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Phyllotaxis from a single apical cell

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Abstract

Phyllotaxis, the geometry of leaf arrangement around stems, determines plant architecture. Molecular interactions coordinating the formation of phyllotactic patterns have mainly been studied in multicellular shoot apical meristems of flowering plants. Phyllotaxis evolved independently in the major land plant lineages. In mosses, it arises from a single apical cell, raising the question of how asymmetric divisions of a single-celled meristem create phyllotactic patterns and whether associated genetic processes are shared across lineages. We present an overview of the mechanisms governing shoot apical cell specification and activity in the model moss, *Physcomitrium patens*, and argue that similar molecular regulatory modules have been deployed repeatedly across evolution to operate at different scales and drive apical function in convergent shoot forms.

Convergent evolution of phyllotactic patterns in plants

Phyllotaxis – the regular arrangement of leaves around stems – is a primary determinant of plant architecture and amongst the most striking and elegant natural patterns [1]. Deciphering how the periodic emergence of leaves is coordinated in space and time at the apex of growing shoots constitutes an enigma that has puzzled biologists, mathematicians and physicists for centuries [2]. Besides some early studies in ferns [3], most experiments investigating the cellular and molecular mechanisms of phyllotaxis have been carried out in flowering plants, including *Arabidopsis thaliana* (thale cress), *Solanum lycopersicum* (tomato) and *Zea mays* (maize) [4]. In these plants, leaves bulge out at the periphery of the shoot apical meristem, a multicellular structure located at the shoot tip and that contains a stem cell niche at its center. The phyllotactic pattern is defined by the number of leaves forming at a given node (j) and the divergence angle between consecutive leaf initiation events (α). Phyllotaxis is frequently spiral with a divergence angle close to the golden angle ($j = 1, \alpha \approx 137.5^\circ$) as in *Arabidopsis* or tomato, but it may also be distichous ($j = 2, \alpha = 180^\circ$) as in maize, or reflect a few other arrangements [4,5]. Similar phyllotactic patterns are described in ferns, lycophytes, leafy liverworts or mosses but in comparison with flowering plants, they emerge from meristems with a simpler structure [6–9]. An extreme example is seen in bryophytes whose meristem is composed of a single apical stem cell (**Figure 1**). Evidence from phylogeny and the fossil record suggests that leaves (e.g. fern fronds, lycophyte microphylls) or leaf-like structures (e.g. bryophyte phyllids) evolved multiple times independently, which implies that despite compelling morphological resemblance phyllotaxis is a convergent feature of major plant groups [10]. The continuing development of the moss *Physcomitrium patens* as a model system beyond flowering plants offers unprecedented opportunities to investigate how phyllotaxis can emerge from a single apical cell and whether shared mechanisms underlie morphogenesis from unicellular and multicellular shoot apical meristems [11,12].

Apical cell shape and cleavage directs phyllotaxis

The production of phyllids (hereafter named leaves) in the moss gametophore (i.e., the gametophytic leafy shoot, hereafter named leafy shoot) is directly determined by

the activity of a single apical cell. Each asymmetric cleavage of the apical cell produces a leaf initial and perpetuates the apical cell itself to sustain shoot growth, as evidenced by live imaging of physcomitrium shoot buds [9]. The relationship between apical cell division angle and resulting phyllotactic patterns has mainly been inferred from histological observations of fixed specimens [6,13,14]. In some moss species, the apical cell is lenticular with two alternating division planes that produce opposite segments ($j = 1, \alpha = 180^\circ$), resulting in distichous phyllotaxis like in *Fissidens* (**Figure 1A, 1B**). In most species, the apical cell is tetrahedral, resembling an inverted pyramid, and produces leaves by successive rotating divisions of three cutting faces. Divisions parallel to apical cell side walls distribute leaves at fixed circumferential positions around the stem ($j = 1, \alpha = 120^\circ$), resulting in tristichous phyllotaxis like in *Fontinalis* (**Figure 1C, 1D**). Divisions may also be oblique to the side walls and successive leaves will be positioned at a slightly broader angle ($j = 1, 120^\circ < \alpha < 180^\circ$), resulting in spiral phyllotaxis like in *Physcomitrium* or the hair cap moss, *Polytrichum* (**Figure 1E, 1F**) [6,9]. Other less common scenarios have also been described, but in all cases, direct measurements of apical cell geometry, orientation of successive apical cell division planes and leaf divergence angles are still needed to quantitatively understand the functional link between cell division orientation in single-celled meristems and phyllotactic patterning [6,14,15]. To date, the molecular mechanisms controlling apical cell division patterns also remain elusive. However, the existing data suggest that understanding how the geometry and division plane orientation of the shoot apical cell are specified in the first place from the gametophore initial cell (i.e., the leafy shoot precursor cell) holds some of the keys to decipher how moss phyllotaxis is established and perpetuated in development.

Geometry distinguishes shoot initials

In physcomitrium, leafy shoots spring up from a network of filaments – the protonema. A small proportion of protonemal side-branches (c. 2-5%) becomes leafy shoots, while the majority gives rise to other filaments [16,17]. Although both leafy shoot and filament initial cells emerge as a localized swelling at the distal end of protonemal cells, several geometrical features allow them to be distinguished before the first division [9,18,19]. In comparison with filament initials, leafy shoot initials have a more

bulbous and irregular shape, a greater width and a wider division plane angle relative to the growth axis of the filament from which they emerge (**Figure 2**) [9,20]. These features might be related to distinct cellular growth modes: filamentous cells elongate by tip growth while leafy shoot initials might expand by diffuse growth [9,21]. Genes encoding cell wall remodeling proteins, including cellulose synthase (CESA), xyloglucan endotransglycosylase, expansin, cellulose synthase-like D (CSLD) and pectin methylesterase (PME) enzymes, are differentially expressed between filamentous cells and nascent shoot buds, which suggests that the different geometries and growth properties could result from different wall compositions [22]. Indeed, disruption of the *PpCESA5* gene leads to severe morphological defects in the leafy shoot, but not in the filaments [23]. Although apical cell specification is not affected in *ppcesa5* knock-out mutants, leaf emergence is impaired due to cellulose deficiency and associated defects on cell expansion, growth anisotropy and cytokinesis. This reveals a shared feature with flowering plants as *CESA* genes also play a key role in shoot morphogenesis and phyllotaxis in arabidopsis. *CESA1* and *CESA3* genes are expressed throughout the shoot apical meristem, and corresponding mutants have smaller shoot apical meristems with fewer cells and increased surface curvature than wild-type, which affects the distribution of phyllotactic angles in the inflorescence [24]. Other arabidopsis cell wall related genes such as *CSLD*, *PME*, *Xyloglucan α -Xylosyltransferase* or *α -xylosidase* are also essential for shoot apical meristem maintenance, leaf emergence or phyllotactic patterning, but their contribution in plants with single-celled meristems has not been explored so far [25–28]. Furthermore, it remains to be tested whether controlled changes of wall composition in the side-branches of moss filaments may be sufficient to trigger specific cell growth mode and fate transition.

Stem cell regulators and microtubules dictate division plane orientation

The apical stem cell governing physcomitrium leafy shoot morphogenesis is established by a series of four oblique divisions from the shoot initial cell (**Figure 2**) [9]. In contrast, filament initials divide exclusively perpendicular to the growth axis, which suggests that cell geometry might subtend division plane orientation, as described in other plant tissues [29–31]. Live imaging of shoot initials has revealed

that the angle of the first three divisions is robust, indicating that division plane establishment is tightly controlled and crucial for phyllotaxis [9,32]. This is also suggested by mutants where apical cell formative divisions are defective and leafy shoot development cannot proceed normally [33], such as *Defective Kernel1 (DEK1)* [34,35], *No Gametophores 1 (NOG1)* [18], *Targeting Protein for Xklp2 (TPX2)* [36] or mutants in the CLAVATA (CLV) signaling pathway [37].

Cytoskeleton reorganization is an integral feature of plant cell division [38], and the study of the microtubule-associated protein TPX2 has provided direct evidence for a role of the cytoskeleton in cell division plane positioning and shoot morphogenesis. The hypomorphic *tpx2-5* mutant produces stunted gametophores with obvious leaf patterning defects. This phenotype is explained by the abnormal displacement of the mitotic spindle (i.e., a microtubule-based structure that guides chromosome segregation in the two daughter cells) towards the basal side of shoot initials and the consequent mispositioning of the phragmoplast (i.e., an array of microtubules arranged perpendicular to the division plane that accompanies cell plate expansion towards the plasma membrane fusion site during cytokinesis) and, hence, the oblique division plane (**Figure 2**). This mechanism is dependent on actin microfilaments, as treatment with the actin inhibitor latrunculin B suppresses spindle position defects of *tpx2-5* mutants [36]. The gametosome, a cytoplasmic microtubule organizing center that forms in prophase, also plays a key role. Transient oryzalin treatment of shoot initials causing specifically depolymerization of gametosome microtubules is sufficient to alter spindle and division plane orientation [32]. Unfortunately, it has not been shown whether gametosome disruption affects development at later stages. Altogether, this shows that the transient deployment of microtubule-based structures is needed to tilt the division plane in shoot initials. In this context, it remains to be seen whether microtubule-dependent division plane orientation is prescribed by initial cell shape, mechanical forces, or both, as proposed in other plant tissues [39,40].

Beyond its structural role, the proper positioning of the cell division plane is likely needed to compartmentalize positional cues involved in the establishment of asymmetric cell identities. For example, the graded distribution of CLAVATA3/EMBRYO SURROUNDING REGION-RELATED (CLE) peptides may

provide positional information to specify distinct cell fates in arabidopsis [41,42]. Interestingly, transgenic mosses in which *PpCLE* expression has been silenced rarely form shoots with more than one leaf. The first and second division plane of *Ppcle* mutant shoot initials deviate from a normally oblique orientation and are often parallel to each other, which is not seen in wild-type plants (**Figure 2**). Altered division plane orientation is also reported in *Physcomitrium clv1a1b* double mutants and *receptor-like protein kinase 2* (*Pprpk2*) mutants and affects shoot morphogenesis at various stages. This role for CLV signaling is conserved in evolution as arabidopsis *clv1/barely any meristem 1* (*bam1*)/*bam2/bam3* mutants show abnormal periclinal divisions in root tissues [37]. Components of the CLV signaling pathway have first been identified as regulators of stem cell homeostasis in the arabidopsis shoot apical meristem. Perturbation of CLV activity in flowering plants typically leads to a variety of phenotypes ranging from moderate meristem enlargement to fasciation and massive cell overproliferation, correlating with a gradual increase in stem cell number and activity [43–46]. Such increase in meristem size perturbs leaf divergence angles in arabidopsis *clv3-2* mutants that transit through decussate and whorled phyllotactic patterns over their lifetime [47]. Overproliferation of ectopic apical stem cells was observed in moss *Ppclv* mutants, and exogenous treatment with PpCLE peptides is sufficient to suppress cell proliferation in wild-type shoots. Thus, *CLV* genes exert key roles in leaf initiation and positioning in mosses and flowering plants and may operate at different scales, through regulation of stem cell homeostasis, cell identity and division plane orientation. In moss, the analysis of *PPCLV* mutants highlights the functional link between cell division orientation and shoot architecture, and further work is needed to determine how PpCLV signaling controls microtubule-dependent division plane orientation.

Hormonal interactions control apical function

In addition to cell geometry and the above-mentioned molecular players, the phytohormone auxin influences microtubule distribution [48] and can act on division plane orientation control, as proposed in arabidopsis [49]. Whether a similar mechanism operates in unicellular meristems is unknown, but several lines of evidence indicate that auxin-based shoot patterning is a shared characteristic of

mosses and flowering plants (**Figure 3**). Pharmacological perturbations of polar auxin transport in tomato and arabidopsis [50–53] and genetic studies of auxin influx and efflux carriers have established a prominent role for auxin transport in phyllotaxis control [54,55]. Computational models and 4D quantitative imaging using auxin biosensors have allowed to define further that auxin transport functions in phyllotaxis by self-organizing zones of lower auxin concentration that act as inhibitory fields around organs, a mechanism that may account for the diversity of phyllotactic patterns observed in flowering plants [56–61]. Radioactive auxin transport assays have been used to assess the conservation of polar auxin transport beyond flowering plants [62,63]. These experiments have failed to detect long-range unidirectional auxin movement in the leafy shoot of four different moss species [62], and it has been proposed that auxin could flow by a diffusion-like mechanism in *physcomitrium* [64]. Nevertheless, treatment of wild-type moss with the polar auxin transport inhibitor, *N*-1-Naphthylphthalamic Acid (NPA), and/or the synthetic auxin, 1-Naphthaleneacetic acid (NAA), causes a range of developmental defects such as stunted shoots with fewer and narrower leaves, suppression of leaf outgrowth or loss of apical cell activity [65]. Canonical *PIN-FORMED* (*PIN*) genes are conserved in *physcomitrium*, and *PINA* and *PINB* are highly expressed in the gametophyte [65–67]. *pinA pinB* double mutants have twisted leaves and reduced leaf cell proliferation, resembling plants that accumulate auxin. They also display hypersensitivity to exogenous auxin, which causes phenotypes also seen in wild-type moss treated with both NPA and NAA, such as growth termination. This led to the proposal that PIN proteins sustain apical function and rhythmic leaf production by exporting auxin out of the apical cell and newly formed leaves [65]. Thus, both in mosses and flowering plants, PIN-mediated auxin transport regulates meristem function and leaf development.

Local auxin biosynthesis is also essential to shoot apical meristem activity in arabidopsis. Notably, PLETHORA (PLT) transcription factors determine lateral organ position by controlling auxin levels [68,69]. *plt3/5/7* mutant plants display abnormal phyllotaxis with an increase of successive 180° divergence angles. Transcript levels of *YUCCA1* (*YUC1*) and *YUC4* auxin biosynthetic genes are reduced in *plt3/5/7* mutants, PLT5 directly promotes *YUC4* expression, and heterozygous *yuc1 yuc4* double mutants tend to have distichous leaf arrangement [68]. It is worth stressing

that YUC-dependent auxin biosynthesis more likely occurs in developing primordia [60] than in the meristem center as previously proposed [68]. In *Physcomitrium*, four *APB* (for *AINTEGUMENTA*, *PLETHORA* and *BABY BOOM*) genes are orthologous to *arabidopsis* *PLTs*, and their expression is induced by auxin and associated with leafy shoot initials [16]. Quadruple *apb* mutants are unable to initiate leafy shoots and ectopic *APB4* expression raises shoot number, suggesting that *APBs* are necessary and sufficient for apical cell specification. *APB4* might regulate auxin biosynthesis [16], but this has not been tested at the transcriptional level.

Cytokinin promotes leafy shoot initiation in moss (**Figure 3**), probably by converting receptive cells to shoot apical cells, and this effect is enhanced by auxin [16,64,70,71]. Although *APB* expression is not regulated by cytokinin, *abp* quadruple mutants fail to respond to the synthetic cytokinin BAP, suggesting that auxin-induced *APBs* are required for cytokinin-dependent shoot initiation in moss [16]. Studies of *arabidopsis* *histidine phosphotransfer protein 6* (*ahp6*) and *abphyl1* (*abph1*) phyllotactic mutants have highlighted the importance of auxin-cytokinin crosstalk in flowering plant shoot development [72,73]. *AHP6* is activated by MONOPTEROS/AUXIN RESPONSE FACTOR5 and hence acts downstream of auxin to set the pace of organ production [72]. Unlike wild-type maize plants, *abph1* mutants display decussate phyllotaxis ($j = 2$, $\alpha = 90^\circ$), and this is caused by the disruption of a cytokinin-inducible type A response regulator (ARR) [74]. NPA treatment restricts *ABPH1* expression in the shoot apical meristem, *ZmPIN1a* expression and auxin levels are reduced in *abph1* mutants, and cytokinin promotes *ZmPIN1a* expression in an *ABPH1*-dependent manner. These results emphasize the importance and possible conservation of auxin-cytokinin interactions at the shoot apex of land plants and support a probable involvement in the regulation of moss phyllotaxis (**Figure 3**) [73].

Concluding remarks and future perspectives

Although shoot architecture diversified independently in the gametophyte and sporophyte generations of the plant life cycle [75], analogous shoot forms and phyllotactic patterns arose by convergence in both generations and their development is underpinned by a series of deep homologies (defined in [76] as “the sharing of the genetic regulatory apparatus that is used to build morphologically and

phylogenetically disparate (...) features”), as illustrated throughout this article (**Figure 3** and references therein). Further work is now needed to elucidate how conserved hormonal regulatory modules pattern phyllotaxis from single apical cells and how polarity cues are set at the single-cell level and translated into asymmetric divisions (see **Outstanding questions**). The concept of inhibitory fields introduced above comes notably from surgical experiments on fern meristems [3]. It is thus of particular interest to question whether auxin-based inhibitory fields exist in other spore-producing plant species, possibly at the scale of one or a few cells. Beyond mosses, these questions have started to be addressed in *Selaginella kraussiana* in which NPA treatment perturbs phyllotaxis and causes shoot apex termination [63], but the lack of a lycophyte transformation method hinders functional genomic analyses. Recently, an efficient genetic transformation procedure has been established for the fern *Ceratopteris richardii*, opening the possibility to bridge a huge gap in our understanding of plant form evolution [77]. The development of more spore-producing plant models will be pivotal to explore the mechanisms of phyllotaxis control in a broader evolutionary context and in meristems with increasing complexities [78].

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Figures

Figure 1. Phyllotactic patterns in mosses

(A, B) A single lenticular apical cell produces distichous phyllotaxis ($j = 1$, $\alpha = 180^\circ$) in *Fissidens taxifolius*. (C, D) A single tetrahedral apical cell produces tristichous phyllotaxis ($j = 1$, $\alpha = 120^\circ$) in *Fontinalis antipyretica*. (E, F) A single tetrahedral apical cell produces spiral phyllotaxis ($j = 1$, $120^\circ < \alpha < 180^\circ$) in *Polytrichum commune*. Spiral phyllotaxis is also typical of the model moss *Physcomitrium patens*. (A, C, E) Numbers indicate the sequence of leaf emergence, from the youngest “1” to the oldest visible leaf. In (C), the leaves numbered “3”, “6”, “9” and “12” are not visible. (B, D, F) “AC” marks the apical cell and numbers indicate the relative order and position of leaf emergence, “1” being the youngest leaf. Arrows indicate parastichies, j corresponds to the leaf number per node and α is the divergence angle. Image credits : (A, E) Hermann Schachner, <https://commons.wikimedia.org>, (C) Hugues Tinguy, <https://inpn.mnhn.fr>.

Figure 2. Asymmetric divisions underpin tetrahedral apical cell specification and proliferation in the moss *Physcomitrium patens*

(A) In *physcomitrium*, the gametophore initial cell (i.e., the leafy shoot precursor cell) develops from the protonema (i.e., the filamentous tissue produced by spore germination) and is characterized by an irregular and oblong shape (drawing to the left of the arrow). In wild-type (WT), the first division of the leafy shoot initial is oblique and produces apical (marked by an asterisk) and basal domains with asymmetric identities. In abnormal situations this division may be symmetrical and perpendicular to the growth axis (*Pp**cle* mutants) or skewed toward the basal side of the initial (*tpx2-5 hm* mutant), which may affect or prevent leafy shoot apical cell specification or lead to conspicuous leaf patterning defects. (B) In WT, following the first division of the shoot initial (i), the apical domain (marked by an asterisk) undergoes a series of oblique divisions (ii-iv) leading to the formation of the tetrahedral apical stem cell (coloured in green) at the origin of spiral phyllotaxis. (A) adapted from [9,36,37], (B) redrawn from [9] with permission from corresponding author.

Figure 3. Hormonal cues control apical function in moss and flowering plant leafy shoots

(A) Interacting auxin and cytokinin-dependent regulatory modules control shoot apical meristem function and phyllotactic patterning in *Arabidopsis thaliana*. Mutated forms of genes in bold are associated with phyllotaxis defects. (B) Gametophore apical cell specification and activity are regulated by at least partly similar regulatory modules in *Physcomitrium patens*. Mutants in genes shown in bold are unable to form gametophores or have apical cell and leaf developmental defects. Molecular interactions are inferred from the following references : (i), [54]; (ii), [50,51,56–58]; (iii), [79]; (iv), [69]; (v), [68]; (vi), [72]; (vii), [37,47,80]; (viii), [65]; (ix), [81]; (x), [16]; (xi), [82] and involve the following genes : *AUXIN RESISTANT 1 (AUX1)*, *LIKE AUXIN RESISTANT 1 (LAX1)*, *PIN-FORMED (PIN)*, *AUXIN RESPONSE FACTOR (ARF)*, *MONOPTEROS (MP)*, *PLETHORA (PLT)*, *YUCCA (YUC)*, *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN (AHP6)*, *ARABIDOPSIS RESPONSE REGULATOR (ARR)*, *CLAVATA3 (CLV3)*, *AINTEGUMENTA*, *PLETHORA* and *BABY BOOM (APB)*, *CLAVATA3/EMBRYO SURROUNDING REGION-RELATED (CLE)*. Molecular interactions occurring outside the shoot are not mentioned in the figure, e.g. auxin regulation of PLTs in the arabidopsis root [83]. Dashed lines in (A) indicate the indirect regulation of *CLV3* expression by cytokinin, via *Type-B ARR* and *WUSCHEL (WUS)*, and of cytokinin signaling by *CLV3* via *CLAVATA1* and *WUS*. In moss shoots (B), *PpCLE* and cytokinin have antagonistic developmental effects, but evidence for a direct or indirect interaction between *PpCLE* and cytokinin is lacking. Auxin biosynthesis, signaling and transport components are shown in blue, and cytokinin

494 signaling components are shown in green. Double lines indicate auxin-cytokinin
495 signaling crosstalks.