Chapter

Mosses: Accessible Systems for Plant Development Studies

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Abstract

Mosses are a cosmopolitan group of land plants, sister to vascular plants, with a high potential for molecular and cell biological research. The species *Physcomitrium patens* has helped gaining better understanding of the biological processes of the plant cell, and it has become a central system to understand water-to-land plant transition through 2D-to-3D growth transition, regulation of asymmetric cell division, shoot apical cell establishment and maintenance, phyllotaxis and regeneration. *P. patens* was the first fully sequenced moss in 2008, with the latest annotated release in 2018. It has been shown that many gene functions and networks are conserved in mosses when compared to angiosperms. Importantly, this model organism has a simplified and accessible body structure that facilitates close tracking in time and space with the support of live cell imaging set-ups and multiple reporter lines. This has become possible thanks to its fully established molecular toolkit, with highly efficient PEG-assisted, CRISPR/Cas9 and RNAi transformation and silencing protocols, among others. Here we provide examples on how mosses exhibit advantages over vascular plants to study several processes and their future potential to answer some other outstanding questions in plant cell biology.

Keywords: bryophyte, moss, model organism, plant development, regeneration, cell polarity, reprogramming, asymmetric division, stem cell, water-to-land, 2D-to-3D

1. Introduction

1.1 Mosses in context

Mosses are plants that belong to the Bryophytes, a cosmopolitan sister group of vascular plants with the last common ancestor between 400 and 500 million years ago [1, 2]. As a mostly avascular lineage, Bryophytes, that include mosses, liverworts and hornworts, thrive in mostly moist niches near the surface and stay compact (<10 cm), with some neovascularised exceptions that grow up to 65 cm [3–5]. Their cosmopolitan distribution in a variety of biotopes including moist and arid environments, can be explained by unique adaptations like drought, freezing and salinity tolerance [4, 6, 7]. Mosses' life cycle is dominantly gametophytic (the photosynthetic and growing phase is haploid), and the size and architecture of their organs is smaller and simpler than that of vascular plants, with leaf-like structures (phyllids) and sexual organs (antheridia and archegonia) of often only one cell of thickness, stem-like structures of circa ten cells and spore-bearing containers (sporangia) of single-cell spores [4, 8–10].

This miniaturised body renders mosses accessible systems for the study and dissection of cell and molecular aspects of plant biology that require close monitoring in time and space [11]. Such studies greatly benefit of the accessibility to single cells in a multicellular context. For instance, asymmetric and directional cell divisions are key life developmental tools to build an organism, but we lack understanding on how these processes are exactly controlled and regulated [12]. Importantly, these developmental drivers are shared between mosses and vascular plants, and to some extent with animals, and associated gene functions seem to be highly conserved despite the long period of independent evolution [13].

In the last two decades, mosses have gained high interest in plant research, with *Physcomitrium patens* (Hedw.) Mitten becoming a central model system. *P. patens* organelle genomes were sequenced in 2003 (plastids) and 2007 (mitochondria) and nuclear genome in 2008, with the latest revision in 2018. In addition to this, a myriad of genetic tools has emerged that allow close study of all processes of this moss as a representative of this lineage of the Bryophytes.

Hereby, we present how mosses, thanks to their simplified body plan and genetic networks in development, and with special focus in *P. patens*, can become cornerstone model organisms to study several developmental processes that determine plant architecture of most land plants [11]. We selected a number of outstanding developmental processes that pose central research questions in developmental biology of plants and that started to be investigated in mosses in the last years. These processes are introduced in a bottom-up approach, with special attention to their molecular and cellular basis, going from the early stages to the final plant organisation, chronologically. The essential and most used tools available to investigate these aspects of mosses are briefly described to facilitate the initiation into this model system. Finally, we show how the moss revolution has recently started with the rise in moss genomes sequenced and increase in moss research with additional species and questions beyond *P. patens*.

1.2 Moss morphology and life cycle

As most land plants, mosses have alternating generations between the haploid gametophyte and diploid sporophytes. However, unlike vascular plants, mosses spend most of their life cycle in the gametophytic stage, in which most of the organism asexual development, including photosynthesis and growth, occurs. The sexual organs eventually develop at this stage to give rise to the embryo after fertilisation, that produces the sporophyte over the gametophyte. This fruiting stage is diploid until haploidisation in spore formation takes place [11, 14].

Starting from a spore, the first developmental stage of the moss is the chloronema, a chloroplast-rich and single cell-wide filamentous tissue that serves for initial colony expansion, early photosynthesis and nutrient absorption. The cells are slightly elongated (~80 μm long), and the intercellular cell walls are oriented perpendicular to the growth direction [15]. This filament eventually transitions to caulonema, a quick-growing filamentous cell type that have underdeveloped chloroplasts at early stage, with longer and narrower cells (~250–300 μm long) that grow twice as fast, and with oblique intercellular cell walls [15–17]. This tissue has exploratory purposes and is favoured in stressful, light-poor, and nutrient-poor conditions, possibly with the aim of finding more suitable conditions [18, 19]. Caulonema can transition again to (secondary) chloronema [17]. The filamentous tissues are collectively referred to as protonemata and can laterally grow and divide to branch as new filaments. The protonemata grow in a mat-like fashion that shapes the two-dimensional (2D) developmental stage of mosses where the growth is confined in a plane of few millimetres of thickness.

Sometimes, the lateral cell outgrowth (the cell initial) gives rise to a bud cell instead of a branch cell, which is the beginning of gametophore development and the transition to three-dimensional (3D) growth [20]. The identity of the cell initial can be predicted by the division plane angle, implying that identity is determined before division (**Figure 1**). Currently, the list of known genes involved in the path selection and division plane orientation is growing, but the early determinants of bud formation and branching remain unknown [18, 21]. The bud grows by well-defined asymmetrical and oriented divisions to form the gametophore, the leafy shoot-like plantlet of mosses that ultimately bears gamete-producing organs.

The bud basal cell gives rise to a new type of filamentous tissue, the rhizoids, with pigmented, caulonema-like morphology. They function as anchorage to the ground to stabilise the up-growing gametophore and contribute to nutrient and water uptake, similar to roots and root hairs of seed plants, but with the tissue complexity of root hairs [22, 23]. The bud apical cells divide in precise directions to give rise to oriented phyllid initial cells with a particular phyllotactic pattern (i.e. lateral organ organisation around the shoot; e.g. spiral) to develop the gametophore.

The apical cells eventually arrest their proliferation, or terminally give rise to sexual organs (firstly antheridia, and later archegonia) under autumnal/spring conditions: short day (8 h), low light (20 μmol/m^2/s) and low temperature (15°C) [24, 25]. Despite the asynchronous development of male and female gametangia, this moss is self-fertilising, and thus tends to genetically self-isolate [26]. Flagella-driven spermatozoids (male gametes) move towards the archegonial venter in liquid water and fertilise the egg cell to give rise to a diploid zygote. The zygote will subsequently develop, via an embryonic stage with a new 2D-to-3D transition, into the sporophyte, that consists of the foot (the interface with the gametophyte) and a short stalk (seta) with a terminal capsule. In the capsule, meiosis gives rise to up to few thousands of haploid spores [8, 26].

The first documented ecotype, known as 'Gransden' (United Kingdom, 1962), has reduced rates of sporophyte formation, probably due to long asexual propagation in laboratories [27]. In many laboratory lineages, it has become self-sterile, rendering it unattractive for studies dependant on sexual reproduction. On the contrary, the more recently isolated ecotypes 'Villersexel' (France, 2003) and

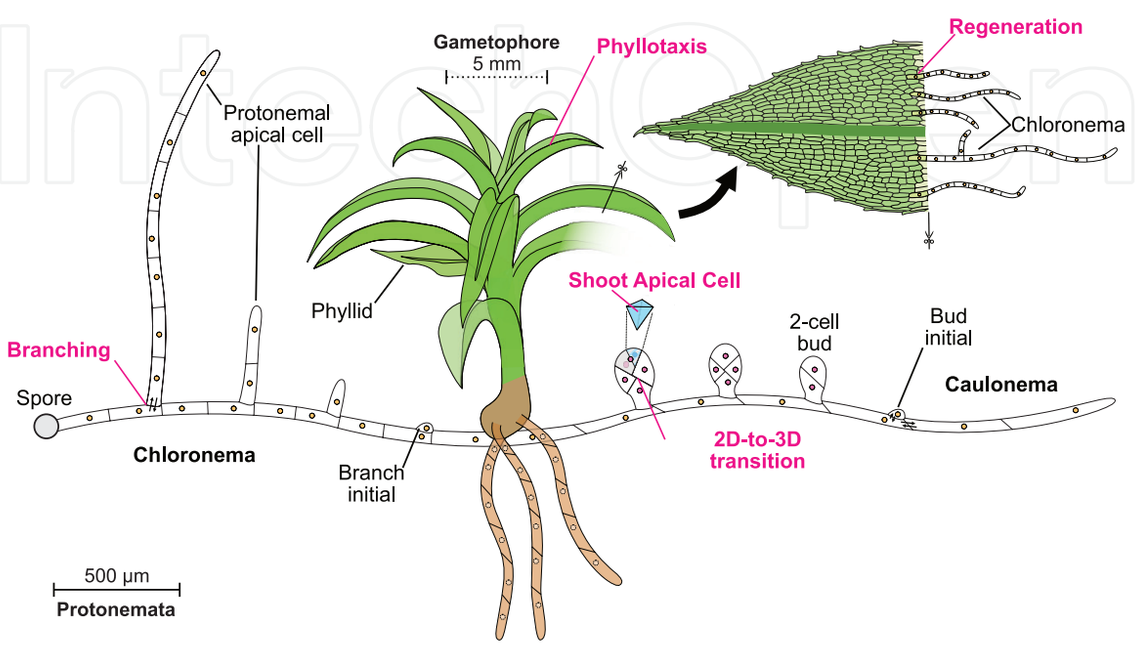

Figure 1.
Scheme of morphology and location of different developmental processes.

'Reute' (Germany, 2006) have 15 times more sporophytes (77% of total gametophores), indicating a high fertility rate [26]. Despite these differences, all ecotypes can be propagated asexually in identical conditions from any tissue thanks to the high regeneration rate of this moss, through which explants will redifferentiate into chloronema and initiate the life cycle from there [28, 29].

2. Outstanding developmental processes

Several essential developmental processes are shared between all land plants, including angiosperms and bryophytes. Strikingly, in mosses many shared processes take place with a simplified set of genes and sometimes in single cells. Therefore, the underlying genetic regulatory networks of development are easier to study. In this section, we highlight some of these processes and their unique ease of study in the moss *P. patens*.

2.1 The 2D-to-3D transition

The ability to structure organs in three dimensions (3D) was an essential feature for water-to-land transition. Many aquatic plants develop in a homogeneous environment, whereas land plants faced a highly distinct environment at ground surface level (a plane) and at the air/soil axis (perpendicular to this plane). This required land plants to develop specific tissues to efficiently grow in each dimension and cope with new challenges [20].

From a physiological perspective, the transition from 2D-to-3D growth in mosses consists in the development of complex structures such as the gametophore that grows out of the surface plane where protonema thrive, exhibiting negative gravitropism and positive phototropism [30–32]. The development of the gametophore relies on the ability of the moss to define different organisation in each dimension of space. The basal units from which this spatial assembly takes place are ultimately single cell primordia, from which organs emanate [33]. To this end, a cell must spatially sense intrinsic and extrinsic signals and transduce them to subcellular structures that pave the way to division plane positioning and subsequent asymmetric and oriented cell division. However, the molecular basis of spatial sensing and its transduction to organisation, action and maintenance of the division machinery has not been fully elucidated yet [12].

Cellularly, the 2D-to-3D transition in a moss occurs during bud formation (**Figure 1**). When a cell initial is formed in protonemata, in 5% of the cases it has a bud initial cell identity instead of a branch initial identity. Each bud initial swells and undergoes divisions oriented in all three axes of space [34]. This transition is supported and maintained by a different genetic and molecular machinery than that of protonemal development, and is the molecular basis of 3D growth. These proteins are specifically present from the first cell of the bud and onwards (e.g. DEK1, NOG1), and remains active in subsequent proliferating organs (e.g. shoot apical meristem, phyllids, gametangia) [13, 34–36]. In general, gradients or local clusters of proteins, peptides, nucleic acids, and hormones can be signals, sensors and/or actuators in developmental processes, and what is upstream of the cascade of 2D-to-3D transition remains a mystery. Some actors that are already on the radar include oscillations of auxin and cytokinin concentrations, ROP and SOSEKI proteins and CLE/CLV peptides-receptors, and they have been pinned down to different moments of the process and at different locations [12, 37, 38]. However, how do they coordinate their activity remains elusive and an active area of study.

In vascular plants, the 2D-to-3D transition occurs only once in their lifecycle, during embryogenesis, where orthologs of the essential moss 3D machinery genes

are also expressed [34, 35]. This event is confined in the endosperm during embryogenesis and its observation in seed plants requires seed and ovule microdissection, which makes *in vivo* monitoring difficult [39]. Furthermore, knockouts of some of these genes are lethal in this early stage. On the contrary, *P. patens* exhibits both growth fashions (2D and 3D) simultaneously and frequent transition events (bud formation) during all its vegetative stage in each colony (months), and deletion or functional mutants are non-lethal due to the indefinite growth character of the remaining 2D tissues [34, 35].

These features provide a privileged seat for *in vivo* long-term tracking and subcellular study of molecular markers, gene expression and protein localization that allows to shed light to the necessary cellular events required establish 3D growth in single cells to build a full plant.

2.2 The shoot apical meristem/cell (SAM/SAC) assembly

The protagonist of 2D-to-3D growth transition is the formation of the shoot apical meristem (SAM), a region at the apical growth side responsible for the continuous formation of the aerial organs of the plant, including leaves and reproductive organs [40, 41]. The histology of the SAM in angiosperms describes a central zone with stem cells that self-renew, divide and radially differentiate into peripheral cells that determine organ initiation at specific locations (e.g. by placement and establishment of new leaf primordia) [42]. While angiosperms present multicellular stem cell centre SAMs during their sporophytic phase, bryophytes present an unicellular structure, the shoot apical cell (SAC), both in their gametophytic and sporophytic phase (**Figure 1**) [43]. Despite the differences, both SAM/SAC share a common organisation, with stem cell(s) at the centre, surrounded by regularly differentiating tissue [44].

The mechanisms that establish and maintain these pluripotent stem cell(s) in the SAM/SAC is unknown. It involves spatial sensing, cell-to-cell communication, and asymmetric and oriented cell divisions, which render mosses attractive systems for their accessibility. *De novo* establishment of SAC is especially easy to study in mosses because it occurs once per bud formation (hundreds of events per colony) and in sporophyte development after egg cell fertilisation (on top of ~77% of gametophores). These SAC establishment events consist of one relatively exposed cell, that is easy to monitor during several division rounds for weeks [16, 26]. Angiosperms present a SAM and numerous equivalent lateral meristems, sometimes big and manageable (e.g. cauliflower meristems), but in general their study requires dissection for visualisation and it consists of complex multicellular structures that complicate characterisation.

The developmental origin of the sporophytic SAC in *P. patens* is either a *de novo* SAC establishment after egg fertilisation or a gametophytic SAC redefinition [45, 46], and in any case the genetic and signalling basis and developmental mechanisms of its establishment seem conserved between angiosperms and bryophytes [22, 47].

The genetic make-up in both taxa has shown to rely on auxin response through AUXIN RESPONSE FACTORS (ARFs), cytokinin signalling by ARABIDOPSIS RESPONSE REGULATORS (ARRs), CHASE domain-containing histidine kinases (CHKs) and CYTOKININ OXIDASE/DEHYDROGENASE (CKX), local coordination through several transcription factors families like CLAVATAs (CLVs), CUP-SHAPED COTELYDONS (CUC), LATERAL ORGAN BOUNDARY (LOB) and signalling peptides like CLAVATA3/EMBRYO SURROUNDING REGIONRELATED (CLE) and chromatin modification by Polycomb Repressive Complex 1 and 2 (PRC1,2), among others [48]. Although many key factors have conserved roles in SAM formation and maintenance in seed plants and mosses, some important factors in angiosperms, like the key regulator of stem cell maintenance WUSCHEL,

are not found in *P. patens*. These kind of differences can be insightful in defining the basic network to maintain *stemcellness*, tailoring a SAM and help understanding cell identity switch and organ formation [44].

2.3 Phyllotaxis from a cell

The most noticeable outcome of the shoot apical meristem/cell (SAM/SAC) activity is the organised and oriented initiation of leaf primordia along the stem, which leads to a unique geometric pattern of leaves and shoot branches named *phyllotaxis* [49]. In land plants, phyllotaxis may be defined by both genetic and environmental factors (light abundance, wavelength intensity ratio, etc.). For instance, leaf organisation can be adapted by shade avoidance syndrome [50]. However, only the genetic factors are shown to play a role in organ primordium location determination. A phyllotactic pattern is quantified by a fraction in which the denominator is the number of organs of the same type until the same orientation repeats (e.g. in *P. patens*, every fifth phyllid lies almost exactly below or above the first) and the numerator is the number of turns it takes (e.g. two turns in *P. patens*). This ratio (2/5) is then the fraction of a turn (e.g. 2/5 x 360°) or angle between two consecutive organs. When the angle between organs tends to the golden angle (137.5°, with fractions of turn derived from the Fibonacci sequence: 2/5, 3/8, 5/13, etc.), a spiral pattern emanates. Different angles can be observed in different species, like e.g. the 180° angle that gives rise to a distichous (or alternate) pattern or 120° for a tristichous pattern. Both Arabidopsis and *P. patens* follow a spiral pattern [43, 49, 51–53].

However, the pattern arises from essentially different SAMs/SACs: in angiosperms, phyllotaxis derives from a multicellular system with well-reported oscillating auxin peaks around the SAM growth axis, whereas *P. patens* effectively generates a pattern from a single apical cell (SAC). During the first division rounds in bud formation, the initial cell divides asymmetrically and gives rise to an inverted tetrahedral SAC with three lateral faces (**Figure 1**) [24, 54]. An oriented cell division of the SAC produces a new central SAC and a peripheral derivative cell, the merophyte, which develops into the future phyllid and a portion on the stem. The change of the stem cell division plane orientation in the SAC in each round results in a spiral phyllotactic pattern of the phyllids [55], which requires some unknown round-to-round cue to achieve rotation. Surprisingly, the rotation direction or chirality of the division orientations appears to be randomly determined, showing both clockwise (S) and counter clockwise (Z) patterns, yet there is high frequency of switch from one to the other (antidromy) in branches of gametophores of other moss species [56].

The limited understanding of the origin and underlying molecular mechanisms of this rotating pattern and derived phyllotactic pattern is largely confined to the sporophyte of the evolutionarily recent group of flowering plants (angiosperms). The available transcriptomic data of bud and tip cells and gametophores (or 3D shoots) may provide more insight in the transition from uniplanar to triplanar meristematic growth in moss [48].

Aligned with the phenotypic similarities of moss and angiosperm phyllotactic patterns, several factors known from Arabidopsis have also been found in mosses, including receptor signalling genes involved in shoot meristem size and patterning, hormone biosynthesis genes, transcription factors that control cell-specific mechanism of developmental pattering, chromatin remodelling complexes and cell cycle [48]. Many of these factors are essentially executive and likely controlled by some spatial sensing machinery. Comparing them with new contributors or absent members in the minimal regulatory network of mosses may help unravelling the

fundamental elements that trigger the orientation-specification machinery that greatly impacts plant architecture in all land plants, including relevant crops.

2.4 Regeneration

Most described processes in this chapter require cell identity acquisition and maintenance. In certain circumstances (e.g. wounding), differentiated plant cells can reprogram to become new stem cells, divide and redifferentiate for organ *regeneration* [57, 58]. *P. patens* is an excellent system to investigate cell reprogramming and regeneration due to its fast and broadly occuring cell pluripotency [59].

In most tissue cultures of other plant species, exogenous hormones (e.g. auxin and cytokinin) are required to induce callus formation and plant regeneration [60]. However, in moss, cells are capable of regenerating from protoplasts or excised phyllids into new protonema filaments in the absence of exogenous hormones (**Figure 1**) [61]. This implies that the whole regeneration toolkit is present in mosses and can be endogenously activated on demand, which makes them different from other taxa (e.g. angiosperms) [62].

When a phyllid is excised, cells neighbouring the cutting edge can reprogram from somatic cells to protonemal stem cells, which can then start a new life cycle [14]. This regeneration process is easy to study in mosses for several reasons: firstly, cell identity conversion can be easily tracked with protonema stem cell reporters [29]; secondly, aside of the simplicity of *in vivo* observation (see section *Imaging*), the unistratose (i.e. single cell-layered) phyllid simplifies single-cellular extractions (e.g. laser ablation) for single cell *omics* and other high precision studies [63]. Interestingly, the result of the reprogramming cascade is timely visible 48 hours after cutting, which also allows large scale collection of excised tissue for time-course tracking of gene expression evolution during regeneration activation [64].

The mechanistic studies of the cell fate acquisition can benefit from this simple cell type conversion in comparison to other model systems used in cell reprogramming investigation (e.g. regenerative callus or Arabidopsis roots that consist of multiple cell types that possess different tissue identity) for its minimality and event frequency [65, 66]. In angiosperms, regeneration is often reduced to localised stem cell pools (e.g. the base of leaves), takes longer to establish, and it is multicellular and asynchronous at the explant level [58, 67].

Previous studies have taken advantage of the abovementioned features to investigate gene expression profile during phyllid cell reprogramming, which revealed that genes involved in stress, proteolysis, and hormone signalling pathways are induced from 6 to 24 h after cutting [64]. Some genes have been demonstrated to play essential roles in moss leaf reprogramming, including *Cyclin-dependent kinase A (CDKA),* found to link cell cycle reactivation and other cellular responses that promote cell outgrowth as a new protonema filament [29]. Similarly, the outgrowth of reprogrammed protonema cell requires *WUSCHEL-related homeobOX 13-Like (WOX13L)* genes and *Cold-Shock domain Protein 1 (PpCSP1)*, induced in the cells facing the cutting edge within 24 h [68, 69]. Finally, an AP2/ERF transcription factor *STEMIN1 (STEM CELL-INDUCING FACTOR 1)* was discovered to induce cell reprogramming in moss leaves without excision or wounding [70]. These studies have identified new pieces in the puzzle of cellular reprogramming, and future studies will aim to unravel mechanisms behind the cell identity conversion and reprogramming.

One interesting feature of moss regeneration is inhibition of neighbouring cells. The necessary cell–cell (apoplastic e.g. Ca^{2+}-mediated) or cell-to-cell (plasmodesmata-mediated) communication makes regeneration an attractive developmental

process to study this cell crosstalk [71–73]. The phytohormone ABA is a key responsible of the dynamic regulation of the permeability of plasmodesmata in response to changing environments, such as wounding. Control in plasmodesmata pore size can influence the signalling molecules that can pass through or can be blocked in particular cells, which can have a direct effect in development of the processes mentioned until now [74, 75].

2.5 Hormone regulation

The signalling pathways and functions of plant hormones are substantially conserved in *P. patens*. Given the differences in physiological structures and relative evolutionary positions between angiosperms and bryophytes, mechanistic studies of hormone regulation in mosses can bring new insights in the hormone regulatory networks of all plants that resolve current questions.

Three plant hormones—auxin, cytokinin and strigolactone—have shown to regulate shoot branching patterns (phyllotaxis) and activation in angiosperms. Auxin moves down the main shoot of angiosperms to inhibit branch development, while cytokinin promotes branching. In addition to branching, auxin is the key molecule in the control of plant growth and development, and promotes organ differentiation [76, 77]. Exogenous application of auxin or its inhibitors results in irregular cell shapes and inhibit lateral organ formation, for instance in shoot apical meristem (SAM) maintenance. The understanding of hormone regulation and signalling in angiosperms progresses slowly due to tissue and gene network complexity. In *P. patens* interfering with auxin transport via the auxin efflux protein PIN-FORMED (PINs) knock-outs reveals the same effects on SACs as that have been observed in Arabidopsis SAMs. Also, the interaction between core components in auxin signalling and their response to auxin in *P. patens* is also conserved when compared to Arabidopsis [78–80]. Furthermore, exogenous application of auxin leads to termination of gametophore and differentiation into rhizoids, as it happens with shoots and roots in Arabidopsis [81]. In protonema cells, PIN-mediated auxin transport is essential for the chloronema-to-caulonema transition. When PINs are overexpressed, tip auxin levels deplete, which results in cell fate transition inhibition, while the PIN knock-out mutants show a faster transition from chloronema to caulonema [82]. Despite of these similarities, it has been shown that mosses may not weave their architecture with PIN-based transport as angiosperms do. On the contrary, they require bi-directional auxin transport to generate the observed patterns of shoot branching, as was confirmed by modelling and empirical evidence [83]. It derives that plasmodesmata-based transport may play a key role, which renders cell-to-cell communication essential in plant architecture definition and has not been reported in angiosperms [83].

Cytokinins and strigolactones also influence plant architecture, both in angiosperms and mosses. The levels of cytokinin are high and precisely distributed in the central stem cell region of SAM in angiosperms to maintain stemcellness [84]. In the root apical meristem, auxin and cytokinin act antagonistically in meristem size control, but its levels, distribution and interaction in *P. patens* single apical cell environment are unknown. Despite that, both hormones are present in this moss and are likely to play a role. The application of high concentrations of cytokinin in culture causes ectopic shoot formation and inhibition of leaf formation [83, 85]. Also, in gametophore development, cytokinin inhibits rhizoid formation by opposing auxin, like in roots of Arabidopsis. As expected by this homologous functionality, the mutants that stimulate cytokinin degradation lead to a strong increase of rhizoids in both number and length [86]. The last mentioned hormone, strigolactone, is reported to inhibit shoot branching in angiosperms and its localisation is

restricted to the base of shoots. The same compound is able to stimulate the pattern of shoot branching in *P. patens*. In filamentous tissues, strigolactone is produced to inhibit chloronema branching and to regulate the colony extension [15, 87].

As shown, many processes that define plant architecture are regulated in similar ways both in angiosperms and mosses. However, mosses offer a reduced gene network and regulation complexity that facilitates the analysis of hormone functions in the related developmental processes. Furthermore, subcellular and tissue-level transport and distribution of hormones can be best visualised in their simple plant bodies. In such plant models, new hormone functions will prove to be easier to study and translate to agronomically relevant plants.

3. Protocols and tools

Many valuable online resources with information on protocols, stocks, tools and genetic information have been exhaustively compiled elsewhere [11]. Hereby, we provide some additional information and summary of the essentials of research in *P. patens*.

3.1 Imaging

3.1.1 Accessibility

The small size and simple architecture of moss organs allows detailed microscopic visualisation easy to accomplish in almost all tissues. The strings of cells in protonema and their branching is trivial to closely visualise, and the transition to gametophores can be well tracked until the stem-like centre becomes slightly thicker than a dozen of cells and grows out of the plane. From it, the leaf-like structures (phyllids) have only one cell of thickness except in the midrib and can be tore apart for up-close visualisation. The terminal sexual organs (antheridia and archegonia) have a 3D structure that is easy to fully dissect or directly visualise due to their monolayered sack structures. The subsequently developed spore-bearing containers (sporangia) are full of single-cell spores [4].

This miniaturised body renders mosses accessible systems for the study and dissection of cell and molecular biology that require close monitoring in time and space. Such studies greatly benefit of accessibility to single cells in their context for observation of subcellular responses *in vivo,* e.g. protein localization, cytoskeleton rearrangement, and cell divisions along the developmental progress in a better resolution than most other multicellular plant tissues.

3.1.2 Reporter or marker lines

In *P. patens*, fluorescent marker lines that label different organelles (e.g. ER, chloroplasts, mitochondria, peroxisomes, Golgi apparatus, vacuoles, and nucleus) are available. In addition, given the predictable division patterns of the protonema tip cells, *P. patens* has been extensively used to investigate mitosis. Therefore, marker lines containing fluorescently labelled proteins such as several microtubule-associated proteins relevant to cell divisions were generated for mitosis imaging. Other published reporter lines show the concentration of the hormone auxin (DR5, GH3 and R2D2), cell identities, like protonema-specific proteins (RM09 and RM55) or mature rhizoids (RSL1,2), and developmental switches such as 2D-to-3D transition markers. In **Table 1**, references to all these reporters are indicated.

Visualised	Fusion/Target	Purpose	Reference
Nucleus	NLS4-GFP-GUS	Nuclear localisation	[21]
Endomembrane system		Endoplasmic reticulum	[11]
	α-1,2-mannosidase	Golgi apparatus imaging	[88]
	Targeting signal type 1 (SKL)	Peroxisome imaging	[88]
Mitochondria	Cytochrome c oxidase	Mitochondria imaging	[88]
Cytoskeleton	LifeAct	Actin cytoskeleton	[89]
	Tubulin α	Microtubule cytoskeleton	[90]
	MAP65	Antiparallel Microtubule-microtubule contacts	[91]
	Kinesins		[90]
Plasma membrane	SNAP-TM-mCherry	Membrane tracking	[21]
Auxin	pDR5v2:GFP-GUS	Aux. induced fluor.	[92]
	GH3:GFP-GUS	Aux. induced fluor.	
	R2D2	Ratiometric induction	
Protonema	pRM09:NLS4-GFP-GUS	Protonema identity reporting	[29]
	pRM55:NLS4-GFP-GUS	Protonema identity reporting	

Table 1.
Compilation of key molecular reporter lines published in literature to study cell and developmental processes in P. patens.

3.1.3 Microfluidics

Despite the advantageous physiological features of *P. patens*, observing cellular and subcellular processes with high resolution and for long periods of time is challenging. Traditionally, monitoring the intrinsic changes involved in regeneration, tip growth, bud formation, gametophore development and phyllid development, such as cytoskeleton organisation, protein distribution, organelle location, etc. has been done in glass-bottom petri dishes for as long as culture media could sustain, or in coverslip-sandwich sample preparations for up to few hours, due to lack of gas exchange [16, 37, 93–95]. However, the advent of microfluidics for bioimaging offers a new tool to overcome some limitations.

Microfluidic devices are transparent and flexible structures commonly produced using the biocompatible and air-permeable polydimethylsiloxane (PDMS) polymer. In biology, they have been used for study and imaging of cell and tissue development *in vivo*, including 3D development. For instance, it has been beneficial for high-throughput Arabidopsis root research [96]. Remarkably, the growth fashion of the pollen tube and the embryo development in Arabidopsis or the protonemal growth and early gametophore development of *P. patens* are ideal candidates for high-throughput and high-resolution imaging in microfluidic devices with light- and fluorescence-based microscopies [93, 97].

Until now, *P. patens*-tailored microfluidic devices have proven to be a reliable system for the monitoring of previously mentioned processes, as they offer tracking for up to weeks thanks to the air-permeability and possibility to refresh the media by circulating it from a reservoir [16]. Furthermore, it is then possible to introduce chemical agents or co-culture other organisms to image their cytological and gene expression effects on the plant over long periods of time [93]. The close tracking

allows for quantitative cell measurements such as biomechanic parameters, growth rate and size of different tissues, frequency and geometry of divisions, developmental time and pace studies, etc. Microfluidic devices can capture subtle phenotypes of mutant lines for full analysis and high-quality phenotype reporting [16, 93].

3.2 Manipulation

3.2.1 Forward genetics

Some groups carried out forward genetic screening by X-ray or chemical mutagens that generated mutants with hormone resistance or abnormal tropic responses [85, 98]. However, due to the lack of genomic information, the disrupted genes that caused the phenotypes were never identified.

Recently, the completion of genome sequencing and the establishment of its genetic mapping tools removed the obstacles in the forward genetic screening of *P. patens*. To establish genetic mapping, two genetically divergent ecotypes of *P. patens*, Gransden and Villersexel were used [99].

With this genetic mapping resource, in the past few years, researchers started to perform forward genetic screening by treating protoplast with UV light. In such screenings, mutants with phenotypic defects were successfully obtained and the causal lesions were identified by outcrossing and whole genome sequencing. Notably, essential genes that are crucial for growth may not be identified due to the lethality of their knock-outs; therefore, conditional screening was performed to overcome this problem. After the UV light treatment, plants were cultured under different temperatures and in such conditions, plants showed growth defect only in high temperature were selected as a temperature-sensitive mutant [100].

Another screening aimed to discover genes that are essential for the 2D-to-3D transition is also limited by developmental defects, given that mutants in this process cannot produce gametophores necessary for sexual crossing. To overcome this problem, instead of crossing, researchers generated somatic hybrids between Villersexel mutants and Gransden wildtype, which produced diploid sporophytes [34]. Spores released from this hybrid sporophyte exhibited consistent phenotypic segregation ratio with meiosis. Mutant plants generated from these diploid spores were sequenced and genomically mapped to achieve the identification of new crucial genes for moss 2D-to-3D transition (e.g. NO GAMETOPHORES 1 and 2, or NOG1, NOG2).

In addition to UV light, tobacco Tnt1 retrotransposon was used to produce insertional mutations in genic and GC-rich regions [101]. Both PEG- or Agrobacterium-mediated transformations were applied and successfully produced mutants.

3.2.2 Gene identification

P. patens was the first moss fully sequenced. Organelle genomes were sequenced in 2003 (plastids) and 2007 (mitochondria) and nuclear genome in 2008, with the latest fully annotated revision made and genetic mapping obtained in 2018 [47]. This information and the molecular tools available allow targeted mutagenesis to dissect functions of genes of interest. Additionally, full genome structure and SNP variation between four main ecotypes (Gransden, Reute, Villersexel and Kaskaskia) was reported in 2017, completing the toolbox for reverse genetics and bioinformatics research.

3.2.3 Neutral locus integration

The integration of DNA constructs (including promoter, gene of interest and selection cassettes) necessary to produce transformants with stable expression and

Locus	Vector	Purpose	Reference
PIG1 locus	pGX8 pGG626	XVE inducible overexpression XVE inducible RNAi expression	[102, 103]
Pp108 locus	pUGGi	Constitutive RNAi expression	[104]
Pp108 locus	pTH-Ubi-Gate	Constitutive expression by the maize ubiquitin promoter	[105]
Redundant copy of the ARPC2 gene	pTK-Ubi-Gate		
Redundant copy of the ARPC3 gene	pTZ-Ubi-Gate		
PTA1 locus	pT10G	Overexpression by EF1α promoter	[106]
BS213 locus	pMJ1		[107]

Table 2.
A compilation of vectors designed to target proven neutral loci in P. patens.

non-disrupting phenotypes requires targeting of neutral loci that do not intrinsically produce a phenotype when disrupted, often due to gene redundancy. In **Table 2**, there are several standard neutral loci indicated which reportedly showed no visible phenotypes or morphological defects when it is replaced by an entire gene expression cassette.

Currently there are several vector sets released to specifically target neutral loci, that contain their flanking regions homologous to parts of the locus at start and end of the vector. Cloning the gene of interest in between readily allows replacement of the targeted locus with the entire cassette via homologous recombination (see section *Homologous recombination*).

In *P. patens*, the gene loci have three commonly seen annotations in literature. The first and standardised since 2017 (with the third chromosome annotation version) is PpGcX_uuyyyVn.m, where *G* is the genome release version (version 3), *c* stands for chromosome, *X* stands for chromosome number (from 1 to 27), and *uuyyy* is an arbitrary flexible number that indicates the exact locus; *V* stands for version, *n* for annotation version and *m* for locus version. Previous nomenclatures and equivalences can be found elsewhere [108]. In **Table 2**, loci are named as the original publication for traceability.

3.2.4 Homologous recombination

P. patens possesses an extremely high capacity of homologous recombination, which allows researchers to alter moss genomic DNA in any desired endogenous locus [14]. A common workflow is gene deletion by replacement with an antibiotic cassette. Also, protein localization studies with endogenous expression level is easily achieved by fusion of the fluorescent gene reporter sequence right after the target gene. Some vector sets for knock-out and knock-in to edit moss genome have been established and can be requested from several research groups (see **Table 2**) [11, 109]. Due to the ancestral genome duplication events in moss evolution, there is high functional redundancy of several gene families that decrease the risk of unwanted ortholog disruption.

3.2.5 Targeted double strand break and directed repair (CRISPR/Cas9)

The game-changing CRISPR/Cas9 method has proven an efficient and effective tool in *P. patens* to achieve large deletions, localised knock-in and point mutations [110, 111]. Transient transformation, flexibility of selection strategy and easy cloning workflow has rendered CRISPR/Cas9 transformation an established tool for

P. patens research. Two groups developed whole CRISPR/Cas9 platforms independently with high editing efficiencies.

In Nogué's lab, a co-delivery method was developed where each element of the system (Cas9, sgRNA and selection cassettes) was present in a separate plasmid. In this method, Cas9 expression is driven by an actin promoter and ready to use as is. The selection strategy can be chosen freely due to the lack of integration, minding the presence of resistance in the background lines. This method is also suitable for multiple mutations in different genes at once, given that more than one sgRNA plasmid can be simultaneously delivered in one transformation with still sufficiently high efficiencies of transformation [110].

In Bezanilla's lab, a whole set of gateway destination vectors for CRISPR/Cas9 system was developed. The strategy was to design a vector set to finally put all the three essential components (Cas9, sgRNA and selection cassettes) in a single expression plasmid. In both protocols, the sgRNA can be designed and optimised using the online design tool CRISPOR V1 against *P. patens* genome Phytozome V11 [112].

To increase the accuracy of mutations, the CRISPR/Cas9 system is applied with a homology-directed repair (HDR), which allows for seamless knock-in or point mutation in desired sites. The template DNA can be a donor plasmid that harbours homologous fragments or oligodeoxynucleotides (ODNs) [111, 113]. By co-transforming the plasmid or ODNs together with CRISPR/Cas9 and sgRNA vectors, both methods show high accuracy to generate a desired point mutation or scarless insertion with a fluorescence tag at any suitable location of the gene.

3.2.6 RNA interference

Given that moss possesses a relatively big gene family, arguably due to its double genome duplication, the employment of gene deletion strategies might be inefficient to investigate gene functions due to the high redundancy rate (e.g. there are four ROP genes with highly homologous or identical sequences) [38]. For this, RNA interference (RNAi) strategies offer an alternative to overcome this problem, and the procedure has been well-established.

To generate an RNAi construct for a gene of interest, a DNA sequence of 300 to 1000 bp is subcloned in a destination vector. After standard PEG-mediated transformation, the silencing effect can be detected after 24 h and last up to 3 weeks [104]. To avoid lethal effects when constitutively expressing interfering RNA, an oestradiol-inducible RNAi system is available [102]. Coupling RNAi silencing activation with fluorescent reporters facilitates screenings of loss of function phenotypes [104].

3.3 Transformation

Standard transformation protocols have been applicable to *P. patens* for a long time. In **Table 3**, three methods are shown, with key protocol references for their experimental application. The most essential step after transformation is phenotypic characterisation, that is often performed at the colony level (as it is derived from the microscopic phenotypes). Some phenotypes that must be compared with the reference wild type ecotype include colony size, shape, colour, texture, gametophore count and ratio of gametophore number to colony surface. In the microscopic level, protonemal parameters such as chloronema and caulonema cell length, thickness, growth rate and transition and ratio of one to the other are valuable indicators of several hormone and developmental processes. Naturally, the phenotyping should include characteristics associated to the process of study.

Strategy	Highlights	Reference
PEG/Mannitol	One to two round selection, 10% of transformants, 3–4 weeks for stable transformants	[114–117]
Agrobacterium tumefaciens	Four-round selection, 100% positive transformants, 12–16 weeks	[118]
Particle bombardment	Easy to conduct; less used. Transient and stable DNA integration.	[119]

Table 3.
Summary table of the classical and current transformation techniques and reference protocols for application.

4. Beyond *P. patens*

Due to their accessibility, tractability and close yet independent phylogenetic position, the interest in Bryophytes has increased dramatically in the last decade [1]. Beyond *P. patens*, mosses have garnered special interest for their physiology and development, involvement in carbon sequestration, abiotic stresses management and biotic interactions. Eight species have had their nuclear genome sequenced and drafted in the last few years, and at least thirteen others are currently being sequenced [120–127]. Furthermore, the mitochondrial and/or plastid genomes of more than forty other moss species (not cited) has been published in the last six years, which may precede their nuclear genome study as with *P. patens*. This level of knowledge is an essential tool to dissect the molecular basis of processes under study, and the recent and future increase in the availability of this information is going to dramatically accelerate research in mosses, among other bryophytes [128]. In this section, a collection of mosses at the frontier of moss cell and molecular research are highlighted for their ecological relevance, distinct physiology and genetic composition, among others. We believe these will be the next generation of mosses for research that will provide new insights in plant research beyond *P. patens* in the coming years.

4.1 *Ceratodon purpureus* (Hedw.) Brid.

The fire moss *C. purpureus* (Dicranales, Bryopsida) is a cosmopolitan species that thrives in diverse ecosystems, including hostile post-wildfire or heavy metal-contaminated areas, and those with high radiation and freezing temperatures [129, 130]. The life cycle of this moss involves male and female haploid individuals due to the presence of sexual chromosomes. Consequently, it has become a reference for dioecious reproduction and sexual dimorphism, with some developmental differences in sexual and non-sexual features [131, 132]. In 2021, male and female nuclear annotated genomes were published, making *Ceratodon* the third sequenced moss genus [127]. Furthermore, gene targeting in this species has been proven effective, providing all the basic tools for cell and molecular biology research [133]. Importantly, the similarity of growth fashion between *C. purpureus* and *P. patens* will provide a new reference to study the discussed developmental processes in higher depth.

4.2 Hypnales W.R. Buck & Vitt

The Hypnales (Bryopsida) are the biggest and most diverse order of mosses with varied morphology, and mostly exhibit pleurocarpous (i.e. non-erect) growth fashion. It includes *Fontinalis antipyretica* Hedw., *Pleurozium schreberi* (Brid.) Mitt. and *Calohypnum plumiforme* (Wilson) Kučera & Ignatov, the genome sequences

of which have been published in the last two years [123, 125, 134]. *P. schreberi* is attractive due to its documented symbiotic relationships with N_2-fixing cyanobacteria and *C. plumiforme* for bryophyte-exclusive biosynthetic gene clusters research [123, 134]. As pleurocarps, all of them exhibit a non-erect plant architecture that suggests an adapted regulation of stem development.

Remarkably, the common aquatic moss *F. antipyretica* is a globally distributed species and serves as a reference organism for the study of land-to-water habitat reversal and its genetic basis. From the developmental processes' perspective, this moss has a distinct interest due to its tristichous phyllotactic pattern (120° rotation from organ to organ) that can serve as a reference in the investigation of genetic regulation of asymmetric cell divisions in the shoot apical cell at the gametophore apex (see **Figure 1**) [24].

4.3 *Sphagnum* L.

The genus *Sphagnum* (Sphagnales, Sphagnopsida) plays an important ecological role in the climate change situation, as its species are important carbon fixators. For this reason, The Sphagnome Project was created in 2018 in the aim to sequence fifteen species across the genus [124]. At this moment, the genomes of *Sphagnum fallax* and *Sphagnum magellanicum* have been published [124]. From the developmental point of view, *Sphagnum* spp. are attractive due to their branching gametophores and subsequent implications in lateral shoot meristems and phyllotaxis, which is different from *P. patens* and *C. purpureus*. Furthermore, *Sphagnum spp.* do not show rhizoids and some species are mostly or fully aquatic, serving as models for land-to-water reversal. Remarkably, *Sphagnum* spp. do not develop filamentous protonemata as most mosses, but thalloid protonemata (i.e. disk-like, bidimensional), as that of liverworts [135].

4.4 Polytrichopsida Doweld

The moss class Polytrichopsida is the second biggest (~200 species) after Bryopsida (~11500 species), and its species have unique morphological characteristics that make them uniquely interesting for developmental biology [5]. Despite mosses being regarded as avascular plants, some exhibit hydroids and leptoids, a functionally analogous tissue to tracheids and sieve elements of vascular plants that slightly differs morphologically and developmentally. Polytrichopsida has several genera with such structures, in some cases underdeveloped and, in others, completely functional, like in *Polytrichum* and *Dawsonia*, with up to 65-cm tall gametophores [5, 136]. This distinct characteristic suggests that xylem-like structures evolved independently and thus the genetic and molecular machinery necessary to its development may have similar origins to that of fern, gymnosperm, and angiosperm vasculature. Another attractive feature of several genera of Polytrichaceae is the perpendicular lamellae on the unistratose phyllids, that represents an increase in leaf complexity and has proven to be an alternative evolutionary path for increased photosynthetic capacity.

Despite its potential to provide valuable insight, genetic tools have barely been developed for this taxon, but full mitochondrial and plastid genomes have recently been published for *Polytrichum commune*, a cosmopolitan species ~10 cm long that has a complete stem vasculature [137, 138].

4.5 Other mosses

The desert moss *Syntrichia caninervis* Mitt. (Pottiales, Bryopsida) is the first outstanding example of dessication-tolerant moss to have its genome sequenced [126].

This genome will provide tools to dissect the development of the unique sub-micron structures of its phyllids that stimulate water capture and what genes are involved in the asymmetric growth and divisions necessary for this structure [139].

The heavy metal-tolerant moss *Scopelophila cataratae* (Mitt.) Broth. (Pottiales, Bryopsida), capable of thriving in copper-rich environments, has had its genome drafted (unpublished, 2016) and CRISPR/Cas9 mutagenesis demonstrated [121].

Funaria hygrometrica Hedw. (Funariales, Bryopsida) is evolutionary close to *P. patens*, and they have virtually identical morphology at the gametophyte generation, but remarkably different sporophyte generation. *F. hygrometrica* has a longer seta, different mechanisms and regulation of spore release [140]. However, the level of difference in their transcriptome is unexpectedly high and transcripts seem to be shifted in expression time but not in sequence. The recently published genome may allow investigate how time-shifted expression of regulators impacts morphology [120].

5. Conclusions

We have shown how mosses are an increasingly relevant model group to plant developmental biology due to their distinct accessibility at physiological and molecular level. Their evolutionary distance with agronomically relevant plants does not diminish their potential to help to understand fundamental questions of development that remain unsolved, given that most essential regulatory networks are conserved. This has been shown in the hormonal regulation of branching and shoot and root development, regeneration, the establishment of shoot apical cells and the genetic make-up in 2D-to-3D transition. The utility of mosses has convinced the scientific community to the point of promoting the sequencing of eight new species to explore other physiological processes in the last few years. We have also shown how *Physcomitrium patens* is a workhorse in cell and molecular biology of plants, and provided evidence that it is likely to become a standard tool of plant developmental biology together with a number of other mosses.

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