moniliformopses, and between the moniliformopses-spermatophyte clades found by the two studies, has no real support. Moreover, using a combination of extant and extinct taxa, and of molecular and morphological characters, Rothwell and Nixon (2006) showed that Pryer et al.'s (2001) moniliformopses is not a well-supported clade. In a competing hypothesis based on extinct and extant taxa and using morphological characters, Rothwell (1999) proposed that seed-free euphyllophytes form a paraphyletic grade at the base of the seed plants, with sphenopsids nested as the sister group of an exclusively extinct pteropsid clade. Thus, for the moment, as pointed out by Rothwell and Nixon (2006), the phylogenetic structure of the euphyllophyte tree remains controversial. A phylogeny based on the synthesis of phylogenetic trees supported in the analyses by Rothwell (1999) and Hilton and Bateman (2006) is presented by Tomescu (2009).

In this chapter, I present a comparative survey of vegetative sporophyte features emphasizing the differences between the various extant lineages of seed-free vascular plants and between those and seed plants, and I review the state-of-the-art knowledge of developmental genes in the sporophytes of the seed-free lineages of vascular plants. Evolution is the result of changes in developmental characteristics which are controlled by genes. Understanding evolution is therefore a matter of understanding the molecular genetic pathways that control development, and the

way these pathways have changed over time. The taxonomic patterns of distribution of shared and divergent developmental pathways point to phylogenetic relationships which reflect evolution. A deeper understanding of these pathways in seed-free plants is crucial for improved phylogenetic resolution, especially at deep nodes, and would, thus, greatly illuminate our understanding of evolution.

6.2 Six Sporophyte Body Plans

The seed plant sporophyte body plan originates in a cotyledonary embryo with two poles of growth, the radicle and the epicotyl. This polarization of the embryo results in a bipolar growth pattern that produces a positively geotropic root system, which branches laterally from the endodermis, and a negatively geotropic shoot system with axillary lateral branching. In contrast to the shared seed plant sporophyte body plan, growth patterns in the various groups of seed-free vascular plants produce six different sporophyte body plans. These are characteristic of the lycopodiales, selaginalleales, isoetaleans, psilotopsids, sphenopsids, and pteropsids. The body plans of these six groups are differentiated by organography, growth polarity, and branching characteristics (Table 6.1).

The sporophytes of seed-free vascular plants originate in noncotyledonary embryos, which may or may not (i.e., psilotopsids and isoetales) have an embryonic root pole. Even when present, the embryonic (primary) root is short-lived and growth of the mature sporophyte proceeds in a unipolar pattern exclusively from the shoot apical meristem which produces both positively geotropic roots (which are all adventitious) and negatively geotropic shoots. An exception from the unipolar growth pattern seen in the majority of seed-free plants is found in *Isoetes* and its fossil relatives (isoetaleans). Growth of isoetalean sporophytes is bipolar, with both poles of growth represented by shoots. Studies of living and fossil isoetaleans have shown that dichotomous branching of a unique embryonic pole of growth early in development produces the two poles of growth of the mature sporophyte. One of these two poles produces the shoot system, whereas the other produces a positively geotropic rooting system, the rhizomorph. The rhizomorph is, thus, a modified shoot bearing appendages ("rootlets") that are leaf homologs. The evidence leading to these interpretations has been assembled and discussed thoroughly by Rothwell and Erwin (1985). Interestingly, these rhizomorph appendages have co-opted some of the root developmental programs leading to the development of a root cap and apical dichotomous branching.

The selaginellales present another particular body plan that adds a fourth organ to the common list of three vegetative organs of the plant sporophyte (stem, leaf, and root). The rhizophore is a naked axis, which branches apically and dichotomously and mixes characteristics of roots and stems (Jernstedt et al. 1994; Lu and Jernstedt 1996; Imaichi 2008). Rhizophores originate exogenously from stem branching points, they lack a root cap and can sometimes produce leaves, all of which represent stem characteristics, but most usually they bear roots. In addition

Table 6.1 Seed-free vascular plants exhibit six different sporophyte body plans defined by organography, growth polarity, and the branching mode of shoots and roots (first four columns of the table). Other features that distinguish the different lineages are the structure of apical meristems and stele morphology. Based on Campbell (1911), Eames (1936), Paolillo (1963), Bierhorst (1971), Ogura (1972), Rothwell and Erwin (1985), Gifford and Foster (1989), Lu and Jernstedt (1996)

	Stem-leaf-root organography	Polarity of growth	Shoot branching	Root branching	Shoot apical meristem	Root apical meristem	Shoot/axis stele type
Lycopodiales	Present (primary embryonic root, then exclusively adventitious roots)	Unipolar	Apical dichotomous	Apical dichotomous	2–3 and sometimes multiple apical cells	Multiple apical cells	Protostele (actinostele, plectostele)
Selaginellales	Present (primary embryonic root, then exclusively adventitious roots) Rhizophore Ligulate leaves	Unipolar	Apical dichotomous	Apical dichotomous	Single apical cell in some species, 2 or more apical cells in others	Multiple apical cells (in root and rhizophore)	Protostele (actinostele, plectostele)
Isoetaleans	Present Roots absent Rhizomorph: shoot modified for rooting Ligulate leaves	Bipolar	Apical dichotomous	–	Single apical cell, but more often multiple apical cells	–	Protostele (medullated+)
Psilotopsids	Absent	Unipolar	Apical dichotomous undifferentiated axes		Single apical cell	Single apical cell	Protostele (medullated)
Sphenopsids	Present (primary embryonic root, then exclusively adventitious roots)	Unipolar	Lateral, non-axillary	Lateral	Single apical cell	Single apical cell	Equisetostele Protostele*

Pteropsids	Present (primary embryonic root, then exclusively adventitious roots)	Unipolar	Apical dichotomous	Lateral	Single apical cell most often; some with multiple apical cells	Single apical cell most often; some with multiple apical cells	Protostele Siphonostele Eustele+
Spermatophytes	Present (primary embryonic root long lived)	Bipolar	Lateral, axillary	Lateral	Multiple apical cells, stratified apical meristem	Multiple apical cells	Eustele Protostele+

+Stele morphology known exclusively in extinct representatives of the lineage

to rhizophores, the selaginellales are also characterized by the presence of ligules on leaves, a feature that they share with plants in the isoetalean clade. The ligule is a specialized flap of tissue that develops on the adaxial side of each leaf. The function of the ligule has not been entirely elucidated, although it has been proposed that it keeps the leaf and sporangium primordia, as well as the shoot apical meristem, moist by secreting a mucilage that contains carbohydrates and proteins (Bilderback 1987; Gifford and Foster 1989).

The psilotopsids are characterized by a very simple body plan consisting of a system of undifferentiated branching axes bearing sporangia (grouped in synangia) and unvascularized (*Psilotum*) or vascularized (*Tmesipteris*) appendages that lack a definite arrangement. This body plan has been interpreted either as representing the plesiomorphic condition seen in the earliest vascular plants, or as an instance of extreme evolutionary simplification of the typical stem-leaf-root organography; the different competing interpretations are evaluated by Stewart and Rothwell (1993). The second interpretation received support from molecular phylogenetic studies (Pryer et al. 2001; Schneider et al. 2009) that have places psilotopsids among the seed-free moniliformopses (but see discussion of the validity of moniliformopses as a group above). Grounds for rejection of that interpretation are the complete absence of roots (even of an embryonic root pole) in *Psilotum* and *Tmesipteris*, and the lack of some or all defining leaf characters in the structures interpreted by some as reduced or modified leaves in *Tmesipteris* and *Psilotum*, respectively. Another intriguing side of the psilotopsid story is the complete absence of a fossil record for the group. This could be invoked in support of a secondarily derived simple organography, young age of the lineage and, hence, of the molecular phylogenetic placing of psilotopsids among crown-group euphyllophytes. However, such a placement would still imply anywhere between 136 and 56 million years of undocumented fossil history, based on the age of the oldest unequivocal fossils representing lineages proposed as sister groups to the psilotopsids in molecular phylogenetic studies – Early Cretaceous *Equisetum* (Stanich et al. 2009) and Late Paleocene *Botrychium* (Rothwell and Stockey 1989). Based on all the available evidence, I consider the organography of psilotopsids to reflect the same simple level of organization as that of the earliest vascular plants.

6.3 Embryogeny

Early work on seed-free plant embryogeny emphasized the orientation of the plane of zygotic division with respect to the archegonium, as well as cell lineage relationships between the two resulting cells, the octants of the early embryo, and the organs of the mature embryo and sporophyte. However, as early as the 1930s it became increasingly clear that such relationships are highly variable within major groups (as discussed by e.g., Wardlaw 1955; Johnson and Renzaglia 2009). A more interesting direction of focus in studies of embryogeny concerns the correlations between the polarity of the embryo and the polarity of the mature sporophyte.

In angiosperms, the division of the zygote is asymmetrical and produces a suspensor cell and a proembryo cell. A derivative of the suspensor cell, the hypophysis, subsequently gives rise to the radicle which later forms the root pole of the mature sporophyte. Derivatives of the proembryo produce the epicotyl which forms the shoot pole of the mature sporophyte. It can therefore be said that the fundamentally bipolar longitudinal patterning of the angiosperm sporophyte is determined by the first division of the sporophyte phase, the division of the zygote. If the same was true of seed-free plants, whose sporophytes exhibit a fundamentally unipolar structure, then we would expect the embryos of those plants to develop just one pole of growth in their earliest stage.

The embryos of all seed-free vascular plants have in common the foot, a temporary structure that is only indirectly involved in sporophyte growth by transferring nutrients from the maternal gametophyte to the embryo during early sporophyte development. Aside from the foot, the development of the embryo and the morphology resulting from it are highly variable among the different lineages, and even within lineages. Experiments on filicalean pteropsids have shown that embryos developed in incomplete archegonia or outside the archegonia fail to establish the normal set of embryonic organs (DeMaggio 1982). This suggests that the archegonium and the gametophyte provide cues crucial to polarity establishment in the embryo.

The psilotopsids, whose simple organography lacks roots altogether, conform to the concept of a unipolar embryo: their embryos have a foot and only one pole from which the axis grows. Interestingly, the embryo goes through a stage characterized by unipolar organization in the isoetaleans as well, although the mature body plan of those plants is determined by a bipolar growth pattern. As has been demonstrated in some fossil representatives (arborescent lepidodendrales), a unique embryonic pole of growth undergoes dichotomous division early in embryogeny to produce the two poles of growth that form the mature sporophyte body – the aerial shoot and the rhizomorph (Phillips 1979). Based on this, the isoetaleans can be regarded as primarily unipolar and only secondarily bipolar. However, early unipolar organization is not evident in *Isoetes* nor in some of the fossil lepidodendrales (reviewed by Stubblefield and Rothwell 1981), the embryos of which do not go through a unipolar stage and directly develop two poles of growth after the first leaf primordia (including those of leaf homologs for the rhizomorph pole) are produced. Given the embryogenetic pattern documented in the arborescent lepidodendrales, the embryogeny of *Isoetes* could reflect the fact that the unipolar stage of the embryo is skipped altogether as a result of extreme reduction in this genus, which undergoes very little elongation throughout its sporophytic life.

The lycopodiales, selaginellales, sphenopsids, and pteropsids, all of which have unipolar growth as mature sporophytes, have embryos that develop two poles of growth, a shoot pole and a root pole, aside from the foot. However, this lack of correlation between the polarity of the embryo (bipolar) and that of the mature sporophyte (unipolar) is only apparent. In these plants, the earliest polarity event, which precedes the establishment of the embryonic shoot and root poles, and sometimes occurs as early as the two-celled embryo stage, is the specification of two domains of which only one is involved in polar growth and generates the entire mature structure of the

sporophyte, while the other forms the foot. Thus, at this stage, the embryo is unipolar. In some groups (lycopodiales, selaginellales and some ophioglossalean and marattialean pteropsids), the division of the zygote specifies a suspensor cell and a proembryonic cell. Although this embryogenetic pattern seems similar to that of seed plants, the suspensor of seed-free plants has no direct developmental contribution to the polarity of the mature sporophyte. The proembryonic cell follows the typical developmental pattern involving specification of a foot (contiguous with the suspensor) and a polar growth domain. Therefore, the unipolar structure of the early seed-free plant embryo is not affected by the presence of a suspensor. Moreover, it is possible that the suspensor in seed-free plants is just a modified region of the foot (Johnson and Renzaglia 2009). Based on their taxonomic occurrence, the suspensors of lycopodiales + selaginellales, pteropsids, and seed plants have likely evolved independently.

In light of the primary unipolar condition of the embryo in lycopodiales, selaginellales, sphenopsids, and pteropsids, the presence of a shoot pole and a root pole in the later embryos of these lineages may not reflect bipolar organization; a transitional bipolar condition intercalated between the unipolar early embryo and the unipolar mature sporophyte would be difficult to justify developmentally. The most parsimonious interpretation is that the embryonic root pole that develops in the later embryo in these groups is the first adventitious root produced by the embryonic shoot pole, mirroring the pattern of development of the mature sporophyte. If that were the case, then specification of the embryonic shoot pole should precede that of the root pole. A review of embryogenetic patterns (Wardlaw 1955; Bierhorst 1971 and personal observations) shows that this is the case in lycopodiales, selaginellales, and *Equisetum*. The pteropsids, much more diverse taxonomically, exhibit a diversity of embryogenetic patterns. *Botrychium*, some marattiales and some filicales (*Actinostachys, Todea*) differentiate an embryonic shoot pole prior to the root pole, whereas the embryos of *Ophioglossum*, some marattiales and most filicales specify root and leaf primordia prior to the shoot pole. However, even in embryos of this latter category, the embryonic root is ephemeral. Furthermore, the early establishment of vascular tissue connecting the embryonic root and first leaf primordium mirrors the close developmental relationship seen between leaves and adventitious roots in these pteropsids.

Together, these lines of evidence support the hypothesis that the embryos of lycopodiales, selaginellales, sphenopsids, and pteropsids are unipolar, just like the adult sporophytes, and that the ephemeral embryonic root, when present, represents the first adventitious root produced by the embryonic shoot apex. A similar point of view was expressed by Kaplan and Groff (1995) who viewed the primordial embryonic body of pteropsids as a shoot meristem bearing the embryonic leaf and root primordia as laterally emergent organs.

6.4 Apical Meristem Structure

The growing tips of seed-free vascular plants are characterized by unstratified apical meristems in which one or several apical initials, present at the surface of the meristem, are responsible for tissue generation. This plesiomorphic vascular plant

character is contrasting with the stratified nature of most seed plant apical meristems, which are differentiated into regions distinguished by their mode of growth (predominantly anticlinal cell divisions in the tunica, and divisions occurring in various planes in the corpus); some gymnosperms represent exceptions with unstratified apical meristems. The classic, textbook example of seed-free plant apical meristem is the apical cell-based meristem, where a single apical cell, usually tetrahedral (three cutting faces), but sometimes wedge-shaped (two cutting faces), generates all the tissues of the axis. However, meristems with multiple apical initials are present in several seed-free lineages.

Meristems with a single apical cell are found in lycopsid stems (some *Selaginella* and *Isoetes* species), in the axes of psilotopsids and in the stems and roots of sphenopsids, and most pteropsids (Table 6.1). Apical meristems with more than one apical initial are more frequent than single cell meristems in the lycopsids: stems and roots of the lycopodiales have two to three or more apical initials; in *Selaginella*, rhizophores and roots have multiple apical initials, and the stems of most species have two or more apical cells; the shoot apical meristem has multiple initials in most *Isoetes* species and the linear rhizomorph meristem always consists of many initials. Among the pteropsids, apical meristems with multiple initials are found in the stems of some ophioglossales and in the stems and roots of older marattialean plants and of osmundaceous filicales. Thus, apical meristem structure shows a significant divide between lycopsids, characterized principally by meristems with multiple initials, and psilotopsids + seed-free euphyllophytes, in which single apical cell meristems are predominant. Given this divide between the two major clades of vascular plants, and the lack of information on the structure of apical meristems in early vascular plants, it is difficult to infer whether the common ancestor of vascular plants had meristems consisting of single or multiple initials at the tips of its undifferentiated dichotomous axes.

6.5 Branching

The mode of shoot branching sets apart the seed plants, characterized by axillary branching, from seed-free plants, most of which have apical dichotomous branching. An often overlooked aspect of this major divide is that axillary branching is inextricably connected to the presence of leaves, whereas apical branching is entirely independent of leaves. In seed plants, all branches (except for adventitious branches) occur in leaf axils where they develop from axillary meristems. The fact that axillary branching is highly canalized developmentally in this way suggests that it may be controlled by the same mechanism across all seed plants. In angiosperms, axillary branching is underwritten by a mechanism that shows strong association with the adaxial domain of leaf development and controls the retention of meristematic capacity in a group of cells located in the leaf axillary region, as they become detached from the shoot apical meristem. These cells may then assume a meristematic identity at a later moment, or they may never lose the meristematic identity conferred by origination at the shoot apical meristem (Bennett and Leyser

2006). Molecular genetic studies of gymnosperm branching have yet to confirm the degree of conservation of this mechanism. Apical branching, on the other hand, proceeds by direct dissection of the apical meristem, whether it is single-celled or it has multiple initials. In single-celled meristems, it can also arise by differentiation of the apical cell and dedifferentiation of two new apical cells from protodermal cells on either side of it (Kato and Imaichi 1997).

Axillary branching is also thought to occur in rhizomes of *Botryopteris* and *Helminthostachys* of the ophioglossales (Petry 1915; Kato et al. 1988). In these taxa, meristems derived directly from the shoot apical meristem are found in leaf axils, where, upon injury of the rhizome apex, they develop into branches connected to the rhizome stele by two vascular traces that diverge from the two sides of the leaf gap. It is unclear whether this mode of branching is controlled by the same mechanism documented in angiosperms, but current understanding of plant phylogeny suggests that rather than indicating a close relationship between ophioglossales and the extinct progymnosperms, as suggested by Kato et al. (1988), the similarity of the two branching modes is due to convergent evolution, and thus, does not imply homology.

In both apical and axillary branching, branches are specified at the shoot apical meristem and arise from the outermost cell layers (exogenous branching). In contrast, *Equisetum,* and possibly, its fossil sphenopsid relatives, exhibit a very different mode of branching. Branching in *Equisetum* is lateral (as opposed to apical) but nonaxillary; it is, nevertheless, correlated to leaf production in that branches occur at nodes and are, therefore, always associated with leaves. However, instead of developing in leaf axils, *Equisetum* branches are produced between the bases of adjacent leaves in a whorl. More importantly, in *Equisetum*, branches are not specified at the shoot apex and there is evidence (Stutzel and Jaedicke 2000) that their origin may be endogenous (i.e., from tissues beneath the outer layers).

Another type of branching, documented in extant and extinct filicalean pteropsids (Troop and Mickel 1968; Tomescu et al. 2008), is epiphyllous branching. Sometimes referred to as epipetiolar branching, it consists of the production of stems on leaves, usually close to the base of the petiole, but not in the leaf axil. Epiphyllous branching is regarded more as a form of reiteration and, therefore, not included in general discussions of the main modes of shoot branching. However, its common occurrence in certain taxa (e.g., dennstaedtioids and the extinct *Botryopteris, Psalixochlaena, Kaplanopteris, Anachoropteris*), as well as the fixed positions in which it arises, suggest that epiphyllous branching is a constituent aspect of the regular developmental program of those taxa.

While the mode of shoot branching draws a sharp divide between seed plants and seed-free plants, branching in roots sets apart lycopsids from the euphyllophytes. In lycopsids (lycopodiales and selaginellales), root branching is apical dichotomous, from apices with multiple apical initials. In euphyllophytes, the roots branch exclusively laterally and branch roots are not produced directly by the apical meristem. Euphyllophyte root primordia arise endogenously, by dedifferentiation of one or several primary meristem or parenchyma cells of the parent root which form the apical meristem of the branch root. In seed plants and sphenopsids, the layer in

which branch roots originate is the pericycle, but in pteropsids branch roots originate from the endodermis (von Guttenberg 1966). Interestingly, despite the sharp divide in root branching mode between lycopsids and euphyllophytes, all root-bearing plants share the same endogenous mode of production of adventitious roots from the stem pericycle or endodermis. The only exception is *Selaginella* in which the roots are borne at the tips of rhizophores; there, they arise from inner cells of the rhizophore apical meristem (Lu and Jernstedt 1996; Kato and Imaichi 1997).

6.6   Radial Patterning of Sporophyte Axes

The stele of stems and roots consists of the primary vascular tissues (xylem and phloem) and associated ground tissues (pith, pericycle, leaf gaps, interfascicular regions) (Beck et al. 1982; these authors provided the latest in-depth discussion of the stele concept and classifications). Three main types of stele are traditionally distinguished based on the anatomy of mature tissues: protosteles, in which the vascular tissues form a central rod (these include actinosteles and plectosteles); siphonosteles, in which the vascular tissues form a hollow cylinder with gaps produced by leaf divergences (these include solenosteles, with no more than one leaf gap at any level, and dictyosteles, which can have two or more overlapping leaf gaps in some regions); and eusteles, consisting of discrete vascular bundles (sympodia), which can be more or less interconnected (the atactostele of monocot stems is included here).

Among extant vascular plants, seed plant shoots are characterized exclusively by eusteles, whereas seed-free plant shoots have protosteles (lycopsids, psilotopsids, some pteropsids) or siphonosteles (pteropsids) (Table 6.1). However, when fossils are taken into consideration, this distinction becomes blurred: some of the early seed plants (pteridosperms, e.g., *Elkinsia*; Rothwell et al. 1989) had protosteles and some seed-free plants (e.g., *Archaeopteris*; Beck 1960) had eusteles. Among sphenopsids, the extinct sphenophyllales had protosteles, but *Equisetum* and the extinct Calamitales have a unique stele morphology that is similar to a siphonostele at the nodes and to a eustele in the internodes, and for which a fourth stele type, the equisetostele, has been coined by Rothwell (1999). The roots of all plants have protosteles.

The anatomy of the mature sporophyte can be misleading in terms of stele morphology, when taken outside the context of development. In psilotopsids, for example, the anatomy of primary meristems at the apex of axes provides a better criterion that the anatomy of mature tissues for interpretation of the stele type. In these plants, the stele consists of a hollow, ribbed cylinder of vascular tissues, with pith-like nonvascular tissue at the center, and has the appearance of a siphonostele. However, a look at the arrangement of primary meristems at the tips of psilotopsid axes reveals that the center is occupied by procambium, the primary meristem from which vascular tissues differentiate; in psilotopsids, the procambium differentiates into vascular tissues around the periphery and into parenchyma at the center. Thus,

the "pith" of psilotopsid axes is, developmentally, vascular tissue "gone wrong" and the stele is a protostele (referred to as a "medullated protostele" to account for the presence of parenchymatous tissue at the center). Moreover, in the sporophytes of some pteropsids stele morphology is size- and age-dependent, as it transitions from an early protostelic state to a siphonostele (e.g., Thompson 1920). Also, although eusteles are thought to be absent in filicalean pteropsids, some siphonostelic taxa exhibit stele morphologies that resemble the eustelic condition quite closely (White and Weidlich 1995; Karafit et al. 2006).

These examples raise important questions not only about the way we classify stele morphologies but, more importantly, about our understanding of evolution as reflected in development, that such classifications should ultimately mirror. Furthermore, stele morphology is only the most prominent aspect, historically, of a more general feature of plants, the radial patterning of tissues in sporophyte axes (stems and roots). Since the radial patterning of tissues results from coordinated patterns of cell division and differentiation in the apical meristem, could studies of development help us reach a better understanding of commonalities and differences between vascular plant lineages and, ultimately, of phylogenetic relationships and evolution? Could we base our understanding and classification of stele morphology on the geometry of developing vascular tissues in apical meristems?

In the apical meristem region, the procambium forms a solid rod at the center of axes which have protosteles in their mature regions, and it forms a hollow cylinder (with ground meristem in the middle) in axes whose mature regions have siphonosteles. In the eustelic stems of angiosperms, provascular meristematic tissue forms a hollow cylinder (residual meristem) from which procambial strands of the stem sympodia and leaf traces differentiate (Esau 1977). The patterning of the vascular tissues of shoots is also influenced, to different degrees depending on the plant lineage, by leaves and their vascular supply (leaf traces). In the leafless psilotopsids, the vascular tissues are patterned exclusively by the apical meristem of the axis. In lycopsids, there seems to be little influence of leaves on the patterning of vascular tissues and the morphology of the stele, as suggested by the presence of provascular tissue above the level of the youngest leaf primordia (Gifford and Foster 1989). Pteropsids present a wide variety of conditions, as shown by experimental and comparative studies of the development of vascular tissues in shoot apices (Wardlaw 1944, 1946; White 1984) – there are species in which the leaves have a major influence on the establishment of the vascular pattern, while in other species the influence of leaves is less substantial. The eusteles of seed plants seem to be mostly an expression of the production of vascular tissues supplying the leaves, as suggested by experiments (summarized by Steeves and Sussex 1989) which showed that as a result of obliteration of leaf primordia, vascular tissue differentiation in shoot apices is arrested at the stage of hollow cylinder of provascular tissue (residual meristem). The relationship between development of the shoot apical meristem and the geometry of tissues in the shoots of *Equisetum* is still poorly understood, partly due to pronounced modularity, which makes it difficult to follow the differentiation of tissues longitudinally. All of these emphasize the need for renewed efforts in documenting the developmental anatomy of radial patterning in sporophytic axes.

Another aspect of radial patterning is the specification of a boundary layer that is continuous throughout the axes, where it forms the endodermis (in most plant roots and many seed-free plant stems) and starch sheath (in seed plant stems), and into the leaves, where it forms the vascular bundle sheaths (Tomescu 2008). The boundary layer separates the stele from the other tissues in the axes, and the leaf veins from the mesophyll. Beck et al. (1982) advocated the exclusion of the endodermis as a morphological criterion from treatments of stele morphology. However, the boundary layer separates clearly distinct domains in radial patterning and is, therefore, important in understanding this aspect of development and evolution. For instance, recent studies (Stewart and Tomescu 2009 and unpublished results) have shown that there are several patterns of endodermis development in terms of the origin of the endodermal layer (from procambial or ground meristem initials) and the identity of the sister layer produced by the periclinal division that generates the endodermis. The taxonomic distribution of the different patterns in the roots of euphyllophyte is congruent with phylogeny, providing support for some clades. Accumulating evidence suggests that a shared mechanism (the *SHR-SCR* positive feedback loop; Cui et al. 2007) is responsible for the specification of boundary layers throughout the plant body in seed plants (Tomescu 2008). Given the emerging diversity of endodermis development patterns present in seed-free plants, it will be interesting to see how these different patterns correlate with the molecular mechanisms that control boundary layer development in different sporophyte organs and in different vascular plant lineages.

Among the extant seed-free plant lineages, secondary growth is present only in *Isoetes*, in which a secondary meristem (cambium) produces secondary vascular tissue, consisting of both xylem and phloem cells, toward the center of the stem and rhizomorph, and parenchymatous secondary cortex toward the outside (Paolillo 1963; Gifford and Foster 1989). Secondary growth also characterized several extinct lineages, including the lepidodendralean isoetaleans, the sphenophyllalean and calamitalean sphenopsids, and the progymnospermous pteropsids. These represent independent evolutionary origins of secondary growth, and Rothwell et al. (2008) have provided evidence for parallel evolution of the regulation of secondary tissue production by polar auxin flow in isoetaleans, sphenopsids, and lignophytes (progymnosperms + seed plants). On the other hand, the idea that some ophioglossales may undergo secondary growth was dispelled by Rothwell and Karrfalt (2008), who showed that the radially aligned tracheids of *Botrychium* are the result of a particular type of primary growth rather than being produced by divisions of a secondary meristem.

6.7 Leaf Development

The leaves of plant sporophytes are lateral appendages that share four defining features: vascularization, determinate growth, bilateral symmetry (adaxial-abaxial polarity), and a definite arrangement (phyllotaxis). However, they are not homologous

across all vascular plants, a situation reflected in the early popular classification of leaves into microphylls (subsequently equated with lycopsid leaves) and mega-phylls (later equated with euphyllophyte leaves). Modern phylogenies based on broad sampling of extant and extinct plant lineages (Rothwell 1999; Hilton and Bateman 2006; Rothwell and Nixon 2006; synthesized in Tomescu 2009) indicate that leaves may have evolved independently up to ten times in vascular plants – one origin for lycopsid leaves and up to nine among euphyllophytes –, supporting the abandonment of the simplistic microphyll-megaphyll dichotomy in favor of a more sophisticated approach for understanding the evolutionary origins of leaves (Tomescu 2009). Among extant euphyllophyte lineages, there is strong evidence for independent evolution of leaves in at least two lineages (seed plants and pteropsids + sphenopsids), according to the moniliformopses phylogenetic hypothesis (Pryer et al. 2001; Friedman et al. 2004). Phylogenies that include fossils support indepen-dent evolution of leaves in the seed plants, the sphenopsids, and the pteropsids. Although evidence for this has yet to be produced, differences in leaf morphology suggest that the ophioglossales, marattiales, and leptosporangiate pteropsids may represent three independent instances of leaf evolution.

Lycopsid leaves are usually small, with a simple lamina and a single vein, although extant plant diversity and the fossil record include exceptions to each of these three characteristics (Tomescu 2009); the selaginellales and isoetaleans share the presence of a ligule on the adaxial side of their leaves (discussed under Sect. 6.2). It is generally agreed, based on phylogeny and the fossil record, that lycopsid leaves share a common origin and are homologous (Kenrick and Crane 1997; Friedman et al. 2004), but the nature of the precursor structures and evolutionary processes that generated the lycopsid leaf is still debated. Three competing hypotheses propose that lycopsid leaves evolved either by vascularization of enations (small, lateral flaps of tissue on the undifferentiated axes of early vascular plants that lacked vascular tis-sues and a definite arrangement), by sterilization of sporangia, or by reduction of branching systems (Stewart and Rothwell 1993; Kenrick and Crane 1997), but none of these hypotheses has received unequivocal support to date.

In the absence of a demonstrated common mechanism of evolution, no defining characteristics are available presently to define the lycopsid leaf as a synapomorphy. Two features that may be synapomorphic are the mode of origination of leaf primor-dia and the relationships between stele architecture and phyllotaxis (Tomescu 2009). The leaves of *Selaginella kraussiana* have been shown to originate from a small number of initials specified in the outermost (protodermal) layer of the shoot apex (Harrison et al. 2007). This characteristic, although not documented exhaustively, has been nevertheless recorded in other lycopsids (von Guttenberg 1966) and differenti-ates the clade from pteropsid euphyllophytes, whose leaves originate from larger groups of primordium founder cells, which are usually specified in the protodermal, as well as underlying layers of the shoot apex. Another study (Gola et al. 2007) has shown uncoupling between stele architecture and phyllotaxis in *Lycopodium*. If docu-mented in other lycopsids, this characteristic that contrasts the much tighter connec-tions between stele architecture and phyllotaxis present in euphyllophytes, could represent another defining synapomorphy for lycopsid leaves.

In the psilotopsids, morphological evidence provides the strongest support for interpretation of organography as consisting of leafless undifferentiated axes, in spite of alternative interpretations that have been put forward. The two genera included in the group, *Psilotum* and *Tmesipteris*, have somewhat different morphologies. *Psilotum* sporophytes consist of a system of profusely but irregularly branched below-ground axes bearing rhizoids; some of the below-ground branches produce above-ground axes that exhibit regular, three-dimensional dichotomous branching and bear minute enations and synangia. Two alternative interpretations have been proposed to explain the organography of *Psilotum* as a derived state of a pteropsid-type body plan, a hypothesis which found support in the placement of psilotopsids among the hypothetical moniliformopses in "extant-only" phylogenies (Pryer et al. 2001; Schneider et al. 2009). In one of these interpretations, Bierhorst (1971, 1977) has argued that the above-ground axes are homologous to leaves (fronds) attached to a rootless rhizome represented by the below-ground axes. However, the absence of important leaf-defining features in the above-ground axes argues against this interpretation: they lack a definite arrangement (phyllotaxis) on the below-ground axes and they lack bilateral symmetry; additional grounds for rejection of this interpretation have been provided by Kaplan (1977). A more popular interpretation proposes that *Psilotum* axes are stems that bear highly reduced leaves represented by enations (Gifford and Foster 1989). However, the lack of any vascularization and of a definite arrangement in the enations of *Psilotum* make this interpretation untenable. In *Tmesipteris*, the above-ground axes bear appendages that have been interpreted as leaves because they are flattened in a vertical plane and, thus, exhibit apparent bilateral symmetry. However, unlike leaves, the appendages of *Tmesipteris* lack a definite arrangement and are vascularized by strands that are radially symmetrical, suggesting axial rather than leaf similarities.

The whorled leaves of *Equisetum* are highly reduced, single-veined, and fused at nodes, forming sheaths. There is good evidence in the fossil record that the leaves of *Equisetum* and, probably, of all the sphenopsids, have evolved by reduction of branching systems (Stewart and Rothwell 1993). Leaf development in *Equisetum*, like most other features of this genus, is unique. The shoot develops in a very regular pattern, by production of vertically stacked series of three derivatives, each cut from one of the three cutting faces of the apical cell. Each derivative produces a group of cells (merophyte) by transversal and vertical (periclinal and anticlinal) divisions. Each group of three merophytes, corresponding to a series of three derivatives of the apical cell, produces one node and one internode of the stem. The leaf initial cell is specified as the outer cell of the second highest cell tier of the merophyte, when the merophyte is four cells thick (vertically). When leaf growth is initiated, the leaf initial cell cuts off a leaf apical cell to the outside, by an oblique anticlinal wall (von Guttenberg 1966). Thus, like in the lycopsids, leaves originate in the outermost cell layer in *Equisetum*.

Most pteropsids have relatively large leaves with complex venation and, often, complex architecture, also known as fronds. The leaves originate from relatively large numbers of initials (except in taxa with diminutive mature sporophytes, such as *Azolla* or *Salvinia*) specified in the protoderm and underlying layers of the apical meristem, and phyllotaxis is strongly correlated with stele architecture.

All these features make pteropsid leaves similar to the leaves of seed plants, with which they have been traditionally and misleadingly grouped together under the now obsolete name of megaphylls. Today, there is general agreement based on the fossil record and phylogenetic analyses that pteropsid and seed plant leaves evolved independently (Friedman et al. 2004; Tomescu 2009). Moreover, an earlier hypothesis of partial homology of leaves in the two groups at the level of their hypothesized precursor structures, determinate lateral branching systems (Kenrick and Crane 1997), has become less tenable in light of a fossil study that demonstrated divergent pathways of acquisition of leaf-defining features, and therefore, independent evolution of leaf developmental mechanisms in pteropsids and seed plants. In the study, Sanders et al. (2009) compared an early filicalean pteropsid (*Psalixochlaena antiqua*) to an early seed plant (*Elkinsia polymorpha*) and showed that while the leaf precursor appendages of filicales acquired adaxial-abaxial polarity before determinacy, those of seed plants acquired determinacy prior to evolving adaxial-abaxial polarity.

The independent evolutionary origins of pteropsid and seed plant leaves are also reflected in the differences in developmental characteristics between their living representatives. Pteropsid leaves grow from an apical cell for a significant part of their development; their maturation is strictly acropetal and the apex retains a meristematic character for the entire duration of leaf development. In contrast, apical meristematic activity is much more limited in the leaves of seed plants, in which growth is much more diffuse throughout the leaf surface. Furthermore, leaf maturation in seed plants is acropetal in early stages, but once the tip matures, the final phases of tissue differentiation and maturation proceed basipetally (Kaplan and Groff 1995). Finally, the independent evolution of pteropsid and seed plant leaves is also supported by significant differences in the expression patterns of genes that control leaf development in the two groups (Tomescu 2009).

6.8 Developmental Genes

Few, if any, developmental processes in the seed-free plant sporophyte are understood at the genetic level. Homologs of genes involved in major developmental programs in seed plants, and especially angiosperms, have been nevertheless isolated in different seed-free vascular plants. However, the coverage of different lineages is very uneven (Table 6.2). The most studied species has been *Selaginella moellendorffii*, which is also the only seed-free plant with a completely sequenced genome to date. Several other *Selaginella* species, most prominently *Selaginella kraussiana*, have contributed data, and Floyd and Bowman's (2007) seminal work that sought to reconstruct the developmental tool kit of land plants and its evolution, considerably expanded the number of gene families isolated in *Selaginella*. The only other seed-free plant that has undergone significant study of developmental genes is the pteropsid, *Ceratopteris*. The lycopodiales, isoetaleans, psilotopsids, sphenopsids, and the eusporangiate (ophioglossales, marattiales) and hydropteridalean (marsileaceae, salviniaceae) pteropsids have received little attention. Analyses of expression patterns

Table 6.2 Developmental genes identified in seed-free plants

	KNOX	ARP	HD-ZIP	MADS-box	AP2/ANT	FLO/LFY	CUC	WOX	CLV	PIN	TCP	GRAS	EPFL	
Lycopodium	+++	◆		++										Svensson et al. (2000); Svensson and Engstrom (2002)
Selaginella	+++	◆	+++	++	+		+	+	+	+	+	+	+	Tanabe et al. (2003); Harrison et al. (2005); Floyd and Bowman (2006); Floyd et al. (2006); Prigge and Clark (2006); Floyd and Bowman (2007); Rychel et al. (2010)
Isoetes						+								Himi et al. (2001)
Psilotum			+			+								Himi et al. (2001); Floyd et al. (2006)
Ophioglossum				++										Munster et al. (2002)
Botrychium						+								Himi et al. (2001)
Angiopteris						+								Himi et al. (2001)
Osmunda	+++	+++												Harrison et al. (2005)
Ceratopteris	◆	+++	+	+++	+	++								Kofuji and Yamaguchi (1997); Munster et al. (1997); Hasebe et al. (1998); Aso et al. (1999); Himi et al. (2001); Axtell and Bartel (2001); Sano et al. (2005); Floyd et al. (2006); Prigge and Clark (2006)
Anogramma	+++													Bharathan et al. (2002)
Matteucia		+				+								Himi et al. (2001)
Marsilea														Prigge and Clark (2006)
Equisetum						+								Himi et al. (2001)

+, presence of gene; ++, organ-level expression pattern characterized; +++, tissue-level expression pattern characterized; ◆, studies of gene function

are limited to a subset of the taxa and genes studied (Table 6.2). Most of these analyses only addressed expression patterns at the whole sporophyte level, seeking to characterize differences between organs, sometimes with added detail differentiating meristematic from mature organ segments. Although informative, data produced by such expression studies provide only circumstantial evidence for inferences on gene functions. However, a few studies using in situ hybridization documented tissue-level expression patterns, much more informative for functional interpretations, for several genes in *Selaginella* and pteropsids (*Ceratopteris, Osmunda,* and *Anogramma*). Gene functions have been addressed by only two studies to date, one using ectopic expression in *Arabidopsis* to characterize the functions of *Ceratopteris* class 1 KNOX (KNOX1) genes (Sano et al. 2005), and the other using complementation of *Arabidopsis* mutants to document the functions of an *AS1* ortholog (an ARP gene) in *Selaginella* (Harrison et al. 2005). Both studies also documented tissue-level expression patterns of the respective genes using in situ hybridization.

6.8.1   KNOX and ARP Genes

KNOX1 genes have been identified in *Selaginella kraussiana* and pteropsids (*Osmunda regalis, Ceratopteris richardii,* and *Anogramma chaeophylla*) (Table 6.2); at least one class 2 KNOX (KNOX2) gene is present in *Selaginella kraussiana* and in *Ceratopteris richardii*. In angiosperms, KNOX1 genes are involved in meristem formation and maintenance, positioning of leaf primordia within the shoot apical meristem, and internode elongation (Floyd and Bowman 2007). In *Selaginella*, two KNOX1 genes are expressed one in the shoot apical meristem (in the cells subtending the apical cells, but not in leaf primordium initials) and the other in internodal regions (Harrison et al. 2005). This expression pattern is also seen in some KNOX1 genes in angiosperms, in which it was suggested to have evolved independently of *Selaginella* (Harrison et al. 2005). As an alternative interpretation, Floyd and Bowman (2007) proposed that the common ancestor of extant vascular plants had one KNOX1 gene that covered both patterns of expression and which was duplicated independently in lycopsids and angiosperms, following divergence from their common ancestor; each of the two copies then became subfunctionalized for one or the other of the two functions independently in the two lineages.

   KNOX1 genes have similar patterns of expression in the three pteropsids studied – they are expressed in the shoot apical meristem and in leaf primordia (Bharathan et al. 2002; Harrison et al. 2005; Sano et al. 2005). A study in *Ceratopteris* (Sano et al. 2005) showed that KNOX1 genes are also expressed in procambium at the shoot apex and along the margins of young developing leaves (at the tips and in associated provascular tissue of pinnules). These patterns of expression are largely consistent with those documented in seed plants for KNOX1 genes. Indeed, the results of over-expression of *Ceratopteris* KNOX1 genes in *Arabidopsis* suggest that the angiosperm

and pteropsid proteins have similar functions in meristem development and leaf architecture (Sano et al. 2005). The KNOX2 gene is expressed in all sporophyte tissues in *Ceratopteris*, like in the angiosperms (Sano et al. 2005).

ARP genes, expressed in the leaf primordia of all vascular plants studied to date, are thought to induce determinacy of growth by promoting cell fate determination. Among seed-free plants they have been identified in *Osmunda regalis*, *Selaginella kraussiana*, and *Selaginella viticulosa*. A *S. kraussiana* ARP gene has been shown to rescue the *Arabidopsis as1* phenotype (albeit in a dosage-dependent manner), which suggests conservation of function between lycopsids and angiosperms (Harrison et al. 2005).

The antagonistic interactions of KNOX1 and ARP genes are considered one of the major determinants of leaf development (Harrison et al. 2005). In angiosperms, KNOX1 genes are expressed in the shoot apical meristem, but are downregulated in the leaf primordium initials, where ARP genes are expressed. Once the leaf primordium is initiated, KNOX1 expression resumes in the primordia of species that produce compound leaves, but not in those of species with simple leaves. The patterns of expression of KNOX1 and ARP genes in pteropsids and *Selaginella* apparently fit the angiosperm model. KNOX1 genes are downregulated in the leaf primordia of *Selaginella*, and they are expressed in those of pteropsids, where they have been hypothesized to delay determinacy and, thus, promote complex architecture (Harrison et al. 2005). However, there are differences in both groups compared to the angiosperm model. In pteropsids, the transient downregulation of KNOX1 genes in leaf primordium initials is absent (this situation has also been documented in one angiosperm, *Medicago*; Di Giacomo et al. 2008). Furthermore, ARP genes, expressed only in the leaf primordia in angiosperms, are expressed in the leaf primordia, as well as the shoot apical meristem, where they are coexpressed with KNOX1 genes, in pteropsids (*Osmunda regalis*) and *Selaginella kraussiana* (Harrison et al. 2005).

These differences in the expression patterns of KNOX1 and ARP genes between major plant lineages suggest that vascular plants do not share a unique type of KNOX1-ARP antagonistic interactions. These interactions, although important in meristem patterning and leaf development, vary in their degree of antagonism and appear to be modulated differently in the different lineages, which is congruent with the multiple independent origins of leaves in vascular plants (Tomescu 2009). The expression of KNOX1 genes in the shoot apical meristems of selaginellales, pteropsids, and seed plants suggests that the function of these genes in meristem development may have been established early on in the common ancestor of all vascular plants. The fact that ARP genes are also expressed in the apical meristems of selaginellales and pteropsids suggests that this may be another plesiomorphic feature likely to have characterized the earliest vascular plants prior to the divergence of lycopsids and euphyllophytes. It will be interesting to characterize the functions of the KNOX1 and ARP genes in the seed-free plants and to find out whether they are shared between lineages.

6.8.2   HD-ZIP Genes

Class III HD-ZIP genes have been identified in *Selaginella kraussiana*, *Selaginella moellendorffii*, *Psilotum nudum*, and two pteropsids (*Ceratopteris richardii* and *Marsilea minuta*), but their expression patterns have been characterized only in *Selaginella* (Table 6.2). The two genes identified in *Selaginella* have different expression patterns (Floyd and Bowman 2006; Floyd et al. 2006). One is expressed in apical cells and below the shoot apical meristem at the center of provascular strands (which differentiates into xylem), as well as in differentiating protoxylem tracheids in both stem and leaves. The other HD-ZIP III gene is expressed on the adaxial side of expanding leaves where the ligule develops and conducting tissue first differentiates; its expression domain extends toward the leaf apex and into the stem procambium but is restricted to the outer layers of provascular tissue that differentiate into phloem and pericycle. These two patterns of expression seem to indicate complementary roles of the two genes in the patterning of the shoot apex and the vascular tissues of the stem and leaves (Floyd et al. 2006). These expression patterns have been partially confirmed by another study of *Selaginella* HD-ZIP III genes (Prigge and Clark 2006), which also found them expressed in regions of the shoot apical meristem associated with leaf initiation, suggesting a role in leaf initiation, as well as in the developing vascular tissue of roots.

In seed plants, HD-ZIP III genes are involved in shoot apical meristem formation and growth, vascular patterning and determination of leaf adaxial polarity (Floyd and Bowman 2007). The patterns of expression of HD-ZIP III genes in *Selaginella* and their apparently complementary roles in the development of the shoot apex and of tissues within vascular strands, suggest that stem and leaf vascularization are separated spatially and temporally, and are possibly initiated by two different genes (Floyd and Bowman 2006). These have potential implications for the evolution of development. Since *Selaginella* HD-ZIP III genes seem to not be involved in leaf polarity, but they are likely implicated in shoot apical meristem functioning and vascular tissue patterning, these two functions shared with the seed plants are probably plesiomorphic and were present in the common ancestor of all extant vascular plants (Floyd and Bowman 2007). The difference between lycopsids and seed plants, where HD-ZIP III genes are major players in the establishment of leaf adaxial-abaxial polarity, is congruent with the independent origins of leaves in the two lineages and may support the hypothesis of origination of lycopsid leaves from enations (Floyd and Bowman 2006).

Aside from HD-ZIP III genes, HD-ZIP genes belonging to subfamilies I, II, and IV have also been identified in *Ceratopteris richardii*, where they are expressed in both gametophytes and sporophytes, not associated with any sporophyte organ in particular, but expressed with different intensities in the different sporophyte parts (Aso et al. 1999). This is thought to indicate that those genes are not required for organ identity, but are likely involved in developmental and physiological processes common to the sporophyte and gametophyte phase.

6.8.3 MIKC-type MADS-box Genes

MADS-box genes have been identified in many eukaryotes (including plants, animals, and fungi) and MIKC-type MAD-box genes include most of the organ identity genes implicated in the development of the angiosperm flower. Among the seed-free vascular plants, MIKC-type MADS-box genes have been identified in lycopsids (*Lycopodium annotinum*, *Selaginella remotifolia*,and *Selaginella moellendorffii*) and pteropsids (*Ophioglossum pedunculosum* and *Ceratopteris richardii*) (Table 6.2), but none are orthologs of the angiosperm floral organ identity genes (Munster et al. 1997, 2002). Their patterns of expression have been documented at the organ level so only very general inferences have been made regarding potential functions, except for *Ceratopteris* in which tissue-level expression patterns have been reported.

In *Lycopodium*, several MADS-box genes of the LAMB2 group are expressed in all vegetative and reproductive parts of the sporophyte (with different levels of expression for different genes and tissues), whereas *LAMB1* is expressed exclusively in developing strobili (Svensson et al. 2000; Svensson and Engstrom 2002). In *Selaginella*, MADS-box genes grouped in the same clade with the *Lycopodium* LAMB2 genes are expressed in vegetative and reproductive sporophyte tissues except for the rhizophore and root. This has been interpreted as evidence for independent origins of roots in the two lineages (Tanabe et al. 2003).

Most of the *Ceratopteris* MADS-box genes are expressed in both gametophyte and sporophyte, but the levels of expression vary. In the sporophytes, they tend to be similarly expressed in both the vegetative and reproductive organs, with somewhat higher levels of expression in organ primordia and meristems. One gene has nevertheless been shown to be predominantly expressed in roots (Theissen et al. 2000). In situ hybridization studies by Hasebe et al. (1998) showed that *Ceratopteris* MADS-box genes are expressed in the procambium of stems, leaves and roots, as well as in the shoot and root apical meristems, leaf primordia, developing leaf tips and pinnule tips, and sporangium initials. These patterns of expression have led to the assumption that MADS-box genes are involved in cell division in the seed-free plants (Hasebe et al. 1998). MADS-box genes are also expressed relatively ubiquitously in *Ophioglossum*, where of the four genes identified three were expressed in both the trophophore (vegetative) and the sporophore (reproductive) segments of the leaves, and only one had sporophore-specific expression (Munster et al. 2002); the study did not check for expression in the rhizome and roots.

MADS-box gene phylogenies suggest that the last common ancestor of ferns and seed plants had at least two different MIKC-type MADS-box genes that were homologs, but not orthologs, of the floral homeotic genes (Munster et al. 1997). The largely ubiquitous patterns of expression documented in seed-free plants suggest that the functions of MADS-box genes are different from those of flowering plants and more general (Theissen et al. 2000), not exclusively associated with reproductive organs. However, the fact that one *Ophioglossum* MADS-box gene is expressed only in the sporophore segment of leaves, and that some genes are

expressed in *Ceratopteris* sporangium primordia, may indicate that some MADS-box genes with functions in reproduction evolved in pteropsids independently from those of seed plants.

6.8.4   AP2 and ANT Genes

In angiosperms, these genes are involved in flower development, floral organ identity and ovule development. AP2 genes of the euAP2 subfamily have been cloned from *Selaginella moellendorffii* and *Ceratopteris thalictroides*, and ANT genes from *Ceratopteris thalictroides*, but their expression patterns have not been documented (Table 6.2). Based on gene phylogenies and the functions of these genes in angiosperms, Floyd and Bowman (2007) have proposed that the ancestral function euAP2 genes may have been meristem cell maintenance and control of the transition from vegetative to reproductive meristematic development (i.e., repression of transition to a reproductive state). Interestingly, while *Ceratopteris* shares with the seed plants a miR172 binding site on the AP2 sequence, that binding site is absent from the *Selaginella* AP2 (Axtell and Bartel 2005), indicating that regulation of euAP2 expression by miR172 evolved in euphyllophytes after their divergence from the lycopsids (Floyd and Bowman 2007).

6.8.5   FLORICAULA/LEAFY

FLORICAULA and *LEAFY* are positive regulators of floral homeotic MADS-box genes in angiosperms. *FLO/LFY* homologs have been identified in *Isoetes asiatica*, *Psilotum nudum*, pteropsids (*Botrychium multifidum*, *Angiopteris lygodiifolia*, *Ceratopteris richardii*, and *Matteucia struthiopteris*) and *Equisetum arvense*. In *Ceratopteris*, they are expressed in vegetative and reproductive shoot tips and in circinate reproductive leaves, with strongest levels recorded in the reproductive shoot tips. Because this expression pattern is different from that of *Ceratopteris* MADS-box genes, *FLO/LFY* probably have different functions in pteropsids from those of angiosperms, and do not act as positive regulators of the MADS-box genes (Himi et al. 2001).

6.8.6   Other Genes

Floyd and Bowman (2007) identified a series of other developmental genes in *Selaginella*, but their expression patterns are not characterized. Among these are NAC family genes similar to *CUC*; considering the organ separation functions of *CUC* in angiosperms (leaf primordium and leaf lamina lobe delimitation), these

may have had a role in apical branching in the early vascular plants. Homologs of the angiosperm shoot apical meristem maintenance genes *CLV* and WOX-family genes were identified, although among the WOX genes none is a homolog of *WUS*. Homologs of auxin transfer and response genes, *PIN*, *TIR* and ARF genes are also present in *Selaginella*. Of the TCP genes identified in *Selaginella*, none is regulated by miR319 as they are in angiosperm; this indicates that miR319 regulation of these genes implicated in cell division in angiosperms has evolved only in the euphyllophytes or maybe even only in seed plants (Floyd and Bowman 2007). Numerous GRAS family genes are present in *Selaginella*, including homologs of the gibberellic acid signaling regulators DELLA, and of the *SCR* and *SHR* root radial tissue patterning genes. The presence of *SCR* and *SHR* in lycopsids and seed plants, two lineages that diverged prior to the evolution of roots, suggests that these two genes may have been responsible for the radial patterning of tissues in the undifferentiated dichotomous axes of the earliest vascular plants. However, the functional homology of *SCR* and *SHR* across extant vascular plants, and even across extant euphyllophytes, has yet to be proven (Tomescu 2008). Rychel et al. (2010) identified in *Selaginella moellendorffii* genes of the EPFL family, putative ligands that influence stomatal density in angiosperms. Finally, YABBY genes are the only family of major developmental genes that seems to be exclusively present in seed plants (Floyd and Bowman 2007); they were not identified in any seed-free plant to date.

6.8.7 MicroRNA Regulation of Genes

Floyd and Bowman (2004) have shown that the miR165/166 binding site of HD-ZIP III genes is conserved across all embryophytes. This gene regulation mechanism is absent in *Chara*, which suggests that this mechanism may have evolved in the common ancestor of embryophytes. An ancestral function of HD-ZIP III genes seems to be the control of apical growth, therefore the advent of this mode of HD-ZIP III gene regulation shared by all embryophytes may have been important for the evolution of three-dimensional growth in land plant sporophytes (Floyd et al. 2006). However, an alternative interpretation is that HD-ZIP III regulation by miR165/166 was plesiomorphic in the common ancestor of embryophytes and it was lost and possibly replaced by another mechanism, in *Chara* (Floyd et al. 2006).

Axtell and Bartel (2005) found several microRNAs shared by *Selaginella uncinata* and *Ceratopteris thalictroides* with all seed plants; some of these are also shared with the bryophytes (also reviewed by Axtell and Bowman 2008). These document as many microRNA-target interactions that have been constant throughout plant evolution. The fact that all of these deeply conserved microRNAs are primarily involved in developmental programs in *Arabidopsis* suggests that they affected the morphology of plants throughout their evolution (Axtell and Bartel 2005). In some cases, the pattern of shared and derived microRNA-target interactions gives clues to the phylogenetic position of evolutionary events in the control

of development. Such is the case of AP2 control by miR172, shared only among euphyllophytes, and that of miR319 regulation of TCP genes found only in the seed plants (Floyd and Bowman 2007).

6.9 Conclusion

Seed-free vascular plants encompass most of the morphological diversity known among land plants. Six distinct body plans can be defined in seed-free vascular plants based on major vegetative features of the sporophyte (growth polarity, organography, root and shoot branching), and they are characteristic of the lycopodiales, selaginellales, isoetaleans, psilotopsids, sphenopsids, and pteropsids. However, the evolutionary history of all this morphological diversity is not well understood. In fact, except for the major phylogenetic divide between lycopsids and euphyllophytes, the relationships among the major vascular plant lineages remain unresolved to date (Rothwell and Nixon 2006). Furthermore, the developmental anatomy features that have yet to be documented in detail and sampled broadly, from a taxonomic standpoint, are not few. All of these will provide as many improved criteria for comparing morphology and will add resolution to phylogenies of vascular plants.

In this endeavor, an additional layer of information is added by the molecular genetic pathways that control developmental programs and which can be used in addressing questions of morphological evolution. A number of developmental genes characterized in seed plants, and especially in angiosperms, have been isolated in the seed-free vascular plants. An extensive survey by Floyd and Bowman (2007) revealed that most of the known plant developmental gene families are present in mosses, lycopsids, and seed plants, indicating that the developmental toolkit of seed-free vascular plants probably includes members of many developmental gene families present in angiosperms. However, to date, the taxonomic sampling of studies providing information on developmental genes remains sparse – significant numbers of these genes have been isolated only in *Selaginella* among the lycopsids and *Ceratopteris* among the pteropsids. Of the genes identified, few have been characterized in terms of tissue-level expression patterns, and even fewer have undergone functional studies.

The developmental genetic pathways that have received attention are the interactions between class 1 KNOX genes and ARP-type genes, implicated in leaf development, and the MADS-box genes. The little that is known on the functions of these genes suggests that MADS-box genes have much more general functions in seed-free plants and are potentially involved in meristem activity and growth, whereas KNOX1-ARP interactions reveal a complex mosaic of shared and divergent pathways that mirrors the different independent evolutionary origins of leaves. Aside from directly available information on genes in different lineages, inferences can be based sometimes on collateral data. Such is the case of the shared genetic mechanism that controls the development of cells with a rooting function in mosses and seed plants (Menand et al. 2007). The broad taxonomic range of this mechanism brackets, phylogenetically, all seed-free plant lineages which are, hence,

hypothesized to share the same pathway. However, overall, we are far from a complete understanding of the genetic controls of development in the seed-free plant sporophyte, which would enable comparisons among the different lineages and between those and the seed plants.

Functional genetic analyses in seed-free vascular plants are in their infancy. The establishment of transformation techniques for representatives of each of the different lineages is still a remote beacon, but as it gains momentum, it will produce a wealth of exciting new data. The next decade should see more than a few significant advances and new hypotheses in the fields of plant evo-devo and phylogeny and maybe even some paradigm shifts.

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Editors
Helena Fernández
Universidad de Oviedo
Dpt. Biología de Organismos y Sistemas
Area de Fisiología Vegetal
c) Catedrático R Uría s/n
33071 Oviedo
Spain

Maria Angeles Revilla
Universidad de Oviedo
Dpt. Biología de Organismos y Sistemas
Area de Fisiología Vegetal
c) Catedrático R Uría s/n
33071 Oviedo
Spain

Ashwani Kumar
University of Rajasthan
Department of Botany
302004 Jaipur
India

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