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# Design Principles of Branching Morphogenesis in Filamentous Organisms

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The radiation of life on Earth was accompanied by the diversification of multicellular body plans in the eukaryotic kingdoms Animalia, Plantae, Fungi and Chromista. Branching forms are ubiquitous in nature and evolved repeatedly in the above lineages. The developmental and genetic basis of branch formation is well studied in the three-dimensional shoot and root systems of land plants, and in animal organs such as the lung, kidney, mammary gland, vasculature, etc. Notably, recent thought-provoking studies combining experimental analysis and computational modeling of branching patterns in whole animal organs have identified global patterning rules and proposed unifying principles of branching morphogenesis. Filamentous branching forms represent one of the simplest expressions of the multicellular body plan and constitute a key step in the evolution of morphological complexity. Similarities between simple and complex branching forms distantly related in evolution are compelling, raising the question whether shared mechanisms underlie their development. Here, we focus on filamentous branching organisms that represent major study models from three distinct eukaryotic kingdoms, including the moss *Physcomitrella patens* (Plantae), the brown alga *Ectocarpus* sp. (Chromista), and the ascomycetes *Neurospora crassa* and *Aspergillus nidulans* (Fungi), and bring to light developmental regulatory mechanisms and design principles common to these lineages. Throughout the review we explore how the regulatory mechanisms of branching morphogenesis identified in other models, and in particular animal organs, may inform our thinking on filamentous systems and thereby advance our understanding of the diverse strategies deployed across the eukaryotic tree of life to evolve similar forms.

## Introduction

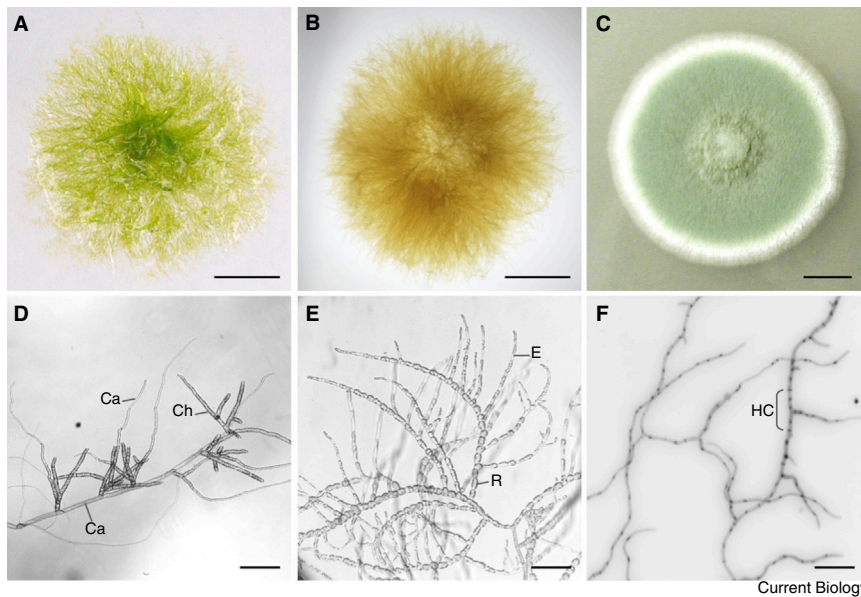
The evolution of multicellularity was a prerequisite to the diversification of complex morphologies in living organisms. The multicellular body plan (as defined in [1]) has three basic variants that can be described by the number of planes that orient cell division, which is typically mirrored in development by the dimensionality of tissue growth. A single cell that cleaves repeatedly in one plane gives rise to unbranched uniseriate filaments, cleavage in two planes gives rise to branching uniseriate filaments or pseudo-parenchymatous tissues, and cleavage in three planes leads to parenchymatous tissues [2–5]. Unicellularity represents the morphological condition preceding the origin of multicellular forms across the tree of life. The unbranched filament is likely the simplest and most ancient variant of the multicellular body plan, and filamentous branching forms represent a key step in the evolution of complex three-dimensional multicellular morphologies [6]. It is noteworthy that despite the constraints imposed by cell division patterns, development may also occur in three dimensions in branching filaments through the establishment of new growth directions when branch points are specified. Multicellularity arose repeatedly in the different eukaryotic kingdoms, leading to multiple independent acquisitions of branched filamentous forms. These forms have been maintained as a dominant (e.g., brown, red and green algae, fungi) or transient state

(e.g., moss and fern protonema) of the life cycle in many organisms, probably because they provide a simple and efficient way for spatial colonization and access to resources under the constraint of a sessile lifestyle (Figure 1) [7].

Branching is also a conspicuous morphological feature of multicellular organisms with parenchymatous bodies of varying complexity, including land plants [8], and numerous vertebrate and invertebrate animal organs such as the lung, kidney, mammary gland, prostate gland, vasculature (blood vessels) and tracheal system [9,10]. The regulation of their morphogenesis has been extensively investigated at the molecular and cellular levels, and exhaustive reviews have been written on these topics (see for example [9–13] for animal organs, and [14–16] for plants). In animals, recent work has successfully combined high-resolution microscopy and computational modeling to gain insight into the three-dimensional organization of branched organs, without extensively addressing the underlying molecular logic. These studies have resulted in the identification of global patterning rules and possible unifying principles of branching morphogenesis [17–21]. This body of research provides a timely opportunity to assess the extent to which these developmental regulatory principles apply beyond the kingdom Animalia.

Hereafter, we focus on major model organisms and related species from three different kingdoms that feature filamentous





**Figure 1. Filamentous body morphology in plant, algal and fungal model organisms.**

Gross morphology of single clones (A–C) and dissected branching filaments (D–F) in the moss *Physcomitrella patens* (A,D), the brown alga *Ectocarpus* sp. (B,E), and the filamentous fungus *Aspergillus nidulans* (C,F). Fungal hyphae were fixed and stained with Hoechst 33258 and Calcofluor to visualize nuclei and septa, respectively. Ca, primary (bottom) or secondary (top) caulonema cell; Ch, secondary chloronema cell; E, elongated cell; R, round cell; HC, hyphal compartment. Scale bars: A, 1 mm; B, 1 mm; C, 1 mm; D, 100  $\mu$ m; E, 150  $\mu$ m; and F, 20  $\mu$ m.

branching growth, including the moss *Physcomitrella patens* (Plantae) (Figure 1A,D; Box 1) [22,23], the brown alga *Ectocarpus* sp. (Chromista) (Figure 1B,E; Box 2) [24–26] and the ascomycetes *Neurospora crassa* [27] and *Aspergillus nidulans* (Fungi) (Figure 1C,F; Box 3) [28]. We present a comprehensive overview of the cellular, biochemical and physical processes underpinning branching control in filamentous branching systems, and reveal that their morphogenesis is governed by shared regulatory mechanisms and design principles, including lateral branching, intracellular control of branch position, local repression of super-numerary branches, apical dominance restricting branch outgrowth at a distance from the filament tip, coordination of branch spacing by a developmental clock, and contribution of mechanical and extracellular or environmental cues (Figure 2). We discuss the universality of these principles in the light of studies from animal organs and plant shoot and root systems when appropriate, and explore further how global approaches introduced above may translate to branched filamentous models and contribute to increase our understanding of branching morphogenesis across kingdoms.

### Fundamental Biological Differences across Kingdoms

At first sight, comparing filamentous branching organisms with branched animal organs may seem unwise since there are significant biological differences between them. From a functional perspective, branching allows filamentous organisms to expand in space throughout their lifetime and sustain resource acquisition in environments where nutrient availability fluctuates. In contrast, branching in animal organs is usually limited to embryogenesis. It occurs in a confined space and typically results in the formation of a network of hollow tubes, called ducts, ensuring organ-specific functions, such as draining fluids away (e.g., mammary gland, kidney) or exchanging gas (e.g., lung). In most cases, branched animal tissue development consists of the ramification of an epithelium (i.e., the outer layer of the duct) embedded in a supporting mesenchyme (i.e., a type of loosely organized connective tissue), and relies on various stereotyped

cell behaviors including cell migration, proliferation, rearrangement and deformation (see [9–13] for a detailed account on the cellular basis of branch formation in animal organs). Meanwhile, plant and fungal filamentous organisms form branches from existing filamentous cells rather than from a multicellular epithelium, and must carefully orient their growth since their rigid cell walls make them incapable of the rearrangements observed in animal tissues (Figure 3).

### Two Ways to Form a New Branch

Despite these differences, the formation of new branches shows remarkable similarities across kingdoms. Branching is characterized by the emergence of a growing structure, which may be a group of cells, a single cell or a sub-cellular compartment, that extends anisotropically, i.e. along a completely new axis of polarity. The addition of new growth axes may occur through two distinct processes: apical branching and lateral branching. Whilst apical branching is encountered only in fungal hyphae, lateral branching is also found in moss and algal filaments and stands out as a major convergent feature between these organisms (Figure 3). In animal organs, “clefing” characterizes the split of a branch tip into two or more new growing tips, while “budding” relates to branches forming from an epithelial surface or on the side of an existing branch [9]. Both branching modes are akin to apical and lateral branching, respectively, and may co-exist in the same organ. The observation that two superficially similar branching modes are shared across kingdoms raises the question of whether underpinning regulatory mechanisms are also common.

### Patterned Intracellular Cues Specify Branch Position

The position of branches at the cell level is often predictable in filamentous organisms. In brown algae, like *Sphacelaria* and to some extent *Ectocarpus*, branches are located at the most distal side of the cell relative to the filament base when emerging from cylindrical cells. A similar distal branching location is observed in cells of upright algal filaments which grow by intercalary cell division [29]. The position of emergence seems therefore unrelated to whether growth occurs at intercalary or apical positions. The distal position of branches is a shared feature with the moss *P. patens* and some studies in filamentous fungi have also revealed a general bias toward the distal end of sub-apical

**Box 1. The moss *Physcomitrella patens*.**

In the kingdom Plantae, filamentous branching forms are mainly observed in green algae, mosses and ferns. Mosses (*Bryophyta sensu stricto*) belong to the bryophytes, are spore-producing plants and constitute the extant representatives of early diverging land plant lineages. The moss *Physcomitrella patens* is amongst the most commonly used plant models for evo-devo, cellular and both reverse and forward genetic studies in plants (Figure 1) [127]. Since the release of its genome sequence over ten years ago [23], a significant number of related publications have provided major insights into the evolutionary history and morphological diversification of land plants (e.g. [4,79–81,128–136]). The life cycle of the moss *P. patens* starts with a transient filamentous developmental phase. Following germination, a spore produces two or more tip-growing filaments, called protonemata, comprising two cell types: chloronema and caulonema (Figure 3A–D). Chloronemal cells contain large round chloroplasts and ensure a photosynthetic function; they are slow growing and divide about every 20 hours. Caulonemal cells contain fewer chloroplasts and are longer than chloronemal cells. They are fast growing, divide about every 7 hours, and forage for water and nutrients [71,137]. Primary chloronemata spread radially across the substrate for five days or so before acquiring a caulonemal identity or ceasing growth. The transition from chloronemal to caulonemal identity may occur within one single cell cycle but is usually a gradual conversion occurring over four to six cells (e.g., see primary filament in Figure 3D) [138]. Side-branch cells form at a regular distance from the tip cell, usually the second sub-apical cell on chloronemata, and the third or fourth sub-apical cell on caulonemata [38,60]. At the fifth position starting from the tip cell, more than 90% of the sub-apical cells have side-branches. Considering all the side-branches, ~70% have chloronemal identity, 1% have caulonemal identity and 2% are leafy shoot buds (i.e., the developmental stage after the filamentous phase in the progression of the life cycle). The identity of other side-branches adapts to local environmental conditions.

compartments [30], although this is not a commonality of all fungi species. Several signals controlling branch position have been identified, including calcium ions, actin, and organelle and cross wall positions.

**Calcium Ions and Actin**

Calcium ions and the actin cytoskeleton determine the sites of branching in *P. patens*, *N. crassa* and several brown algae. Fungal mycelium treated with an ionophore capable of raising the cytosolic concentration of calcium ions shows increased apical branching, and this effect is enhanced by the exogenous supply of calcium ions [31]. Treatment of moss protonemata with papaverine, a calcium channel blocker, leads to severe defects in side-branch formation and patterning, including branch formation on both sides of a single cross wall [32]. Modulation of cytoplasmic calcium levels is thus central to the maintenance of filament polarity and branch patterning, and could affect morphogenesis by regulating actin network dynamics (Figure 3A) [33]. In the brown alga *Sphacelaria* and the giant kelp *Macrocystis*, the deposition of radiating actin cortical patches marks the prospective site of branch emergence [34], and actin depolymerizing drugs impair branching [35]. While *Ectocarpus* and *Macrocystis* belong to sister taxa, actin depolymerizing drugs have not yet shown any significant impact on branch initiation in *Ectocarpus* sp., although subsequent apical growth is largely disturbed (Figure 3E) [36].

**Organelles**

Aberrant nuclear placement may lead to developmental defects in various eukaryotic cell types, and polar nuclear movement is associated with root hair bulging in plant cells [37]. In *P. patens* and closely related species, nucleus position could be sufficient to specify branch position. Branches first initiate as a localized swelling at the distal end of the sub-apical cell, close to the nucleus (Figure 3A) [38]. As the side-branch bulges out, the nucleus migrates across to the base of the branch where the future cell wall will be formed [39]. Similar observations have been made in fern protonemata, and centrifugation experiments have shown that artificially changing nuclear location in the cell is sufficient

to modify branch initiation site [40]. Organelles also migrate to the branching site in many brown algae (Figure 3E) [41,42]. While the position of the nucleus remains unchanged in the initial steps, chloroplasts and mitochondria accumulate where the branch will form, concomitantly with active vesicle trafficking [43]. Further experiments are needed to test whether organelles are sufficient to determine the future branch site or ensure other roles such as providing the energy and materials necessary for branch initiation.

**Cross Walls as Landmarks**

In some filamentous fungi, pre-existing cross walls called septa might provide a landmark for branch emergence (Figure 3I) [44]. *Ashbya gossypii* is a filamentous fungus that bears a close phylogenetic relationship to *S. cerevisiae*. Like *N. crassa*, *A. gossypii* exhibits autonomous nuclear division within hyphal compartments and a rapid rate of hyphal extension [45]. Within a given compartment, nuclear division is more likely to occur proximal to lateral branch sites than it is elsewhere [46]. Notably, this bias is lost in the absence of functional septins, which indeed localize to the base of newly emerged branches [47]. An intriguing feature of *A. gossypii* compartments is the presence of regularly spaced septin ‘inter-region rings’ [47]. Because lateral branches tend to emerge in the vicinity of these rings, they could conceivably represent a marking system that plays a role in specifying sites of branch formation. Although septins are found in various algae and fungi [48], the potential conservation of such a role in species like *N. crassa* or *Ectocarpus* sp. remains unknown. Moreover, examination of growing hyphae in ascomycete fungi such as *A. nidulans* and *N. crassa* has to date failed to reveal overt patterns that make it possible to reliably predict where a new branch will appear. Given the apparent absence of a ‘branch site selection’ mechanism in these species, it seems more likely that branch formation is a stochastic process that shares some similarity with spontaneous polarization as described in budding yeast. It must be noted that, despite the absence of septins in mosses, cross walls contribute to branch patterning and may act as repressive cues, as discussed in the next section [48].

**Box 2. The brown alga *Ectocarpus* sp.**

Despite their gross morphological resemblance with green algae and a shared capacity for photosynthesis, brown algae (Phaeophyceae) have evolved separately from plants and independently acquired photosynthetic organelles through serial endosymbiosis [139]. The last shared unicellular ancestor of the SAR supergroup, or the Stramenopiles (including brown algae and diatoms), Rhizaria and Alveolates, diverged from other lineages at the root of the eukaryotic tree  $\sim 1.6$  Bya [140], but brown algae emerged as ‘true’ multicellular organisms only  $\sim 250$  Mya [36]. Brown algae may develop elaborate morphologies with apico–basal axes and bilateral symmetries. They display specific energy and primary metabolisms [141], cellular structural components [142,143] and genetic features [25]. They grow in environments characterized by a paucity of fresh water, high external hydrostatic pressure, increased viscosity and reduced perception of gravitational forces compared to conditions on land. *Ectocarpus* sp. is a microscopic filamentous brown alga which has emerged as a major model for the study of brown algae over the past 15 years, mainly because of its small size and its ability to grow and reproduce in lab conditions (Figure 1) [24,26]. The *Ectocarpus* sp. life cycle has two slightly anisomorphic multicellular phases, the sporophyte (i.e., the meiospore-bearing phase) and the gametophyte (i.e., the gamete-bearing phase). The sporophytic phase starts with an initial asymmetrical division of the zygote, followed by apical cell elongation and division on both sides along a single growth axis [61,144]. While intercalary growth subsequently takes place in the center of the filament, most growth is ensured by apical stem cells (A cells). Sub-apical cells (E cells) produced by successive apical cell divisions progressively lose their anisotropy and become round (R cells). This centripetal differentiation process results in a cell shape gradient, with highly polarized cells at the tip and round cells in the center of the filaments. About two-thirds of the branches are located on round cells (Figure 3E–G) [61].

**Local Inhibitory Cues Prevent Excessive Branching**

Although it cannot be anticipated with precision where branches will grow out in the fungi *A. nidulans* and *N. crassa*, it is well known that only one side-branch initiates at a time per cellular compartment. A limiting cell branching factor could account for this observation, but so far this hypothesis has received little experimental support in contrast with an alternative explanation whereby newly formed branches produce local inhibitory cues preventing further protrusions in their vicinity [49–52]. This represents another major unifying feature with mosses and brown algae, and is reminiscent in principle of lateral inhibition mechanisms identified in branched animal organs (Figure 2).

**GTPases, GAPs, GEFs and Septins**

During branch formation in *A. nidulans* and *N. crassa*, the asymmetric distribution of a key polarity factor (e.g., GTPases such as Cdc42 or RacA) could be locally amplified and coupled with depletion across the rest of the compartment (Figure 3I). In support of this view, deletion of *cdc42* in *A. nidulans* results in a near complete absence of lateral branching [53,54], whereas deletion of the presumptive Cdc42 GTPase activating protein (GAP) RgaA leads to increased branch formation [54]. Moreover, septins that are associated with branch positioning might play a role in repression of lateral branching [49,50]. In particular, the *A. nidulans* septins AspA, AspB, and AspC each localize to the tips of emerging lateral branches. Deletion of any of these septins results in increased frequency of branch formation as well as aberrant branching patterns, such as the emergence of multiple branch initials from the same hyphal compartment [49,50]. Similarly, septin mutants in *N. crassa* and the pathogenic fungus *Candida albicans* also display hyper-branching phenotypes [51,52]. It is not yet clear how septins might repress lateral branch formation, and these effects could indeed be an indirect consequence of primary defects in tip growth. Nevertheless, in the budding yeast *Saccharomyces cerevisiae*, the Cdc42 GAP Rga1 associates with septins to regulate the timing and pattern of bud emergence [55]. A similar link between the Cdc42/RacA GTPase module and septins in filamentous fungi could repress branch formation by maintaining Cdc42 in an inactive state

and thus preventing recruitment of downstream effectors required for polarized growth (Figure 2I). In the moss *P. patens*, Rho Of Plant (ROP) GTPases and guanine nucleotide exchange factors (GEFs) regulate protonemal cell polarity, as suggested by aberrant cell division patterns observed in plants overexpressing *PpROP2*. However, a shared role of GTPases in branch patterning remains to be properly demonstrated [56–58].

**Interplay between Myosins, Cytoskeleton Components, and Cross Walls**

In *P. patens*, microtubule and actin filament dynamics are perturbed in *ROP* RNA interference lines, which suggests that ROP signaling and cytoskeleton components could interact during branch site determination [58]. Direct evidence that branch positioning cues are tightly linked with short-range branch inhibition mechanisms comes from the observation of *myosin* mutants (Figure 3B). Myosin VIII is a motor protein that moves along actin filaments and pulls microtubules to guide cell division in moss filaments [59]. There are five *myosin VIII* genes in *P. patens* and quintuple *myo8* mutants display aberrant cell morphologies and cell division plane positioning. Mutants also form secondary side-branches earlier than wild-type and have an increased branch density due to multiple branching events from single cells. Together, these results suggest that proper positioning of the cell plate could locally prevent supernumerary branch initiation by controlling the spatial distribution of branching cues. It must be stressed that in *P. patens*, about one-third of branched cells bear two side-branches (Figure 3C) [60]. In contrast, more than one branch emerging from a single cell is rarely observed in other models like *Ectocarpus* sp. [61]. These observations suggest that the action range of the underlying inhibitory system is likely to vary depending on the studied organism.

**Receptor Kinase–Peptide Ligand Molecular Modules**

Branching morphogenesis in animal organs necessitates a molecular dialogue between the different tissue layers involved, and a recurrent theme is the requirement for receptor kinase–peptide ligand molecular modules [9]. Typically, localized ligand sources close to the developing organ (e.g., mesenchyme) are sensed through receptors located on the epithelium and

**Box 3. The fungi *Neurospora crassa* and *Aspergillus nidulans*.**

Like multicellular brown algae, green algae, and plants, most fungi are sessile organisms. However, they lack photosynthesis and their cell wall is made of chitin instead of cellulose. In multicellular forms, the fungal mycelium represents the vegetative part of the life cycle and is composed of interconnected filaments termed hyphae that radiate outward from a founding spore (Figure 1). Within the Dikarya, a major subkingdom of Fungi composed of Ascomycetes and Basidiomycetes, an individual hypha consists of multiple compartments (equivalent to cells) that are bounded by cross walls termed septa (Figure 3H,I). Compartments range from uninucleate to multinucleate in different species. The ability of a hypha to successfully explore the environment and acquire resources depends upon the presence of a functional hyphal tip, which is a distinct feature of the apical compartment (Figure 3H), and the formation of new hyphal tips through branching. As demonstrated in *Neurospora crassa*, slowing of hyphal extension and deformation of the hyphal tip typically precede the formation of apical branches [145]. Coincident with this, the Spitzenkorper also disappears (Figure 3H). The latter is a vesicle trafficking centre that is located just behind the hyphal tip and is composed of vesicles, ribosomes, and microfilaments [146]. Once hyphal extension resumes at its normal rate, two or more hyphae emerge from the deformed hyphal tip with each possessing a Spitzenkorper. In lateral branching, new hyphal tips typically emerge from sub-apical compartments that are separated from the actively growing tip by one or more septa, and branches usually emerge at an acute angle relative to the 'parental' hypha, as shown in *N. crassa* and *Aspergillus nidulans* (Figure 3I). This pattern likely facilitates efficient exploration of the surrounding substrate [117]. In *A. nidulans*, sub-apical compartments appear to be in a state of quiescence whereby growth and nuclear division are arrested [30]. Conversely, in *N. crassa* the compartments appear to remain mitotically active [147]. This contrast presumably reflects differences in the rate of hyphal extension (higher in *N. crassa* than *A. nidulans*) and the coordinate regulation of nuclear division in multinucleate hyphal compartments (autonomous in *N. crassa* vs. parasynchronous wave in *A. nidulans*). In both fungi, apical and lateral patterns of branch formation are not mutually exclusive and can be observed in the same hypha.

attract branch-forming cells [10]. The receptor–ligand-mediated signaling cascade drives the growth of protrusions towards the signal source, and tip cells are characterized by higher receptor activity than the proximal stalk cells. Tip cell selection and identity maintenance are associated with the phenomenon of lateral inhibition, often relying on Notch receptor–Delta ligand signaling [62]. The concept of lateral inhibition describes the ability of a cell to prevent its neighbors from adopting the same fate [62]. In this context, this mechanism inhibits the formation of unneeded additional tip cells within a certain distance from the tip, and thereby contributes to define and extend the branch axis [10]. Short-range inhibitory mechanisms described in filamentous organisms prevent a branched cell from changing its fate again and initiating another branch, and seem similar in principle to lateral inhibition (Figure 2).

A strong asset of computational modelling is to provide explicit frameworks to test *in silico* whether minimal gene regulatory networks are sufficient to recapitulate patterns observed *in vivo*. Using such an approach, it has been proposed that among various tested mechanisms only ligand–receptor-based Turing mechanisms are able to quantitatively account for tip branching patterns in both the lung and kidney, despite differences in the underlying molecular players and regulatory interactions [12,63–65]. The identified mechanism could represent a unifying solution for generating reproducible branching patterns [12]. Although some features of the branching patterns are not explained by the models, and it is not yet clear how ligand–receptor-based Turing mechanisms may be reconciled with the global regulatory principles identified elsewhere (see last section), these results highlight the importance of receptor kinase–peptide ligand molecular modules in branching morphogenesis.

Hundreds of receptor kinases and signaling peptides have been identified in plant, algal and fungal genomes [25,27,28,44,66], but only a small proportion of this expanding repertoire has been assigned a biological function [67] and

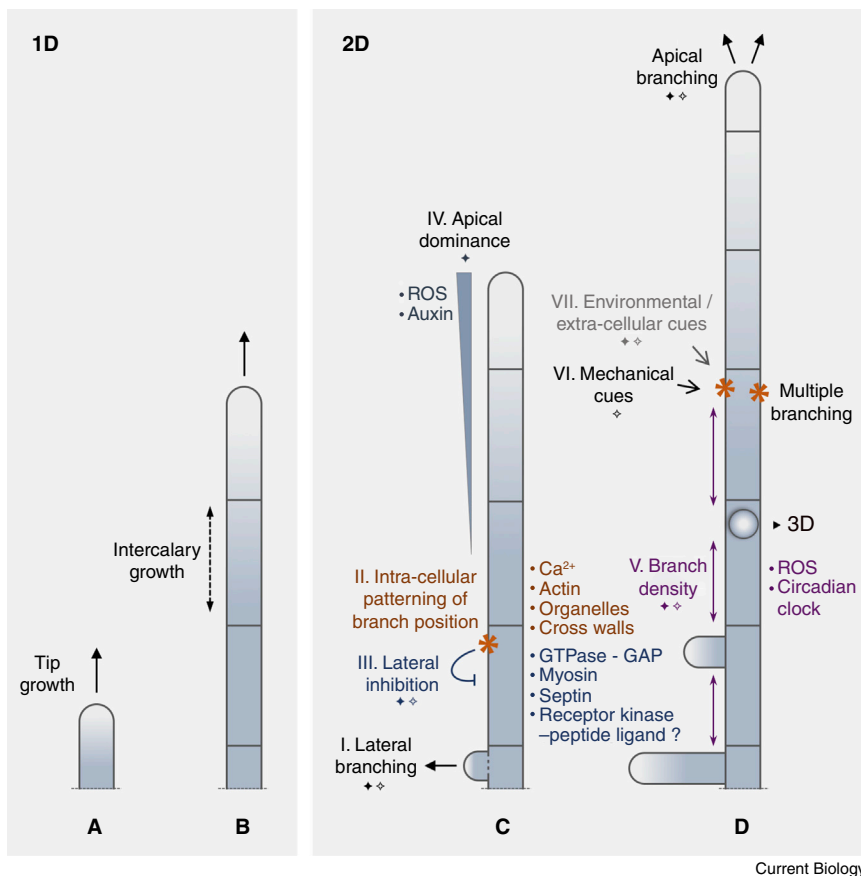
direct evidence for their involvement in filamentous branching morphogenesis is lacking. Yet, a recent study in *P. patens* has demonstrated the role of a CLE peptide–CLAVATA receptor-like kinase pathway in leafy shoot morphogenesis [4]. Leafy shoot initial cells typically bulge out from caulonemal filaments and undergo a series of asymmetric divisions [2]. After initial cell swelling, subsequent cell cleavages are misoriented in *c/e* and *clavata* mutant leafy shoot buds and lead to abnormal leafy shoots. These results suggest that receptor–ligand-based signaling pathways may operate at the sub-cellular level to control developmental patterning in moss. We hypothesize that similar mechanisms could pattern branch distribution in single filamentous cells. At a higher scale, an elegant study in the flowering plant model *Arabidopsis thaliana* has demonstrated that a receptor/peptide-mediated lateral inhibition mechanism ensures the proper spacing of lateral root founder cells at a microscopic level, which translates into the regular positioning of root branches at a macroscopic level [68]. This indicates that the contribution of receptor–peptide pairs to branching form development could be a shared feature of multicellular organisms, and should stimulate further research on this topic.

**Apical Dominance**

Besides short-range mechanisms restricting branch initiation at the cell level, apical dominance characterizes the ability of a filament tip to inhibit the growth of distant lateral branches (Figure 2). This notion finds its origin in vascular plant biology and relates to the control exerted by shoot tips over the outgrowth of lateral buds [69].

**Auxin Inhibitory Gradients**

It is well established that auxin, a small indole-derived signaling molecule, produced in shoot tips inhibits branch development at a distance and is a conserved regulator of apical dominance in land plant leafy shoots [8,70]. In moss, auxin promotes the differentiation of growing chloronemal cells into caulonemal cells



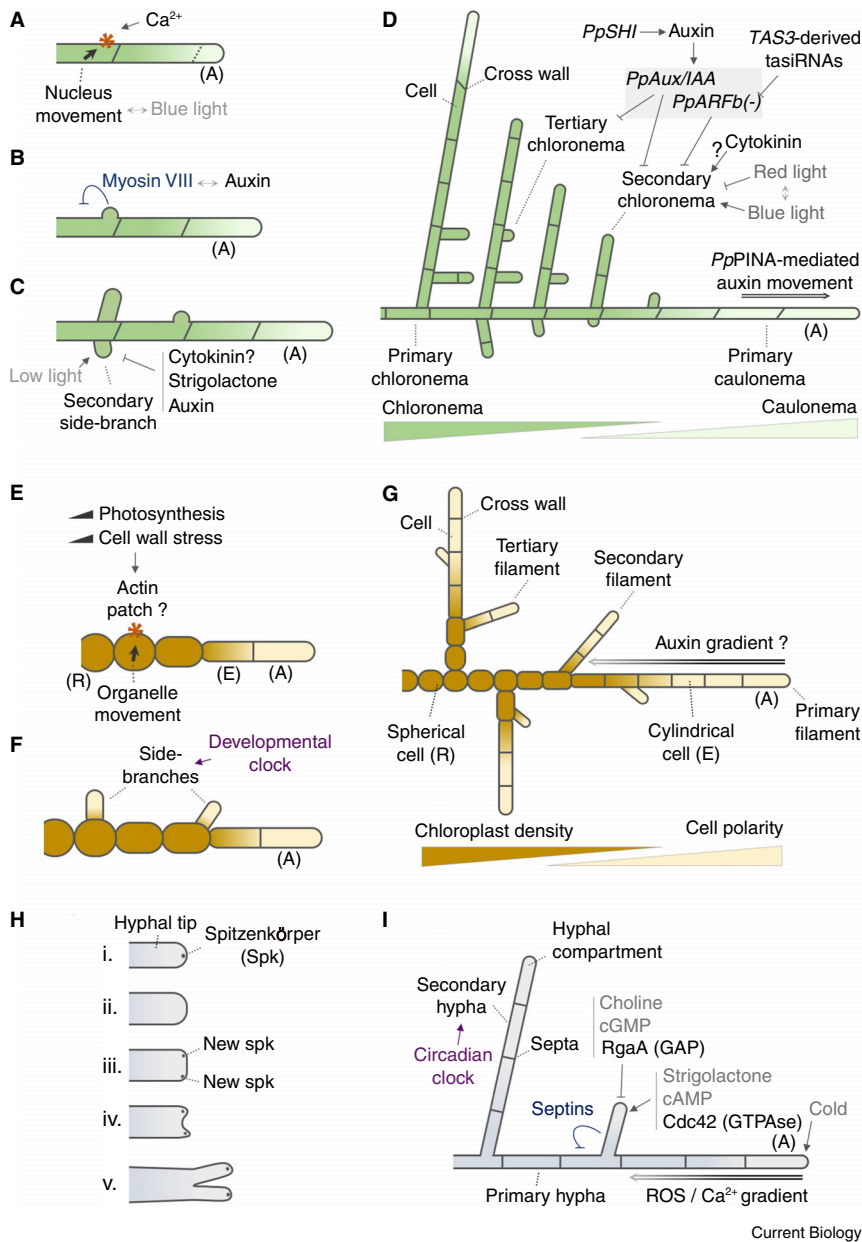
**Figure 2. Shared design principles and key regulatory mechanisms of branching morphogenesis in filamentous systems.**

(A) Filamentous organisms develop by tip (or apical) growth. (B) Apical cell growth and division extend filaments in one direction. Intercalary growth may contribute to the filament axis elongation from non-apical positions. (C) Formation of side-branch cells through lateral branching marks the transition from one-dimensional (1D) to two-dimensional (2D) growth. Apical dominance prevents branch outgrowth at a distance from the apical cell. In cells where apical dominance is ineffective, polarly patterned cues specify the position of the future branch. Lateral inhibition prevents supernumerary branching on branched cells. (D) As the filament develops, further lateral branches form along the main axis and branch density is regulated by various mechanisms. Branch outgrowth in a new direction leads to development in three dimensions (3D). In some species (e.g., *Physcomitrella patens*), several branches may arise from a single cell. Environmental or extracellular cues, and mechanical signals, modulate branch number. Apical branching is integral to the development of some organisms (e.g., filamentous fungi). (A–D) Roman numerals indicate design principles and regulatory cues of branching morphogenesis, and do not reflect the filament developmental stages. Diamonds indicate design principles shared between filamentous systems and plant shoot and root systems (filled diamonds), or branched animal organs (open diamonds).

[71–73], and independent pieces of evidence support a conserved role in filament branching control (Figure 3C,D). Although some reports have failed to detect significant effect of exogenous auxin on side-branch number [60], others have demonstrated that auxin inhibits branching by suppressing both initiation and growth of secondary and tertiary chloronemata. *PpSHI1* and *PpSHI2* genes promote auxin biosynthesis, and negative effects have been observed on branching in *PpSHI* overexpressing lines (Figure 3A) [73,74]. By contrast, the reduction of auxin levels or auxin response tends to promote protonemal branching. Plants treated with the auxin biosynthesis inhibitor L-kynurenine have twice as many branches per caulonema cell [73]. Auxin signaling has been extensively studied in plants, and involves ARF and Aux/IAA transcription factors and TIR1/AFB receptor proteins. Dominant mutations in a degradation motif of *PpAux/IAA* auxin signaling genes lead to a low auxin response and auxin resistance phenotypes [75–77]. Consistently, *aux/iaa* gain-of-function mutants have more secondary and tertiary side-branches. Trans-acting siRNAs (tasiRNAs) function as negative regulators of mRNA targets, and *TAS3* tasiRNAs target *ARF* mRNAs in flowering plants. In *P. patens*, *TAS3* tasiRNA biogenesis mutants show expanded expression domains of repressing *ARFb* auxin signaling genes and lack caulonemal filaments, which suggests that auxin-induced developmental responses are blocked [78]. Accordingly, *TAS3* tasiRNA biogenesis mutants show higher order chloronemal branching than wild-type plants [73,75,78].

Intercellular auxin movement in plants is mediated by plasma membrane targeted transport proteins, including PIN auxin efflux carriers [79,80]. PINA proteins are polarly localized in protonemal cells and seem to display a gradual expression along the filament with the highest level at the tip (Figure 3D) [79]. Although the exact location of auxin biosynthesis sites is not known yet, PINA protein expression pattern suggests that auxin is exported toward the filament apical cell, where it might accumulate and be exuded [73,79]. Protonemata grown in liquid culture export auxin into the medium, and export is enhanced in PINA over-expressors while it is reduced in *pina pinb* double mutants. Consistently, PINA overexpression causes an increase in branch number on caulonemal cells. However, loss of PINA and PINB function was not sufficient to prevent primary side-branch formation. Together, the above data suggest that auxin may coordinate branching along the filament axis via a base-to-tip concentration gradient, indicating that an auxin-mediated apical dominance mechanism might take place in moss protonemata. In contrast with moss filaments, auxin is transported away from the tips in flowering plant and moss leafy shoots [14,15,81], which suggests that base-to-tip auxin gradients could be generated through opposite directions of auxin transport.

Furthermore, the growth defects of the aforementioned *myo8* quintuple mutants are partially suppressed by exogenous auxin treatment [82], suggesting a close relationship between cellular auxin homeostasis and cell division plane positioning. This



**Figure 3. Regulatory mechanisms of filamentous branching morphogenesis in plant, algal and fungal model organisms.**

(A–D) Branching control in the filamentous body (protonema) of the moss *Physcomitrella patens*. (A) Protruding branch (asterisk) on the distal part of a cell relative to the filament base. 'A' indicates the primary filament apical cell. In experiments where only the nuclear region of a cell is irradiated with blue light, branches appear around the irradiated region [148,149]. (B) Lateral inhibition mechanisms prevent supernumerary branch formation on branched cells. (C) Secondary side-branch formation and cues regulating its development. Low white light levels stimulate secondary side-branch formation on primary filaments, high light levels limit chloronema branching [60,74]. (D) Protonema comprising two cell types, chloronema and caulonema, and molecular and environmental cues regulating its ramification. Unilateral low fluence red light inhibits side-branch formation while blue light promotes branching [150,151], while combining red and blue light has a synergistic effect on branching [148]. (E–G) Branching control in the brown alga *Ectocarpus* sp. (E) Detail of a branching filament with R (spherical or round), E (cylindrical or elongated) and A (apical) cell types, and local molecular and mechanical cues specifying branch position. (F) A developmental clock regulates branch density. (G) Filamentous tissue comprising two cell types, marked by isotropic cell shape and high chloroplast density, or anisotropic cell shape and low chloroplast density. A tip-high auxin gradient mediates apical dominance. (H–I) Branching control in the filamentous fungi *Neurospora crassa* and *Aspergillus nidulans*. (H) Apical branching is underpinned by the disappearance of the apical Spitzkörper (i to ii), and the specification of two new Spitzkörper (iii) followed by subsequent emergence (iv) and growth (v) of newly formed filament tips in distinct directions. (I) Branching filament (hypha), and molecular and environmental cues regulating its ramification. 'A' indicates the primary filament apical cell. Lateral inhibition prevents supernumerary branching. Extracellular signals such as strigolactone, choline, cAMP and cGMP impact branching [152,153].

in *Ectocarpus* sp. filaments [89,90]. Immunolocalization experiments support a model where auxin accumulates in apical cells and could be transported via micro-

relationship might be conserved in evolution as auxin affects cell division plane orientation in the *Arabidopsis* embryo [83]. Auxin is also known to activate ROP signaling, induce ROP nanoclustering at the plasma membrane and trigger polar nuclear movement in plant root epidermal cells [37,84,85]. While a similar interplay could contribute to branch positioning in moss protonema, the mechanisms by which local auxin levels are sensed and signaled along the filament axis still have to be elucidated.

In the brown alga *Ectocarpus* sp., support for apical dominance has been obtained by decapitation experiments in which the surgical excision of filament apices promotes branch formation. Importantly, auxin distribution in nature is not limited to plants. Among a wide range of unicellular to multicellular organisms, it is also found in brown algae [86–88]. Exogenous auxin supplied in the culture medium compensates for apex excision

in *Ectocarpus* sp. filaments [89,90]. Immunolocalization experiments support a model where auxin accumulates in apical cells and could be transported via microscopic intercellular channels called plasmodesmata to inhibit lateral branching at long distance (Figure 3G) [90]. However, independent experiments have shown that exogenous auxin may also promote branching in *Ectocarpus* sp. This suggests that cells along the filament probably have different auxin sensitivities, and auxin homeostasis must be locally regulated. Deep phylogenomic analyses have demonstrated that the ARF–Aux/IAA–TIR1/AFB-dependent auxin signaling pathway originated in the common ancestor to land plants and is not conserved in *Ectocarpus* sp. [90–92]. Thus, the transduction of auxin signals in brown algae remains an open question.

#### Branch-Promoting Cues

Although apical dominance is a repressive mechanism, branch-promoting factors have also been identified. Cytokinins constitute an important class of plant growth regulators that usually



have antagonistic roles with auxin. The coordinated action of both types of molecules is essential in a myriad of biological processes, such as branching control in vascular plant shoots and roots [15,16]. In the moss *P. patens*, cytokinins counterbalance auxin activity in the chloronema to caulonema developmental transition [60,93]. Interestingly, contrasting effects have been described with regard to filament branching. While some studies have reported that exogenous cytokinins increase the number of chloronemal cells with one side-branch [60,74,94], but also inhibit secondary side-branch formation [60], others have proposed that cytokinins tend to suppress secondary chloronema development (Figure 3C,D) [77,95]. These effects seem to be highly cell- and concentration-dependent, as a gradient of cytokinin sensitivity has been identified along primary filaments [60,96]. Downstream molecular mechanisms are not known yet, but cytokinins stimulate nuclear migration to the distal end of cells [97], which suggests that, as for auxin, their intracellular distribution may be important in regulating branch position. Cytokinins are also detected in brown algae and could play a role in apical dominance [42,89], but their function has not been reliably investigated due to a lack of functional approaches.

#### ROS and Variation in Apical Dominance Strength

In the fungus *A. nidulans*, apical dominance seems weaker than in *P. patens* or *Ectocarpus* sp., as it mainly prevents the emergence of branches from the apical hyphal compartment [98]. A tip-high gradient of reactive oxygen species (ROS) is required to enforce apical dominance in *A. nidulans*, as well as other species such as the endophyte *Epichloë festucae* (Figure 3I) [99]. The mechanism by which the ROS gradient interfaces with polarity factors and the morphogenetic machinery remains unknown, though calcium could conceivably play a role based on studies undertaken in plant root hairs [100].

Members of the Sphacelariales, a group of brown algae phylogenetically distant from *Ectocarpus*, initiate branches directly on the side of the growing apical cell or immediately behind the apical cell [42]. This suggests that tip-controlled branch inhibition mechanisms are ineffective or missing in these organisms. Variation in apical dominance strength reflects the activity of distinct molecular gradients and therefore constitutes a major source of morphological diversity across kingdoms.

#### Cues from Distinct Sources Control Branch Density

Another manifestation of spatial patterning mechanisms is the distance between neighboring lateral branches, also called branch density (Figure 2). A variety of patterns have been described, depending on the studied organism. For example, in the moss *P. patens*, side-branches tend to form on consecutive cells beyond the barren apical portion of the filament. In contrast, in *Ectocarpus* sp., adjacent branches are spaced apart by 3.5 cells on average. Although this could be regulated by short-range auxin gradient, as proposed in moss leafy shoots [81], stochastic variation of discrete cellular parameters may also account for this pattern. Any change resulting in either a local loss of the cell wall integrity or a temporary increase in turgor could account for the observation that only some cells initiate branching (Figure 3E), as discussed in the following section [61]. Cell shape and polarity may also considerably contribute to the process. For instance, the mutant *knacki* characterized by a reduced ratio of R cells in comparison to wild-type

displays a reduced branching frequency (B. Charrier, unpublished), and the *etoile* mutant that has cell polarity defects (90% of R cells against c. 50% in wild type) displays a high rate of multiple branching per cell [61,101].

Nevertheless, if cell rounding was the major cue for triggering branch formation in *Ectocarpus* sp., the pace of apical growth would be the main regulator of branch patterning, as it dictates the production rate of round cells. The *etoile* mutant reveals that neither the cell shape nor the apical growth rate are sufficient to promote branch initiation. In this mutant, while apical growth stops after the first few cell divisions, branching is maintained at the same pace as in wild type [101], resulting in a bushy phenotype with multiple branches on single cells [61]. This suggests the existence of a developmental clock controlling the rate of branching whatever its position along the filament (Figure 3F).

In *N. crassa*, branching density is maintained regardless of growth conditions, while other morphological traits such as the hyphal diameter and compartment volume are not. The homeostatic mechanism that coordinates branching with growth is dependent upon the integrity of the hyphal tip [102], and a systematic mutant screen revealed that it also requires multiple signaling functions, circadian clock-associated proteins and the control of ROS production [103] (Figure 3I). Although the molecular basis of these requirements is completely unknown, the role of ROS is intriguing, given that they also mediate apical dominance. Moreover, the involvement of circadian clock components could be a shared feature with *Ectocarpus* sp.

Together, this suggests that the regulation of branch spacing is informed by cues from distinct sources and may partly overlap with mechanisms underpinning apical dominance.

#### Mechanical Forces Control Branch Formation

Biophysical studies have established a functional relationship between changes in cell shape and branching. Using *Ectocarpus* sp. as a model, finite-element simulations have shown that specific parameter values of cell turgor, cell wall thickness and cell wall poro-elasticity are sufficient to trigger branching once the cell reaches a spherical shape and a size equivalent to twice the volume of the initial cylindrical cell [104]. This implies that cell turgor remains constant while cell volume increases, thereby requiring an absolute increase in water potential. Round cells are the oldest cells of the filaments, and during differentiation cell shape changes take place together with an increase in chloroplast density and chlorophyll content, which could promote branching by providing the energy necessary for branch emergence. Thus, branch initiation could be determined by a series of events starting by an increase in photosynthetic activity, which would trigger osmolyte accumulation, result in water entry, and promote cell expansion and rounding. Cell size increase would result in higher cell wall stresses [105], ultimately triggering wall protrusion (Figure 3E). This scenario is supported by laser-capture microdissection experiments of *Ectocarpus* sp. filaments showing that expression of photosynthesis-associated light-harvesting complex protein-coding genes increases during cell differentiation, with the lowest RNA level observed in A cells and the highest in R cells [106] (B. Charrier, unpublished). In the brown alga *Sphacelaria*, the number of mitochondria and chloroplasts also increases in branching cells [41]. However, in this case, observed changes are not a time-dependent cell maturation

process as non-branching adjacent cells remain highly vacuolated. Interestingly, cell wall thinning was also observed in several Sphacelariales [107]. Therefore, both cell wall thinning and accumulation of the energetic machinery at the growth location could be important, and perhaps in some cases sufficient, to trigger branch initiation and ensure its further growth. While changes in cell shape and chloroplast number are associated with cell fate transitions in moss protonema, the contribution of mechanical cues to cell branching has yet to be explored.

The involvement of mechanical signals in branching morphogenesis is a shared property with animal organs. For instance, epithelial and mesenchymal tissues feature distinct viscosities, which could generate mechanical conflicts contributing to branch development. Purely physical studies have shown that in a system where a less viscous fluid is displaced by one that is more viscous, the boundary of the two fluids is unstable and creates finger-like patterns [108]. Modelling the epithelium and the mesenchyme as fluids of different viscosities results in branching forms analogous to those observed in real organs [12]. Although experiments in the lung have shown that this effect is not sufficient to explain the robustness of biological patterns [12], the mechanical properties of the tissues certainly help to shape the organ. It has been shown elsewhere that neurons grown on substrates of varying rigidity exhibit different branch densities, providing direct evidence that the mechanical properties of the environment impact on branching morphogenesis [109]. In contrast with algae, mosses and fungi develop on stiff substrates, which could therefore play a role in branch patterning.

### Extracellular Cues Contribute to Branching Morphogenesis

Filamentous organisms are sessile, meaning that they must expand from a fixed location to reach the resources needed for their survival, and have all their cells permanently in contact with the outside. As a consequence, their development must be plastic to allow them to constantly adapt their shape to the local environmental conditions (e.g., light, nutrients, cold) (Figures 2 and 3). A challenge to fully understand the principles of branching morphogenesis in these organisms is to uncouple developmental plasticity from the ‘hard-coded developmental rules’ that specify a robust ontogenetic program invariable between individuals of a given species.

Certain molecules may act both as endogenous and extracellular regulatory cues, depending on their source. For instance, strigolactones are plant growth regulators that control architecture development and promote symbiotic interactions with arbuscular mycorrhizal fungi (AMF) [110]. Mycorrhizae are fungi that engage in a symbiotic association with plant roots. AMF that belong to the Glomeromycetes display increased hyphal branching in response to strigolactones produced by plant roots [111] partly to facilitate the recruitment of fungal partners. The effects of strigolactones on AMF appear to be pleiotropic, and include increases in mitochondrial activity and lipid metabolism and enhanced production of secreted proteins [112]. It is unclear whether strigolactones exert similar effects in other filamentous fungi. In the moss *P. patens*, *ccd8* strigolactone biosynthesis mutants have 40% more side-branch cells on primary chloronema (Figure 3C,D) [113]. Moreover, strigolactones are secreted

by moss filaments into the medium and sensed by surrounding colonies, which inhibits their branching and extension in a similar fashion to *quorum sensing* in bacterial colonies [113]. Although the molecular mechanisms that underlie the response of fungal or moss cells to strigolactones remain unknown, it has been shown that the function of key strigolactone signaling genes identified in flowering plants has diverged in evolution and is not conserved in *P. patens* [114].

Overall, the ability of extracellular signals to regulate lateral branching in filamentous organisms and the underlying mechanism by which any regulation might occur deserve some attention. It is noteworthy that in animal organs branch formation is also regulated by extracellular cues, for example produced by the mesenchyme. In this context, the environment may be considered as ‘internal’ in contrast with filamentous organisms for which it is entirely ‘external’.

### Computational Models to Bridge the Gap between Experimental Models

Recent studies in branched animal organs, but also in other systems such as plant leaves [115], have highlighted the need to image and quantify whole organs through time to understand how shapes emerge. Such global approaches help to extract fundamental morphogenetic properties from their apparent complexity, and to bring out design principles hidden by developmental variability. Notably, the quantitative comparison of observed and theoretical branching forms has led to the conception of simple models accounting for the developmental patterns of various branched animal organs.

### Simple Models of Tip-Driven Morphogenesis Explain Real Branching Forms

Organs such as the kidney or the mammary gland do not display overt structural regularities in their branching architecture [17]. By precisely comparing the 3D branching forms of developing and mature kidneys, it has been revealed that structural stereotypy exists, but is hidden by a global asymmetry induced by differences in branch size in the various kidney regions and local heterogeneities in branching rates [17]. Further analyses revealed that this global asymmetry reflects the self-similar nature of the kidney branching pattern, in which almost every branch tip bifurcation is asymmetric and results in child branches with unequal tip numbers [19]. This pattern is successfully captured by a three-state deterministic model, called the ‘half delay’ model, governed by simple rules defining whether each branch tip matures or bifurcates [19]. Overall tip number increases during kidney development, but tip spacing remains relatively constant, suggesting a tight coordination between tip bifurcation and extension [17]. The half delay model, supported by these observations, has led to a proposal that branching may be locally inhibited by neighboring tips to maintain adequate inter-tip distances and ultimately reach a functional organ form [19].

In contrast with the above example, detailed examination of the mouse mammary gland architecture has revealed that its apparent complexity may result from a stochastic decision process made at the branch tips [20]. It is proposed that all tips explore space randomly and may bifurcate or terminate with an almost equal probability [21]. Irreversible termination is triggered locally when a growing tip comes into proximity with a neighboring branch axis, called a duct. A computational model

of tip-driven morphogenesis integrating these three simple rules, and based on the theory of branching and annihilating random walks (BARW), is able to accurately capture the branching topology of the gland. This model is extended to the kidney and the prostate, and presented as a unifying theory of branching morphogenesis [21]. However, the BARW model contrasts in principle with the deterministic model proposed in the kidney and described above [19]. An independent study in the developing kidney has suggested instead little effect of the formation of nephrons (i.e., the kidney's functional units that comprise the ducts) on branch tip proliferation, and therefore do not fully support the universality of the stochastic tip branching and arrest model [18].

### Global Approaches May Reveal Essential and Shared Design Principles

Irrespective of the proposed model, a common outcome of the above studies is the central role of branch tips and their interaction with the local environment in branching morphogenesis. Along the same line, a filamentous branching system may be viewed as an ensemble of interacting tips requiring systemic growth coordination through local decisions. Empirical observations suggest that branching could be more stochastic in filamentous fungi and brown algae than in mosses, but this has never been formally tested. It has recently been demonstrated in fungi that long-distance propagation of molecular information in response to local perturbations, for example food availability, occurs via specific hyphae and is highly regulated in time [116]. Nevertheless, little is known about how individual tip behaviors affect branching decisions across an entire filament network [117]. At a higher scale, the 3D shoot system of a plant may also be viewed as a collection of interacting tips (called meristems) that must make “coordinated decisions without a centralized processing system” [118]. Functional studies in the flowering plant *A. thaliana* and the moss *P. patens* have proposed that the transport of auxin, either via the PIN-mediated polar auxin transport stream [70,119,120] or a diffusion-like mechanism [81], acts as an integrator of hormonal or nutritive cues to ensure global growth coordination. In these studies, deterministic models of branch initiation by hormonal control are able to capture the branching patterns of real plants. It remains to be tested whether such rules may apply to filamentous systems, notably in *P. patens* and *Ectocarpus* sp. in which auxin is a major morphogenetic cue.

We propose that such problems should be approached through a combination of computational modeling and experimental testing to try and identify further shared design principles linking evolutionarily distant organisms. Finally, we anticipate that this multiscale approach will help to determine the extent of overlap between the variety of branching forms observed in nature and those theoretically plausible, and to test further the long-standing idea that natural selection favors certain architectural types (e.g., self-similar structures [121,122]) and notably the most efficient branching forms for occupying space [7,123,124].

### Concluding Remarks

Although a capacity for branching has been acquired independently in mosses, brown algae and fungi, morphogenesis in these organisms is underpinned by shared design principles

and most notably branch repression mechanisms. Importantly, these principles are not restricted to filamentous organisms. Lateral inhibition also governs branching morphogenesis in animal organs or plant root systems [62,68], and apical dominance represents a major control mechanism of the three-dimensional architecture of plant shoot systems [8,69,70,81,125]. Although it is less clear how long-range inhibitory signaling affects morphogenesis in branched animal organs, this raises the question of whether branching may be a ‘default mode’ and inhibitory cues dominate over branch promoting factors. In support of this view, hyperbranching phenotypes associated with the loss of inhibitory cues are common in genetic screens for branching defects in *N. crassa*, but hypobranching phenotypes are rare [103,126]. Another unifying feature is that branching regulatory cues have acquired analogous morphogenetic properties by convergence in the different kingdoms, but operate through evolutionarily divergent molecular signaling pathways, as exemplified with auxin in *P. patens* and *Ectocarpus* sp. The extent to which this holds true for other identified molecular players has to be investigated, and whether this happened by chance or in response to particular developmental or evolutionary constraints remains an open question.

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