Chapter 2

Usefulness of *Physcomitrella patens* for Studying Plant Organogenesis

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Abstract

In this chapter, we review the main organogenesis features and associated regulation processes of the moss *Physcomitrella patens (P. patens)*, the model plant for the Bryophytes. We highlight how the study of this descendant of the earliest plant species that colonized earth, brings useful keys to understand the mechanisms that determine and control both vascular and non vascular plants organogenesis. Despite its simple morphogenesis pattern, *P. patens* still requires the fine tuning of organogenesis regulators, including hormone signalling, common to the whole plant kingdom, and which study is facilitated by a high number of molecular tools, among which the powerful possibility of gene targeting/replacement. The recent discovery of moss cells reprogramming capacity completes the picture of an excellent model for studying plant organogenesis.

Key words: *Physcomitrella patens*, Bryophytes, Organogenesis, Morphogenesis, Gene Targeting

1. Introduction

In contrast to metazoans, organogenesis in metaphytae occurs postembryonically and throughout the organism's life. Plant organogenesis is an iterative process: it is accomplished through the activity of multicellular meristems, which continuously divide and differentiate to form the basic unit of plant architecture, the phytomer. In shoots, each phytomer corresponds to a leaf and a stem internode, and these units are piled up along the growth axis, frequently displaying phyllotaxis, to give rise to the plant body. As plants cannot move to escape to unfavorable developmental conditions, coordinated divisions and differentiation steps ensuring both plasticity and adaptability are critical for plant life (1).

Bryophytes represent the extant-living descendants of the earliest plant species that colonized earth ca. 470 million years ago (2).
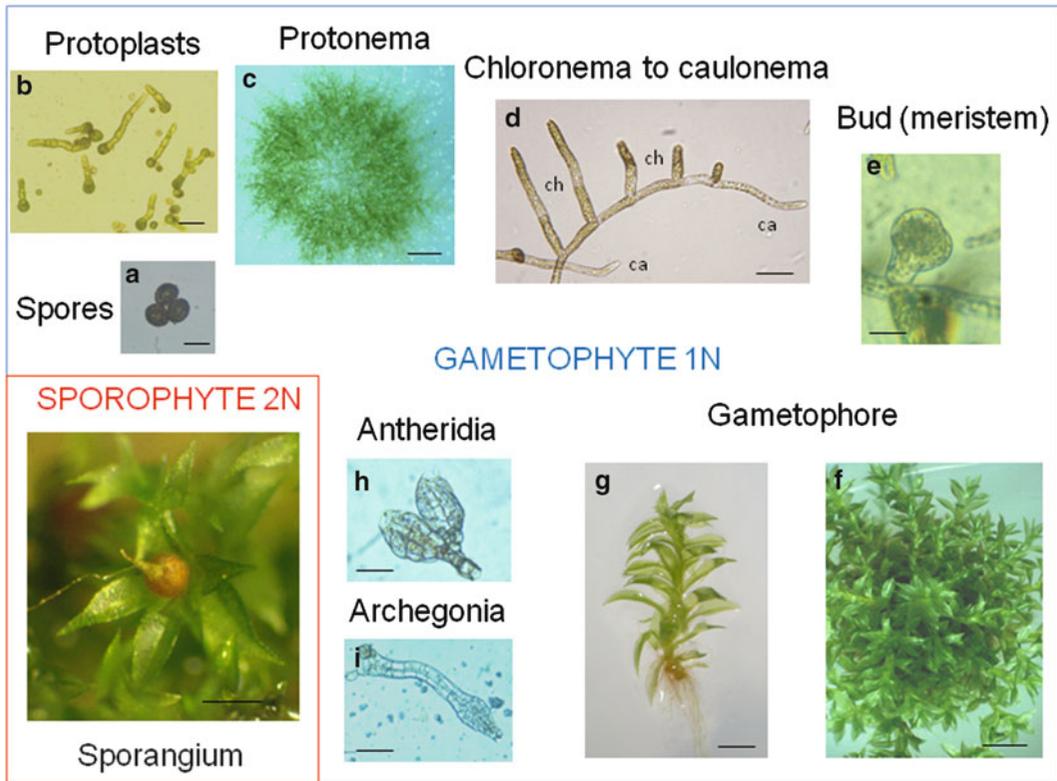
Fig. 1. Spores (**a**) or regenerating protoplasts (**b**) will give rise to a filamentous protonema (**c**) formed by chloronemata and caulonemata (ch and ca in **d**). The chloronema to caulonema transition is visible at the apex of the filaments. Transition to bushy growth is initiated with the differentiation of meristematic buds (**e**) that further develop into leafy shoots (**f**, **g**). Antheridia (**h**) and archegonia (**i**) form at the apex of the gametophores. Water dependent fertilization occurs in the archegonia and the fertilized egg cell gives rise to the brown diploid sporangium in which meiosis occurs and spores differentiate. Scale bars **a**, **e**, **h**, **i**: 20 μm; **b**, **d**: 50 μm; **c**, **g**, **j**: 1 mm; **f**: 0.4 cm.

Despite their relative morphological simplicity, Bryophytes share most of the biochemical, genetic and developmental processes that characterize the biology of modern plants (3–5). Mosses have proven extremely valuable systems to undertake biological research at the cellular level, while the dominance of the haploid gametophyte in the life cycle facilitates genetic approaches. Over the last decade, the moss *Physcomitrella patens* (*P. patens*) has become the reference model in moss biology: the outstanding efficiency of targeted mutagenesis (6, 7) combined with the availability of completely sequenced genome have been essential in its development (8–10).

P. patens development has been extensively reviewed (see (11) and references therein) (Fig. 1). Briefly, a haploid spore germinates to establish a juvenile developmental stage, the protonema. This two-dimensional filamentous protonema is essentially formed by two distinct cell types, the chloronema and the caulonema, that expand by tip growth and multiple divisions of apical and subapical

cells (12). Protonema development is determined by a few single cell developmental transitions that can be modulated by external factors and followed *in vivo* with non-invasive technology (Table 1). It is the most characterized developmental stage of mosses (13, 14) and provides an ideal material to combine molecular, genetic and biochemical approaches to study multiple aspects of plant cell biology. Yet the morphogenetic processes underlying moss protonema development have little analogy with those controlling spermatophyte's organogenesis, except maybe for the differentiation of root hairs and pollen tubes.

The transition to three dimensional bushy growth is determined within single caulonema subapical cells in the protonema (Fig. 1). These cells enter into three cycles of asymmetric cell divisions to establish the bud, a primitive meristem formed by a single tetrahedral apical stem cell (i.e., with three cutting faces). During this developmental transition cell expansion switches from tip growth to diffuse growth. The apical stem cell then continuously divides to form a new stem cell and a basal primordia that will further differentiate into a stem internode and a leaf (15). Subsequent leafy shoot development is achieved by the reiterated differentiation of phytomers from the shoot apical stem cell, as in other land plants. Yet, there is no polar auxin transport in moss shoots (16) and the meristematic activity that generates patterning is confined to a single apical stem cell. In *P. patens*, the adult gametophyte is composed of numerous unbranched leafy shoots carrying leaves displaying phyllotaxis along the stem axis, and of filamentous rhizoids that differentiate from shoot epidermal cells. These filamentous rhizoids constitute the root apparatus of the plant (mosses have no roots) and are involved in substrate fixation and colonization, and in nutrient uptake. Despite the morphological simplicity of the plant, several organs or differentiated tissues can be recognized in leafy shoots. Transverse sections through the stem reveal the presence of an epidermal layer from which basal and mid-stem rhizoids differentiate, of cortically located parenchyma cells and of centrally located solute conducting cells or hydroids (17). Leaf primordia (phyllid) undergo a number of symmetric and asymmetric cell divisions to form a single layered photosynthetic leaf blade and a multilayered midrib. Cells specialized in solute transport differentiate within the midrib (stereids, hydroids, and deuters) but do not directly connect with the hydroids of the stem (17). Stem organogenesis is also a highly coordinated process that is so far poorly documented. In response to environmental conditions (low light and temperature (18)) a bunch of reproductive organs (male antheridia and female archegonia) differentiate at the apex of the shoot concomitantly with the arrest of shoot apical growth. Fertilization between a ciliated antherozoid and the egg cell is water dependent and the resulting zygote further differentiates into an epiphytic diploid sporophyte, the sporangium. Spore mother cells differentiate in the sporophyte, proliferate, and undergo meiosis to give rise

Table 1
Cell types and developmental characteristics of *Physcomitrella patens* protonemal cells

Cell types[a]	Cell division	Cell growth	Light requirement	Primary function	Stem cell capacity[b]	Developmental transitions[c]	Enhancing factors
Spore	24 h		Yes	Stem cell	Yes	Into chloronema apical	
Chloronema apical cell	24 h	5 μm/h	Yes	Stem cell	Yes	Into chloronema apical and subapical	
Chloronema subapical cell	24 h	5 μm/h	Yes	Photosynthesis	Yes/no	Into chloronema apical Into caulonema apical	NH$_4$ Auxin
Caulonema apical cell	8 h	20 μm/h	No	Stem cell	No	Into caulonema apical and subapical	
Caulonema subapical cell	8 h	20 μm/h	No	Nutrients assimilation and colonization	No	Into chloronema II (90%) Into caulonema (5%) Into buds (5%) Into skotonema	NH$_4$ Auxin Cytokinins Darkness
Buds			Yes	Shoot apical stem cell		Into leafy shoot	
Skotonema	8 h	20 μm/h	No		No	Into chloronema apical Into caulonema apical	Light Light
Protoplasts	24 h	5 μm/h	Yes	Stem cell	Yes	Into chloronema apical	Light

[a]In *P. patens*, subapical cells usually undergo 2–4 additional cell divisions that generate the ramified/branched pattern of the protonema, except for dark grown skotonema

[b]Refers to the ability to produce protoplasts that will reinitiate protonemal growth; this capacity decreases with aging in chloronemata. Yet all cell types are able to deprogramming and redifferentiate into a chloronemal apical cell following wounding

[c]Numbers in brackets represent the percentage of each transition in standard growth conditions which roughly corresponds to the fraction of each cell type in the protonema

to ca. 4,000 haploid spores per capsule (for reproductive organs and sporophyte organogenesis see (19)).

All the organogenetic processes described above are highly reminiscent of the basic features that control higher plant development. Yet moss organogenesis is much simpler in terms of cell types and developmental transitions and is frequently initiated within a single cell. This provides outstanding facilities to follow developmental processes at the cellular level. It also may suggest that local and/or positional cues are more important to establish the organogenetic pattern of plant growth than cell-to-cell communication mediated by polar transport of growth factors within multicellular meristems. Here, we first describe why *Physcomitrella* is a good model for cell and molecular biologists and geneticists. Then, we review a number of recent publications describing moss developmental mutants and try to show how this moss could advantageously complement currently used models to understand plant organogenesis. We believe that the moss *P. patens* provides the same advantages and limits to study plant development as those offered by *Caenorhabditis elegans* or *Drosophila melanogaster* to study vertebrate organogenesis.

2. *Physcomitrella patens*, the Green Yeast of Plant Biology

2.1. In Vitro Techniques

In addition to gene targeting facilities (see below), the similarity between moss and yeast extends to laboratory procedures since a *Physcomitrella* culture essentially requires microbiological techniques. For most experiments, moss tissue is cultured on modified Knop's solidified media, while growth on sterilized compost mixture is also possible (see (20) for protocols) (21). In both cases, the life cycle is completed in 2–3 months under standard conditions. Most importantly cell linage and developmental transitions can be followed visually and at the cellular level constantly. *Physcomitrella* is self-fertile but genetic crosses are possible and the recent development of fluorescent tagged lines will facilitate identification of hybrid sporophytes (22). Cultures can be initiated from spores or protonemal fragments, from a suspension of fragmented protonema or from leafy gametophores which have the ability to reinitiate protonemal growth. Thus vegetative propagation is easy at any developmental stage of the moss, which is especially important for the conservation of sterile or developmental mutants. Spores, suspension of protonema or differentiated plants in sealed containers can also be stored up to several years in dark cold rooms for medium to long term conservation. To enable the production of a large amount of homogenous tissue suitable for cellular, metabolic or biochemical analyses, 1-week-old protonemal tissue is collected, fragmented and re-inoculated in a Petri dish to maintain pure protonemal culture (yield is 1 g/week/petri). Such cultures also provide optimal

material for protoplast isolation. Large numbers of protoplasts can be isolated from them (10^6/plate) and these can regenerate with high efficiencies (up to 80%). Importantly protoplast regeneration reproduces spore germination and directly gives rise to differentiated protonemal cells. This significantly reduces the risk of somaclonal variation frequently associated with protoplast regeneration in other plants. Techniques for the establishment of protonemal liquid cultures in batch and bioreactors have also been developed and are applied for the production of biopharmaceuticals (23). Recently fluorescence associated cell sorting techniques have been established for isolated protoplasts that facilitate rapid and quantitative analyses of reporter gene expression (24).

2.2. Targeted Mutagenesis and Other Molecular Genetic Tools

Genetic transformation by polyethyleneglycol-mediated DNA uptake in protoplasts was first reported 20 years ago (25) and remains the best method to transform *Physcomitrella*. Transformation was performed with vectors without sequence homology with the moss genome and the frequencies were low (ca. 2–5 clones for 10^5 regenerants). The amazing efficiency of gene targeting (GT) in *Physcomitrella* was subsequently established (6) which boosted transformation efficiencies (to 1 in 10^3) and the interest of the plant scientific community (26). Efficient GT is the Holy Grail of reverse genetics as it enables the generation of any type of mutations within a genome and *Physcomitrella* ranks No. 1 for GT efficiencies among multicellular eukaryotes (27). The subsequent publication of the complete genome of *Physcomitrella* provided the essential information for developing accurate targeted mutagenesis (8). Over the last 10 years, most GT approaches have used replacement vectors to generate specific mutations in the moss genome. Based on experimental strategies established in mouse embryonic stem cells (28), we have further developed procedures that combines GT with Cre/lox mediated site specific recombination to generate clean mutations. This enables the recycling of selectable markers and the elimination of any vector sequences that could interfere with gene expression in knock-in and complementation approaches (29). Figure 2 describes strategies combining GT with Cre/lox recom-

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Fig. 2. (continued) genotyped by PCR with primers a and b to identify those carrying the expected deletion. In these deleted clones, the entire ORF is replaced by a single LoxP site. (**B**) Gene knock-in strategy. The scheme illustrates the generation of N-terminal knock-in of a fluorescent protein (FP) in YFG. The replacement cassette carries as 5′ targeting sequences 5′ UTS of YFG down to position-30 to -10 regarding the ATG and a translational fusion between an ATG-FP and as 3′ targeting sequences the coding sequence of YFG starting at amino acid 2. Selection of replacement and deletion clones is performed as described above. A single LoxP site will remain upstream of the ATG and the fluorescent fusion of YFG will be driven by its natural chromosomal environment in the final strains. (**C**) Point mutagenesis strategy. The scheme illustrates the conversion of a serine (ser) into an alanine (ala) within exon II of YFG. The 5′ targeting sequence covers exon I and half of intron I and the 3′ targeting sequence covers the second half of intron I and the genomic sequence covering exon II and III. The desired mutation is introduced within exon II in the replacement vector. After Cre mediated elimination of the M+, the final clones carry the ser to ala mutation in exon II and a single LoxP site located in intron I. With similar strategies all kinds of mutations can be generated in the moss genome including promoter exchanges or protein domain shuffling. Targeting sequences positioning and genomic location of the residual LoxP site after CRE recombination are critical but not limiting in the design of the replacement vectors.
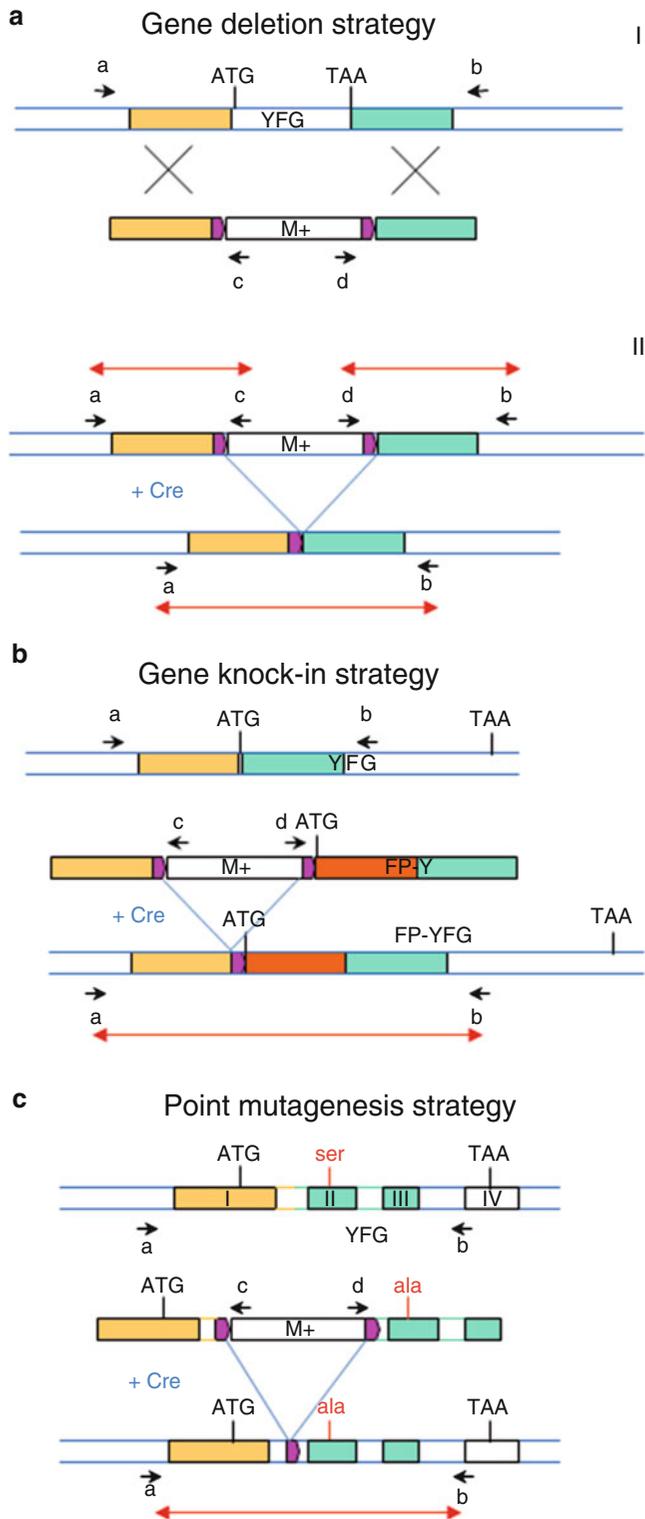
Fig. 2. (**A**) Gene deletion strategy. (I) 5′ (*yellow box*) and 3′ (*green box*) targeting sequences (600–800 bp) are cloned upstream and downstream of a positive selectable marker (M+) flanked by 2 LoxP sites (*violet box*) to build the replacement vector. The targeting sequences are homologous to the 5′and 3′ UTS of your favorite gene (YFG), respectively. Targeted integration by homologous recombination within both targeting sequences (*crosses*) will generate a gene replacement. (II) Antibiotic resistant clones are screened by PCR to identify replacement events using genomic (a, b) and vector primers (c, d). These clones are further submitted to transient expression of the Cre recombinase, single-protoplast derived colonies are regenerated on nonselective medium and then replica plated on selective medium. Antibiotic sensitive clones are further

bination to generate a gene deletion, an N-terminal tagged gene and a point mutation in the moss genome (30). Numerous examples of successful knockouts, gene deletions, knock-in or mutant complementation obtained with these strategies can be found in the articles discussed below. Thus GT facilitates reverse genetics approaches in moss at a speed and an efficiency so far only known for yeast and fungi and allows identification of developmental phenotype or marker lines within 4–6 weeks after transformation.

Efficient GT in *Physcomitrella* also promoted high throughput insertional mutagenesis approaches to establish forward genetic tools. Protoplasts were transformed using either mutagenized cDNA libraries (31) or gene-trap and enhancer-trap systems (32). Numerous developmental mutants have been identified in these collections but only a few of them have been characterized down to the molecular level due to the complexity of transgene integration patterns. They nevertheless constitute valuable resources for further developmental studies. Global gene expression analyses are also possible with the recent development of DNA-microarrays that cover most of the 38,357 predicted genes in the genome.

In addition to GT, gene knockdown can also be obtained via gene silencing techniques such as RNA interference (33), or amiRNA (34, 35). Recently, a system based on RNA interference has been described in order to screen rapidly for temperature sensitive alleles of a given gene (36). For this purpose the authors have co-expressed an RNAi that targets the gene of interest and different versions of an expression construct of the rescuing gene in which residues were mutated and tested for temperature sensitivity (36).

Extensive mutagenesis of the moss genome may require the expression of heterologous genes in overexpression or complementation assays. The introduction of a heterologous gene under the control of endogenous moss regulatory sequences is straightforward with GT and has been successfully used to complement several mutants described below. So far a number of heterologous promoters have been successfully used to drive gene expression in *Physcomitrella*, including the 35S CaMV, the rice actin-1 or the maize ubiquitin-1 gene promoters, or the ABA-responsive wheat *Em* gene and the auxin-responsive soybean GH3-1 promoter (discussed in (37)). A versatile conditional expression system based on the heat-shock-responsive promoter from soybean small HSP17.3b gene was also established and used for example to establish conditional GFP-labelling of microfilaments (38). If working on a haploid genome facilitates genetic analyses, it also impedes the identification of mutants affected in essential genes. The generation of conditional alleles is required to circumvent this situation: this has been recently achieved using the HSP promoter to drive expression of the polycomb gene *PpCLF* to study the gametophyte to sporophyte transition (39).

3. Organogenesis and Cytoskeleton

The principal function of the cytoskeleton is to spatially coordinate basic cellular processes such as cell growth, cell division, cell polarization, subcellular compartmentation, and intracellular membrane trafficking. It thus plays an essential role in organogenesis and we review below some recent studies performed in *Physcomitrella* that provided new insight in this field.

3.1. Actin Microfilaments

Filamentous actin (F-actin) is indispensable for cell viability in eukaryotes. The dynamic equilibrium between globular and F-actin is constantly modulated by regulatory proteins such as actin depolymerization factor (ADF), profilins, formins, or myosin XI. Using RNAi approaches, the groups of Vidali and Bezanilla demonstrated that these genes are essential for *P. patens* development, being required to establish apical growth and cell polarity in chloronemal cells (reviewed and references in (10) and (40)). They have also characterized a quintuple knock-out of Myosin VIII which shows that this small multigene family is required to regulate protonema patterning but is dispensable for the establishment of bushy growth (41).

The ARP2/3 (actin-related protein) complex is a downstream regulator of F-actin nucleation. Its activity is regulated by the SCAR/WAVE complex in response to Rho small GTPase signalling. This highly conserved pathway is essential in yeasts and animal cells, being required for the establishment of cell polarity, localized outgrowth and cell migration. In contrast, loss of function of either complex only affects trichome and pavement cell morphogenesis in Arabidopsis (42, 43). In *P. patens* loss of function of ARP3 or ARPc4 abolishes tip growth but not cell polarization of chloronemal cells and blocks the differentiation of caulonemata and rhizoids (44, 45). The same phenotype was observed in *brk1* mutants (BRICK1 is a component of the SCAR complex) which additionally displayed abnormal orientation of cell division during protonemal development (46). Cytological analyses demonstrate that these complexes localize at the growing tip of protonemal cells in the wild-type, where they control the formation of an F-actin array that is required to establish tip growth. Noticeably leafy shoots develop normally in these mutants, indicating that this regulatory pathway is not required to establish bushy growth. These studies have in a short time brought new insights in the essential role of actin dynamics in cell polarization, tip growth, and plant developmental processes.

3.2. Microtubules

Microtubules (MT) are essential for cell survival and mutations affecting MT dynamics are most of the time lethal in eukaryotes.

In plants, MT form four distinct networks during the cell cycle: (1) interphasic cortical arrays that are required for cell growth and the deposition of cell wall material, (2) the preprophase band (PPB) that defines at the onset of mitosis the future position of the division plane, (3) the mitotic spindle, and (4) the phragmoplast which is required for cell plate formation. In Arabidopsis, loss of function of TONNEAU1 (*AtTON1*) abolishes PPB formation at the onset of mitosis and affects the organization of interphasic MT. The resulting plants display anarchic tissue organization resulting from stochastic orientation of cell divisions and reduced cell elongation, but the differentiation pattern of the plant including phyllotaxis is not affected (47, 48). Loss of TONNEAU1 function in *P. patens* does not affect protonema development but moss gametophores phenocopy the developmental syndrome observed in Arabidopsis (49). Detailed analyses of *Ppton1* hypomorphic alleles further established that the involvement of TON1 protein in the organization of the PPB and of the interphasic MT cortical arrays could be uncoupled, accounting for the defect in the orientation of cell division and in cell elongation observed in both species. Successful reciprocal cross-complementation between Arabidopsis and *Physcomitrella* showed that the function of TON1 has been conserved and was eventually recruited from the gametophyte to the sporophyte during land plant evolution (49). These findings also implicate that proper orientation of cell division is not necessary to establish phyllotaxis in moss, a rather counter-intuitive observation if one considers that meristematic activity is confined to a single stem cell that displays a highly regular pattern of cell division. We have also generated a double *arp3/ton1* mutant to further investigate the extent of overlying function between TON1 and the ARP2/3 complex (Finka and Schaefer, unpublished data). The resulting mutant phenocopies the developmental syndromes of both mutants indicating that the function of these two regulators of the cytoskeleton do not overlap during *P.patens* development (Fig. 3). This work illustrates the plasticity of the moss system since such a mutant would probably be hard to characterize in Arabidopsis.

4. Organogenesis and Growth Factors

Auxin and cytokinin cross talk were early mentioned in moss studies as likely essential for a correct gametophytic development (50, 51). Several recent studies highlight the level of functional conservation of growth factors between *Physcomitrella* and Arabidopsis.

4.1. Auxin

In *Physcomitrella*, early studies have shown that auxin is required for chloronema to caulonema transition, for rhizoid differentiation and for normal shoot development. Auxin resistant mutants unable to

Fig. 3. Top: isolated gametophore from the wild type (WT), *Ppton1* null (TON1-1) and hypomorphic (TON1-2) alleles showing alteration of gametophore development but normal rhizoid differentiation. Bottom: isolated gametophore from *Pparp3* (ARP3) that develops normally but does not form rhizoids and of the double *Pparp3/ton1* mutants. Both the null and the hypomorphic *ton1* phenotype can be recognized in the *Pparp3* background. Scale bar: 0.3 mm.

progress beyond the chloronemal stage have also been isolated (50). Comparative analysis of the moss and Arabidopsis genomes indicates that all basic components for rapid auxin response are found in *P. patens* (52). In Arabidopsis, auxin binds to the TRANSPORT INHIBITOR RESPONSE (TIR1)/AUXIN SIGNALING F-BOX PROTEINS (AFB) which promote the degradation of AUXIN/INDOLE-3-ACETIC ACID (AUX/IAA) repressors and induce expression of auxin-responsive genes. Recent analyses of moss auxin resistant mutants identified mutations in conserved domains of *AUX/IAA* genes and demonstrated auxin-dependent interaction of moss *AUX/IAA* with *AFB* genes (53). This indicates that the molecular mechanism of auxin perception is conserved in embryophytes.

Rhizoid differentiation from shoot epidermal cells is strongly induced by auxin and gametophores grown on 1 μM NAA are leafless with numerous rhizoids (17). It was also shown that the chloronema–caulonema transition is gradual, and that auxin induction of both *PpRHD SIX-LIKE1 (PpRSL1)* and *PpRSL2* genes (transcription factors, see also below) expression is sufficient to promote this transition (54). These results suggest that the involvement of auxin in rhizoid formation may represent an ancient role,

as it has been observed in many earlier-diverging streptophytes, while the more specific role of auxin in chloronema–caulonema transition may have been co-opted within the moss lineage (53).

There is no polar auxin transport in gametophore development (55) but genes homologous to the Arabidopsis auxin efflux carriers *PIN FORMED* (*PIN)* genes have been found in *P. patens* genome and their role is to be unravelled (56). The absence of polar auxin transport suggests an important role for localized auxin biosynthesis during moss development. In Arabidopsis, auxin biosynthesis is positively regulated by SHORT INTERNODE/STYLISH (SHI/STY) proteins and two *SHI* orthologues are present in the moss genome. Overexpressors and single knock-outs of *PpSHI* genes were generated, which consistently displayed increased and decreased auxin levels, respectively (57). In these strains, all the above-mentioned developmental processes were affected in a way that correlated with auxin levels and the involvement of auxin in regulating senescence was further identified. Some of these functions may be analogous in bryophytes and tracheophytes. This study further established a tight correlation between the expression profile of *PpSHI* genes and an auxin inducible reporter construct, suggesting that local auxin production could promote auxin peak formation in organogenetic processes.

4.2. Cytokinin

In *P. patens*, cytokinins (CK) are required for the differentiation of buds from caulonemal subapical and thus for the establishment of bushy growth. Bud overproduction was observed in response to exogenous CK and in *ove* mutants which also displayed altered CK metabolism (21, 50, 58 and see Fig. 4). Noticeably extracellular CK constitute the main CK responsible for bud induction in moss cultures (59). In Arabidopsis, CK signalling is transduced through a two-component system consisting of three phosphorelay signal transducers: His-kinase receptors (HK), a His-containing phosphotransmitters (HPT) and response regulators (RR) that modulate gene expression. Comparative analyses of fully sequenced plant genomes indicated that the complete set of proteins of the CK signalling pathway appeared in the moss genome (60) while initial characterization of the moss PpHK4b CK receptor showed that it exhibits His-kinase activity (61). Further studies of CK induced bud induction in moss will soon provide additional insights on the molecular network regulating CK-induced organogenetic processes.

4.3. ABA

Early physiological studies have shown that abscisic acid (ABA) induces the differentiation of swollen globular drought-tolerant cells, also called brood cells (62) and plays an important role to protect mosses against desiccation, cold or salt stresses. ABA regulated expression of the wheat Em promoter in *Physcomitrella* provided initial evidence for the conservation of ABA response pathways between mosses and seed plants (63). More recent studies
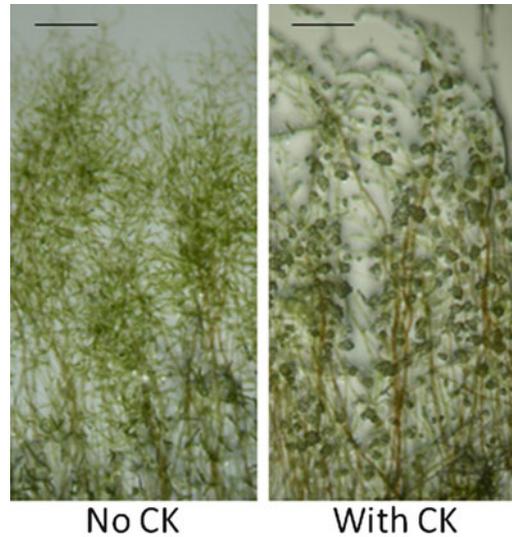
Fig. 4. Over production of buds in response to exogenous supply of CK. Protonema was grown under gravitropic conditions in darkness to enhance caulonemal differentiation. Cultures were transferred after 10 days in light on standard or CK supplemented medium. Images were taken 5 days after transfer to light. Buds differentiate from most caulonemal branch initials while chloronema formation is almost completely abolished on CK. Scale bar: 0.5 mm.

have shown that ABA signalling pathways mediated either by ABA INSENSITIVE (ABI)3-like transcription factors (64–66) or by ABI1-like type 2C protein phosphatase are functionally conserved ((67) and discussed in (68)). These studies also show that *Pp*ABI1 is required for the differentiation of brood cells in response to ABA in *Physcomitrella* (69) while ectopic expression of Arabidopsis *ABI1-1* affects moss development, suggesting a possible role of ABA in leafy shoot and reproductive organ morphogenesis (70). Finally, analysis of *Physcomitrella open stomata-1* (*ost-1*) knock-out and successful complementation of the Arabidopsis *ost1* mutant with *PpOST1* provide functional evidence for the conservation of ABA-mediated regulation of stomatal behavior in embryophytes (71).

4.4. GAs, Brassinosteroids, and Ethylene

As an exception to growth factors mentioned above, no true gibberellin (GA) pathway has been observed in *P. patens* (72, 73) and fully functional brassinosteroid signalling components are only present in vascular plants (74). Yet, *ent*-Kaurene synthase *(PpCPS/KS)* mutants, deficient in the production of *ent*-Kaurene derived GA type diterpenes, show no chloronema–caulonema transition (75). These diterpenes remain to be identified, and would shed light on GA signalling evolution in land plants (76). Finally, the role of ethylene in chloronema–caulonema transition has been early suggested, and sequences for putative ethylene-receptors have been recently found

in *P. patens* genome, but clear evidence for a role of ethylene in this or other moss developmental steps are still lacking (77).

4.5. Strigolactones (SLs)

SL have prior been characterized as key signals of root proximity for both parasitic weeds seeds and symbiotic mycorrhizal fungi, then as a novel class of hormones controlling shoot branching in seed plants (for reviews: (78–80)). We obtained a *Ppccd8* mutant affected in the SL biosynthesis enzyme CCD8 (CAROTENOID CLEAVAGE DIOXYGENASE 8) that, compared to WT moss, fails in arresting plant extension after 3 weeks and shows increased caulonema branching (81). This study led us to propose that SLs or derived molecules are produced by the moss and released in the medium to control moss individual extension (81). Furthermore, the SL concentration would act as a signal for sensing neighbor individuals, an observation reminiscent of the early description of Factor H known to inhibit caulonema growth and possibly determinant in moss community structure in nature (82).

5. Organogenesis and (Nonhormonal) Signal Transduction Pathways

In addition to growth factors, other signals contribute to and/or modify the moss development, by inducing specific transduction pathways.

Light is one of these major regulators of *P. patens* morphogenesis (83): it is necessary for the very early step of spore germination, and later on for all gametophytic development, that responds to both light quality and periodicity. Early studies had shown that red-light induced membrane depolarization of the protonema (e.g., Ca^{2+} fluxes) correlated with side branch initial formation (84). Later on, both cryptochromes (cry) and phytochromes have been shown as photoreceptors contributing to protonema development (85, 86). Moreover, the study of the *Ppcry1a-cry1b* double mutants suggested that cryptochrome (blue) light signals repress auxin signals during moss development (87). Day length affects moss development (in particular short days induce sporophyte development) and several core components of the circadian clock have been found in *P. patens*. However, a recent comparison with Arabidopsis suggests a single feedback loop in moss versus a more complicated clock network in vascular plants (88).

Hexokinases (*HXK*) are enzymes that catalyze the phosphorylation of glucose and fructose, but these proteins may also play a role in sugar sensing and signalling. Since the cloning of the first *PpHXK1* gene, encoding a chloroplastic HXK and the study of the corresponding *hxk1* KO mutant affected in protonema growth (89), ten more *HXK* genes have been characterized in *Physcomitrella* (90). The predicted encoded proteins are more similar to each other

than to hexokinases from vascular plants, which makes this family very interesting for evo-devo studies. Two other protein kinase genes were cloned from *Physcomitrella*, the Snf1-related protein kinase 1 genes *PpSNF1a* and *PpSNF1b*, that may redundantly contribute to moss growth (and hormone regulated?) adaptation to low energy supply conditions (91).

Tip growth regulation requires signal transduction as highlighted by both pollen tubes and root hairs studies from vascular plants. The requirement of *ARABINOGALACTAN PROTEINS* for protonema cell extension has been early demonstrated in moss (92). Recently, *P. patens* KO mutants affected in enzymes responsible for the synthesis of Phosphatidyl Inositol 4,5 diphosphate (PtdIns$(4,5)$P$_2$) (PIPK1 and PIPK2) were obtained (93). The level of PtdIns$(4,5)$P$_2$ were reduced in both single mutants, however only the *pipk1* mutant showed shorter caulonema cells and less developed rhizoids (93), suggesting a role for the PIPK1 enzyme in tip growth. The double KO *pipk1-2* did not develop any caulonema or rhizoid. In a recent survey, homologues of genes encoding proteins involved in *RAC/ROP GTPase*s signalling were identified in the moss genome (94), which characterization should lead to a better knowledge of land plant tip growth control mechanisms.

6. Organogenesis and Transcription Factors

In plants many transcription factors are encoded by members of multigene families that expanded much more dramatically during land plant evolution than during the evolution of animals and fungi (95). In vascular plants, Class 1 KNOTTED1-LIKE HOMEOBOX (KNOX) transcription factors are essential to the regulation of cell division and differentiation in the shoot apical meristem (for review: (96)). The class 1 *KNOX* genes homologues (*MKN* genes), identified in the moss genome, would not regulate the leafy shoot haploid meristem (97). However, these transcription factors are found expressed in the sporophyte where they would regulate sporogenous cells divisions (97, 98). Sakakibara et al. claim that this would correspond to a situation where networks operating in vascular plants sporophyte would not be co-opted from ancestor's gametophyte network (97).

Like caulonema, rhizoid cells elongate by tip-growth. Two types of rhizoids differentiate from *P. patens* gametophore epidermal cells, namely basal and mid-stem rhizoid, and auxin induces their development (17). The *PpHB7* gene (encoding a homeodomain-leucine zipper I transcription factor) is required for rhizoids late differentiation steps, but not for their determination. Its expression is increased following both auxin and CK (6-benzylaminopurine)

treatment (17). Two other transcription factors of the basic-he-lix–loop–helix (bHLH) type have been described as sufficient for rhizoid development, the *PpRSL1* and *PpRSL2* genes (99). Target genes of these transcription factors are yet to be discovered. Both *PpRSL1* and *PpRSL2* genes are induced by auxin, contrary to the Arabidopsis homologous genes (*AtROOT HAIR DEFECTIVE 6* and *AtRSL1*) that do regulate root hairs development (100). Therefore the mechanism of auxin action is different between moss and vascular plants for regulating rhizoid and root hair differ-entiation (100).

The three closely related bHLH transcription factors SPEECHLESS, MUTE, and FAMA regulate a different step in the stomatal lineage: asymmetric divisions, the acquisition of guard mother cell identity, and guard cell differentiation, respectively. Stomata were an ancient innovation of land plants predating even the evolution of leaves and roots. Cross-species complementation between Arabidopsis and *Physcomitrella* show that PpSMF1, one of the two bHLHs of the same subgroup found in *P. patens* pos-sesses dual functionalities while MUTE and FAMA are now each specialized for a single step. A model is proposed where an ances-tral multifunctional transcription factor underwent duplication fol-lowed by specialization to provide the three (now nonoverlapping) functions of the angiosperm stomatal bHLHs (101).

GAI, RGA, SCL (GRAS)-type genes have been recently reported in moss in an attempt to study the evolution of this large family of transcription factors that underwent large diversification (102); a precise role of these proteins in bryophyte development remains to be discovered.

7. Organogenesis and Reprogramming

For more than a decade now, the role of epigenetic factors on developmental processes regulation has been discovered in plants. The predominant gametophytic phase of *P. patens* development was proven very useful for demonstrating the role of chromatin modifiers protein complex (e.g., The Polycomb group complex, see below) and small RNAs.

7.1. Epigenetic Control of Moss Development

The Polycomb group (PcG) complex, involved in the epigenetic control of gene expression profiles, plays a central role in regulat-ing the transition of the female gametophyte to the sporophyte in flowering plants. In *A. thaliana* all PcG complexes comprise the WD40 motif-containing proteins FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and one of the three SET domain proteins CURLY LEAF (CLF), SWINGER (SWN) or MEDEA (MEA). Expression of a PpFIE-GUS fusion protein under

the control of its native promoter indicates that the PpFIE protein accumulates only in gametophyte apical cells, and in cells that undergo fate transition. In the absence of PpFIE, gametophore meristems overproliferate, but fail to develop and to reach the reproductive phase (103). Hence, the essential FIE PcG function in regulating developmental programs along the life cycle was established early in evolution. In another work, PpCLF deletion lines were obtained, where a gametophytic vegetative cell frequently gave rise to a sporophyte-like body which did not form a sporangium but which, with continued culture, branched (104). Sporophyte branching is almost unknown among extant bryophytes demonstrating the role of the PcG complex in sustaining evolutionary innovation in land plants. In the gametophyte, PpCLF represses initiation of a sporophytic pluripotent stem cell, while in the sporophyte, it represses that stem cell activity and induces reproductive organ development.

Most eukaryotes express diverse small silencing RNAs which direct the sequence-specific repression of target RNAs. The accumulation of endogenous 24 nt short interfering RNAs (siRNAs), derived mostly from intergenic and repetitive genomic regions, requires the Dicer family member DICER-LIKE3 (DCL3). Despite the presence of a clear homolog of DCL3 in non-flowering plant, the 24 nt siRNAs are not readily apparent in the small RNA populations of several lineages. Nevertheless, "hotspots" of small RNA production were found in the genome of *Physcomitrella patens* that produced a mix of 21-24 nt siRNAs, which despite their different sizes, are reminiscent of the 24 nt siRNA loci of angiosperms (enriched in transposon content, avoid annotated genes, and densely modified with the epigenetic mark 5-methyl cytosine). *Ppdcl3* mutants failed to accumulate 22–24 nt siRNAs from repetitive regions and displayed an acceleration of leafy gametophore production, suggesting that repetitive siRNAs may play a role in moss development (105). Furthermore, KO mutants for DICER-LIKE1 (DCL1) homologs (*Ppdcl1a* and *Ppdcl1b*) show strong developmental alterations such as growth retardation and/or aberrant cell division and differentiation, and their study pinpoints specific roles for each protein in the transcriptional control of target genes expression, by MicroRNAs (106).

7.2. Moss Organogenesis Versatility

In vascular plants, dissected or wounded tissues can proliferate when treated with exogenous phytohormones to form callus, which can be fated to form shoot or root meristematic tissue bearing stem cells (107, 108). In *Physcomitrella*, when part of a gametophore leaf is excised and cultivated for a few days on culture medium without phytohormone supplementation, leaf cells facing the cut edge change into cells that are indistinguishable from the apical cells of chloronemata. The study of this reprogramming of excised leaf cells showed that cyclin-dependant kinase A (CDKA)

regulates cell cycle progression and acquisition of new cell characteristics in parallel (109). Treatment with a CDK inhibitor or induction of dominant negative CDKA;1 protein inhibited not only cell cycle progression but also tip growth and protonemal gene expression, whereas a DNA synthesis inhibitor, aphidicolin, inhibited cell cycle progression but prevented neither tip growth nor protonemal gene expression. Thus CDKA concomitantly regulates cell division and cellular change in reprogramming differentiated cells to become stem cells in plants.

8. Conclusion

In the recent years the moss *Physcomitrella patens* has emerged as a powerful complementary model system to study plant organogenesis. Recent studies have addressed the relation between moss organogenesis and many regulators of plant development including growth factors, the cytoskeleton, transduction pathways, transcription factors, epigenetic control and dedifferentiation processes. These studies highlight both the similarity and the differences between *Physcomitrella* and Arabidopsis and strongly support the idea that the major developmental innovations that were associated with the colonization of land by plants can be investigated in extant moss species. The unrivalled efficiencies of GT combined with the outstanding cellular and biochemical facilities offered by *Physcomitrella* will certainly contribute to improve our understanding of the evolution of plant developmental processes.

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