Evolutionary crossroads in developmental biology: *Physcomitrella patens*

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Summary

The moss *Physcomitrella patens* has recently emerged as a powerful genetically tractable model plant system. As a member of the bryophytes, *P. patens* provides a unique opportunity to study the evolution of a myriad of plant traits, such as polarized cell growth, gametophyte-to-sporophyte transitions, and sperm-to-pollen transition. The availability of a complete genome sequence, together with the ability to perform gene targeting efficiently in *P. patens* has spurred a flurry of elegant reverse genetic studies in this plant model that address a variety of key questions in plant developmental biology.

Key words: *Physcomitrella patens*, RNAi, Homologous recombination

Introduction

Bryophytes – comprising mosses, liverworts and hornworts – represent three out of the four ancient lineages of land plants (Fig. 1). These early diverging land plants are distinct from other land plant lineages, as they are non-vascular. Furthermore, they use free-swimming motile sperm for fertilization, an attribute shared by the fern lineage but lost in seed plants. In addition, moss plants have a relatively simple morphology, with many fewer cell fates than in flowering plants. Interestingly, gene families encoding much of the basic developmental 'tool kit' identified in flowering plants are conserved in the genome of the moss *Physcomitrella patens* (Floyd and Bowman, 2007; Rensing et al., 2008). During the nearly half billion years since diverging from the moss lineage, flowering plants appeared to have re-purposed much of this tool kit as new developmental processes have evolved.

Using the powerful tools afforded by efficient gene targeting in *P. patens* (Kammerer and Cove, 1996; Schaefer and Zryd, 1997; Strepp et al., 1998), it has become possible to study the roles of many of these genetic pathways in moss. In doing so, we can begin to decipher which developmental mechanisms are elaborations of basic mechanisms that were present in ancestral land plants and which represent novel innovations in the flowering plant lineage. Several *P. patens* developmental pathways will be particularly interesting to dissect in light of what might be learned about important events in land plant evolution.

In this primer, we introduce the *P. patens* life cycle and explain how aspects of this life cycle make this particular moss especially suited for molecular genetic experiments, including targeted deletions, allele replacements and RNA interference (RNAi). In addition, we summarize key findings in *P. patens* in the past 5 years that have propelled the plant developmental field forward.

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The *P. patens* life cycle

P. patens, although not a common moss, is widely distributed in temperate zones. Isolates are available from Europe, North America, Japan, Africa and Australia (Cove, 2005; McDaniel et al., 2010). It is an ephemeral moss that develops in late summer from overwintered spores and grows on banks of ponds, lakes and rivers that have been exposed by lowering water levels (Cove, 2005). It develops sexual organs in the Fall, triggered by lowered temperatures and shortened days (Hohe et al., 2002).

P. patens, like vascular plants, exhibits alternation between generations. Unlike vascular plants, in bryophytes, the gametophyte (or haploid generation), is the larger plant and includes most of the tissues present in the plant (see Glossary, Box 1). *P. patens* germinates from a haploid spore, producing a linear array of cells that branch and generate a filamentous, two-dimensional, network known as protonema (Fig. 2B,C; see Glossary, Box 1). The stem cell for this network is at the apex of each filament, and each filament grows by polarized growth, or tip growth, secreting necessary cell wall components at the apex. The first cell type to emerge from the spore is the chloronema (Fig. 2B; see Glossary, Box 1). These cells can be easily recognized as they contain 50-100 fully developed chloroplasts, and cell plates that form between dividing cells are transverse to the axis of the cell. Subsequent tissue differentiation is dependent on phytohormone levels [see Box 2; for detailed reviews see Cove (Cove, 1992) and Decker et al. (Decker et al., 2006)]. As the plant continues to grow, apical cells transition from chloronemata to caulonemata in an auxin-dependent manner. Caulonemata (see Fig. 2C) are faster growing cells that contain fewer less-developed plastids and cell plates that are positioned at an oblique angle to the long axis of the cell (Fig. 2; see Glossary, Box 1). The branching of protonemal tissue occurs at subapical cells, producing another chloronemal cell.

Moss has a dimorphic gametophyte, with young tissue predominately consisting of protonemata and older tissue of gametophores, the shoot of *P. patens*. As the plant matures, there is a transition in the side branch formation from chloronemal filaments to gametophore initials (Fig. 3), which develop into gametophores (Fig. 2D). This transition is dependent on the phytohormone cytokinin. The side branch initial cell that is fated to become a gametophore apical cell is morphologically distinct from that fated to become a protonemal apical cell. It is more bulbous, and the first division is oblique to the long axis of the cell, compared with the transverse division seen in a protonemal branching cell (Harrison et al., 2009). Analysis of cell division patterns within the gametophore initial has indicated that a single stem cell resides at the apex. This cell divides to produce the cells that go on to form leaflets in a characteristic pattern (Harrison et al., 2009).

At the top of a single gametophore, both male (antheridia) and female (archegonia) sexual organs form (Fig. 2E). Flagellate sperm, known as spermatozoids, are produced in the antheridia and swim to fertilize the egg cell within an archegonium. Moist conditions
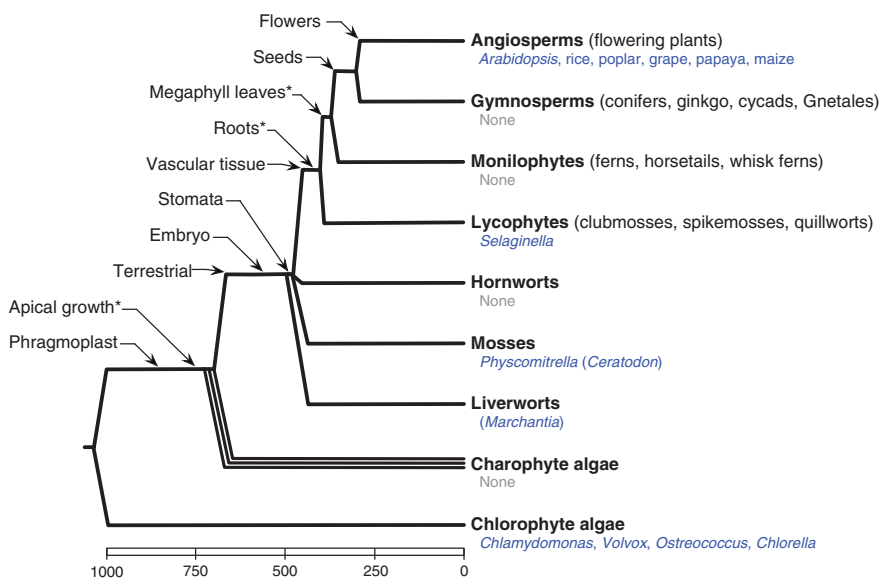
Fig. 1. Phylogenetic relationships among green plants. The dendrogram shows the likely relationships between the major lineages of green plants. The timeline at the bottom indicates the approximate time (millions of years before present) at which lineages diverged. The approximate times at which important morphological innovations appeared are on the left of the nodes. Innovations with asterisks (*) may alternatively have evolved independently in multiple lineages after the point indicated. Plants for which complete genome sequences are currently available are indicated in blue text (with those anticipated in the near future listed within parentheses).

are required for fertilization. After fertilization, the zygote develops into the diploid generation, the sporophyte, which is composed of a short seta topped with a spore capsule (Fig. 2; see Glossary, Box 1). Within the capsule, meiosis occurs and at maturity, ~4000 haploid spores are produced and released into the environment (Engel, 1968).

Some of the key features of the moss life cycle that are particularly suited for genetic studies include the predominately haploid life cycle. Additionally, its simple morphology enables the elucidation of the cellular basis of most observed phenotypes. Before we discuss genetic studies in *P. patens* in more detail, we provide below an overview of the major events in land plant evolution to explain further how studies of *P. patens* could yield important future insights into these events.

Events in land plant evolution
Apical growth
The body plans of all land plants are shaped through the actions of apical meristems, tissues composed of self-renewing stem cells that provide daughter cells for subsequent differentiation (Graham et al., 2000) (Fig. 4). Through decades of research on meristem function in flowering plants, many regulatory mechanisms have been identified (Stahl and Simon, 2009). Most of these are unique to either root or shoot meristem development. However, recently common genetic mechanisms that regulate both root and shoot meristems have been uncovered (Friedman et al., 2004; Stahl and Simon, 2009). These common pathways are good candidates for being conserved within the apical-cell-type meristems of ancestral plants, as well as in present-day mosses. Thus, work with *P. patens* is poised to contribute to our understanding of the basic mechanism underlying apical growth.

Vascular tissue
Vascular tissue is perhaps the most important innovation within land plants, allowing long-distance nutrient transport and providing rigid structural support. The evolution of vascular tissue paved the way for trees to tower over the terrestrial landscape. Mosses are non-vascular. Yet, it has been speculated that specialized conducting cells within moss gametophore 'stems' that transport water or nutrients may be homologous to the provascular cells in

vascular plants [as discussed by Ligrone et al. (Ligrone et al., 2000)]. Provascular cell specification in flowering plants involves the auxin-signaling pathway and specific transcription factors (Ilegems et al., 2010; Rolland-Lagan, 2008). Both the auxin signaling pathway and class III HD-zip transcription factors are conserved in moss (Rensing et al., 2008; Sakakibara et al., 2001). Thus, it will be interesting to learn whether they are involved in the specification of conducting cells in moss.

Alternation of generations
An important difference between mosses and vascular plants is the phase of the life cycle that predominates during the life of the plant (Kenrick and Crane, 1997). Mosses spend most of their lifetime as free-living haploid organisms, while vascular plants spend the majority of their life cycles as diploid sporophytes. Although some developmental mechanisms shaping the sporophyte of flowering plants may perform similar roles in moss sporophytes, others may have first arisen in the free-living gametophyte of an ancestral plant before being activated during the diploid phase as it lost its dependence on the gametophyte.

Flagellate sperm and pollen tubes
Seed plants developed pollen for delivery of sperm to the egg cell, enabling fertilization in the absence of free water. Mosses and ferns, by contrast, have free-swimming flagellate sperm that require moisture for fertilization. Furthermore, moss and fern flagella are structurally distinct, in that they lack outer dynein arms (Hyams and Campbell, 1985; Manton, 1957), proteinacious appendages that are found in the flagella of most eukaryotes. Using comparative genomic as well as functional approaches, moss may provide a useful system with which to analyze the structure and function of flagellar components.

Experimental techniques in *P. patens*
In *P. patens*, much work has focused on reverse genetic approaches for several reasons. First, only *P. patens* and another moss, *Ceratadon purpureus*, can integrate transformed DNA molecules by homologous recombination at high frequencies, enabling gene targeting studies to be performed to analyse gene function (Brucker et al., 2005; Kammerer and Cove, 1996; Schaefer and Zryd, 1997;

Box 1. Glossary

Abscisic acid
A plant hormone that induces intercalary cell division in mosses, producing brachycytes and tmema cells.

Antheridium
Sperm-producing organ.

Archegonium
Multicellular organ in which a single egg is produced.

Auxin
A plant hormone that induces caulonemal formation in mosses.

Brachycytes
Short thick-walled 'brood' cells often formed in chains.

Bryophytes
Land plants consisting of mosses, liverworts and hornworts.

Caulonemata
Colonizing cells of moss protonemata, which have fewer chloroplasts than chloronema and oblique cell plates.

Cell plate
Structure that forms at the equator of the mitotic spindle during cell division.

Chloronemata
First protonemal cells to emerge from moss spores, which contain 50-100 chloroplasts and have transverse cell plates.

Cytokinin
A plant hormone that induces gametophore initials in mosses.

Gametophore
A shoot that bears sexual organs in moss.

Gametophyte
The haploid, gamete-producing generation.

Microphyll leaf
Small leaf with one vein and one leaf trace not associated with a leaf gap.

Phyllid
Individual leaflets in the gametophore.

Plastid
Organelle in the cell of plants and algae responsible for photosynthesis and storage of products, such as starch and secondary metabolites.

Protonema
First stage of development in mosses: filament-like tissue.

Rhizoids
Rooting structures of the moss gametophore.

Seta
The stalk that supports the spore capsule.

Spermatoid
Motile mature male gamete.

Sporangium
Structure in which spores are produced.

Sporophyte
The diploid, spore-producing generation.

Tmema cells
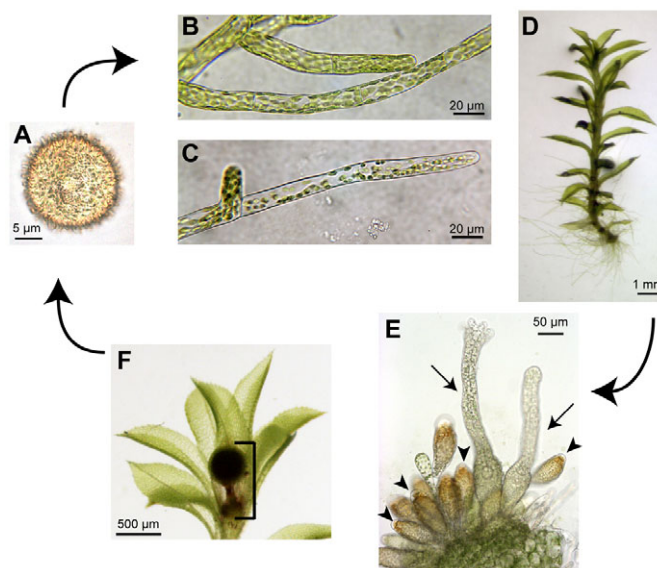Abscission cells that are short-lived and contain very little cytoplasm.

Fig. 2. *P. patens* life cycle. (**A**) A haploid spore germinates into (**B**) chloronemal cells, which continue to grow and differentiate into (**C**) caulonemal cells. (**D**) Gametophores, or shoots, emerge off protonemal filaments and are ultimately anchored by rhizoids that grow by tip growth from the base of the gametophore. (**E**) At the apex of the gametophore, both female, archegonia (arrows), and male, antheridia (arrowheads), organs form. A motile flagellate sperm fertilizes the egg and the (**F**) sporophyte (marked with a bracket) develops at the apex of the gametophore. (A,E,F) Kindly provided by M. Hasebe (National Institute Basic Biology, Okazaki, Japan). (B-D) M.B. (unpublished).

cells in the disrupted area change into chloronemal apical cells producing a new filamentous network (Fig. 5A). As a consequence, mutant strains with a wide range of developmental defects can be maintained indefinitely. Additionally, tissue can be disrupted by cell wall-digesting enzymes, producing a suspension of single cells known as protoplasts. Given osmotically controlled medium, protoplasts rebuild their cell walls and then regenerate into protonemal tissue (Fig. 5B).

Third, transformation of DNA is routine in *P. patens*. It is generally performed by poly-ethylene-glycol (PEG)-mediated transformation of protoplasts (Schaefer et al., 1991). Stable integrants, with the transformed DNA integrated into the genome, can be selected in 4-6 weeks, which is remarkably fast compared with any other plant system. DNA can integrate by homologous recombination (see Box 3) or randomly if the transformed DNA lacks any sequences homologous to the genome. However, the efficiency of generating non-targeted stable transformants is one-tenth of that achieved when mediated by homologous recombination (Schaefer, 2001).

Additionally, many molecular techniques are routine in *P. patens*. *P. patens* has been used as an expression system for purifying complex secreted eukaryotic proteins (Decker and Reski, 2007). Expression profiling at the genome level has been aided by the development of microarrays (Cuming et al., 2007; Richardt et al., 2010). Cre-lox-mediated recombination (Sauer, 1998) is another molecular tool that works efficiently in *P. patens* and has been mostly used to remove a selectable marker from a locus, allowing for subsequent transformation with the same selectable marker (Schaefer and Zryd, 2001). Frequently, transforming DNA

Strepp et al., 1998) (see Box 3). This feature appears to be unique to mosses, with *P. patens* exhibiting a higher frequency of gene targeting compared with *C. purpureus* (Trouiller et al., 2007).

Second, *P. patens* is easily propagated vegetatively. At any developmental stage, if *P. patens* tissues, such as protonemata, gametophores or sporophytes, are mechanically disrupted, then the

Box 2. Evolution of hormone-signaling pathways

Phytohormones, including auxins, cytokinins, gibberellins, abscisic acid (ABA), ethylene, brassinosteroids and jasmonates, are vitally important in regulating morphogenesis and environmental responses in flowering plants. Some of these signaling molecules also regulate moss growth and development. Auxin promotes chloronemal-to-caulonemal cell transition, and has later roles in rhizoid development and in phyllid morphogenesis in *P. patens* and in closely related mosses (Ashton et al., 1979b; Cove, 1992; Johri and Desai, 1973). Likewise, cytokinins regulate gametophore initiation and development (Ashton et al., 1979b; Bopp, 1963), while ABA promotes the production of brachycytes and tmema cells, and dessication tolerance (see Glossary, Box 1) (Decker et al., 2006; Khandelwal et al., 2010).

The availability of the complete genome sequence of *P. patens* allows genes homologous to those encoding components of the hormone-signaling pathways of flowering plants to be searched for. Consistent with phenotypic data, components of the auxin, cytokinin and ABA pathways are encoded in the *P. patens* genome (Paponov et al., 2009; Pils and Heyl, 2009; Prigge et al., 2010; Rensing et al., 2008), as are components of the ethylene and jasmonate pathways (Rensing et al., 2008), although it appears that the brassinosteroid pathway evolved after the moss and vascular plant lineages diverged (M.J.P. and M. Estelle, unpublished). The evolution of the gibberellin pathway is especially intriguing. Although genes similar to those encoding components of a gibberellin-receptor complex are present in the moss genome, the moss proteins apparently do not function as gibberellin receptors (Hirano et al., 2007; Yasumura et al., 2007). Nevertheless, a gibberellin-like diterpene molecule appears to regulate moss differentiation, suggesting that the pathway may have been present in a rudimentary form before the divergence of the moss and vascular plant lineages (Hayashi et al., 2010).

There is extensive crosstalk between the different hormone-signaling pathways in flowering plants, complicating our understanding of their roles in development. Given the apparent reduction in the numbers of hormones regulating moss development and the possibility that some of the crosstalk mechanisms evolved within the vascular plant lineage, *P. patens* research may become instrumental in deciphering certain interactions between hormone-signaling pathways.

may integrate into a locus as multiple tandem copies, which may be reduced to a single copy using Cre-lox recombination (Schaefer and Zryd, 2001).

Coupled with the ease of generating targeted gene replacements, the simple morphology of *P. patens* allows direct microscopic observation of virtually all aspects of moss development (see Figs 2, 3 and 5). Furthermore, as most tissues in moss are only a single cell layer thick, this provides a window into the cellular basis for any observed phenotype. High-resolution fluorescence microscopy has been used to image cytoskeletal dynamics during cell division (Hiwatashi et al., 2008) and during tip growth (Vidali et al., 2010; Vidali et al., 2009b), and to image protein-protein interactions via fluorescence resonance energy transfer (Gremillon et al., 2007). Using confocal microscopy, the cell division events that lead to gametophore formation have been determined (Harrison et al., 2009), allowing for future studies in mutants with defects in this process.

The predominately haploid life cycle of *P. patens*, however, does complicate gene function studies if the gene under investigation is essential. In this case, knockouts cannot be recovered. Knockout studies can also be affected if a gene belongs to a large, functionally redundant gene family. A mutant phenotype might not

be observed unless many family members are knocked out. Fortunately, *P. patens* is amenable to RNA interference (RNAi) (Bezanilla et al., 2003), which provides a method to overcome these disadvantages.

Using RNAi, it is possible to observe terminal phenotypes in *P. patens* 1 week after transformation of the RNAi construct. A rapid, transient RNAi system incorporating a marker for active gene silencing has been developed that effectively allows identification of RNAi-induced phenotypes during the first week of protonemal development (Bezanilla et al., 2005). Additionally, if a gene belongs to a large gene family, it is possible to include regions of sequence from all the family members into a single inverted repeat RNAi construct and thus silence all family members simultaneously (Vidali et al., 2007; Vidali et al., 2009c).

This RNAi system has also been adapted to allow rapid assessments of both gene functionality and the specificity of the RNAi phenotypes. It is possible to co-transform an RNAi construct that targets only untranslated regions along with an expression construct that contains only the coding sequence of the gene. Expression of functional protein-coding sequences should complement specific RNAi phenotypes (Augustine et al., 2008; Vidali et al., 2007; Vidali et al., 2009c). These complementation assays are very rapid, as plants can be analyzed within 1 week of transformation and have enabled rapid screening for temperature-sensitive alleles of key cytoskeletal regulators (Vidali et al., 2009a).

Inverted repeat RNAi constructs have been very successful for transient studies analyzing protonemal development. However, these constructs produce stable transgenic plants at a low frequency, perhaps owing to the presence of the inverted repeat. Thus, to study later stages, such as gametophores and sporophytes, it is preferable to use artificial microRNAs for silencing (Khraiwesh et al., 2008). Stable plants with a range of different expression levels of the targeted gene can be isolated.

Key recent findings and their impact
Tip growth

Owing to the ease of propagation of protonemal tissue, *P. patens* has been a key organism for the study of tip-growing plant cells. As is the case for angiosperm tip growing cells, the actin cytoskeleton is crucial for the growth and development of protonema. In cells, actin exists in a dynamic equilibrium between monomeric and filamentous actin, which is regulated by key actin-binding proteins. Actin filaments are constantly remodeled, by depolymerizing, severing, polymerizing and bundling events. If moss protoplasts are treated with LatrunculinB, an actin depolymerizing drug that drastically reduces the dynamics of the actin cytoskeleton, protonemata are unable to develop. Instead, very small plants consisting of round cells regenerate from the protoplast (Harries et al., 2005). This phenotype is also observed when key regulators of the actin cytoskeleton are silenced (Augustine et al., 2008; Vidali et al., 2007; Vidali et al., 2009c). In particular, class II formins, which are a family of proteins that promote actin nucleation and elongation, work with the small actin monomer binding protein profilin to generate a rapidly elongating cortical array of actin filaments that is concentrated at the apex of protonemal cells (Vidali et al., 2007; Vidali et al., 2009b; Vidali et al., 2009c). The actin depolymerizing factor ADF is crucial for the disassembly of this array (Augustine et al., 2008). Importantly formins, profilin and ADF are essential for moss viability as it is not possible to obtain stably silenced plants (Augustine et al., 2008; Vidali et al., 2007; Vidali et al., 2009c). Additionally, cell death is

observed when ADF and all formins are silenced, as indicated by senescing chlorophyll autofluorescence in silenced plants (Augustine et al., 2008; Vidali et al., 2009c).

Because growth is mediated by the secretion of new cell wall material to the apex of the cell, the current hypothesis is that the dynamic actin array serves as tracks for targeted vesicle delivery to the apex. Recent studies demonstrate that class XI myosins are most probably the motors responsible for vesicle secretion, as silencing of myosin XI results in cells with unaltered actin dynamics that are unable to perform polarized growth (Vidali et al., 2010).

The Arp2/3 (actin-related protein 2/3) complex is another family of proteins required for the nucleation of actin polymerization. Knockout and RNAi studies in *P. patens* have shown that the Arp2/3 complex appears to be important for caulonema formation (Harries et al., 2005; Perroud and Quatrano, 2006; Perroud and Quatrano, 2008). Although protonemata regenerate in mutants that lack subunits of the Arp2/3 complex, these plants are composed mainly of chloronemal-like cells that are smaller than wild type, indicative of a defect in tip growth. However, the tip cells still remain polarized, and plants are able to form gametophores and other later developmental stages (Harries et al., 2005; Perroud and Quatrano, 2006). Interestingly, the *brick1* knockout mutant (Brick1 is an activator of the Arp2/3, complex) has a stronger phenotype, featuring much smaller cells, with impaired cell division planes. Whether Brick mutant cells undergo polarized growth is unclear, as the localization of various apical components is lost (Perroud and Quatrano, 2008). In addition, Brick mutant cells are not as spherical as cells lacking formin function or treated with actin depolymerizing drugs, so it is possible that there is still residual polarized growth. As is the case for Arp2/3 complex knockouts, development continues in Brick mutants with the formation of later developmental tissue stages (Perroud and Quatrano, 2008). Together these studies have, in a relatively short time, identified key molecular players during polarized growth in plant cells, a form of growth that is crucial for growth and development of all land plants.

Small RNAs

Studies in *P. patens* have recently had an impact on the field of small RNAs, revealing a novel pathway that connects microRNAs (miRNA) to the epigenetic silencing of DNA by methylation (Khraiwesh et al., 2010). In mammals, a link between miRNAs and
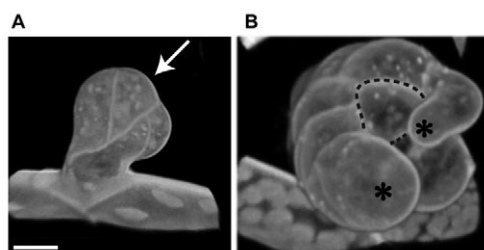

Fig. 3. Simple morphology of *P. patens* gametophore initials. (**A**) A young *P. patens* bud composed of four cells. The cell that is shaped like the triangle is the meristematic stem cell. (**B**) At a slightly later stage in development, the meristematic stem cell is identified by its triangular shape (dashed outline) and the leaflet initials are readily apparent, marked with asterisks. Images are maximal projections of confocal sections of tissue stained with propidium iodide and were kindly provided by D. Brockmann (University of Rhode Island, Kingston, RI, USA).
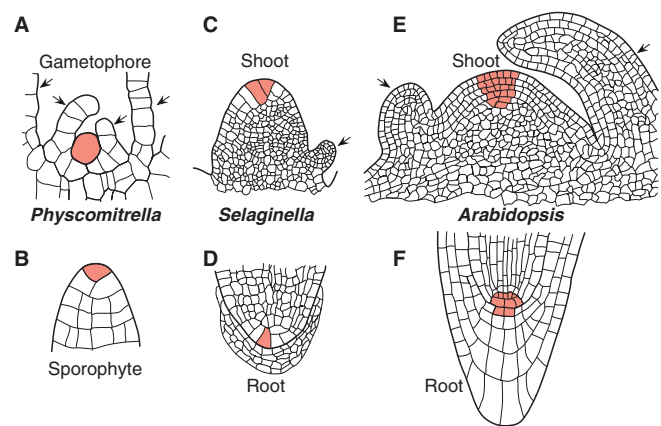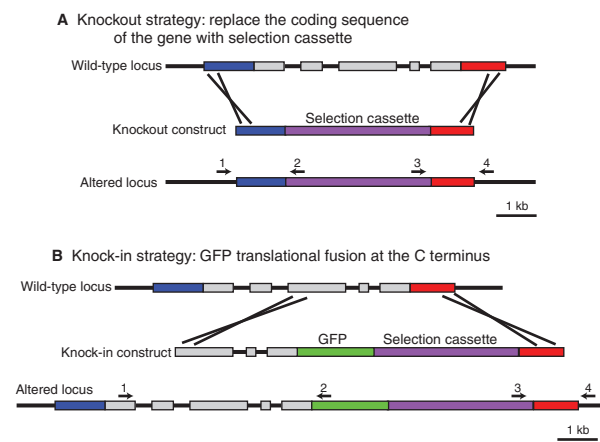

Fig. 4. Apical growth in land plants. A basic apical growth mechanism probably evolved in the algal ancestors of land plants and was elaborated into the apical-cell-type meristems of the: (**A**) moss gametophore; (**B**) moss sporophyte; (**C**) Selaginella (non-seed-plant) shoot; and (**D**) Selaginella root. (**E**) Seed plants produce multicellular shoot meristems and (**F**) root meristems. (Stem cell or stem-cell population are shaded red.) Arrows indicate the (non-homologous) lateral organs produced by each type of shoot: phyllids in A; microphylls in C; and leaves in E. Schematics show longitudinal sections through each apex, drawing on data published by: (A) Harrison et al. (Harrison et al., 2009); (B) Sakakibara et al. (Sakakibara et al., 2008); (C) Harrison et al. (Harrison et al., 2007); (D) M.J.P. (unpublished); (E) Prigge and Wagner (Prigge and Wagner, 2001); and (F) Yamada et al. (Yamada et al., 2009).

transcriptional gene silencing has been recently reported (Gonzalez et al., 2008; Kim et al., 2008). However, the mechanism elucidated in moss is distinct from that observed in mammals (Khraiwesh et al., 2010). In many eukaryotes, small RNAs are processed by a family of proteins known as Dicer. Dicer proteins vary in number between species, and the number of Dicer proteins may reflect the degree of specialization of small RNA function within a particular organism (Meister and Tuschl, 2004). As in *Arabidopsis*, moss has four Dicer-like (DCL) proteins, but moss appears to lack an obvious ortholog to AtDCL2. The deletion of one of the dicer genes, *DCL1b*, in moss results in the normal processing of miRNAs (Khraiwesh et al., 2010). However, subsequent cleavage of the mRNA target is abolished. This leads to a large increase in the amount of mature miRNA complexed with its mRNA target and to the hypermethylation of loci that encode the target mRNAs, leading to transcriptional silencing (Khraiwesh et al., 2010). A similar phenomenon was observed in wild-type plants treated with the phytohormone abscisic acid (ABA; see Glossary, Box 1), suggesting that this pathway is also present under normal conditions (Khraiwesh et al., 2010).

Evolution of gene expression modules

P. patens has also been used to examine the evolution of gene expression modules in the context of the expanding morphological complexity of the sporophyte (see Glossary, Box 1), which occurred during land plant evolution. In seed plants, root hairs are important for nutrient absorption and for anchoring the plant in soil. The *Arabidopsis* bHLH transcription factors *AtRHD6* and *AtRSL1* (*Root hair defective6* and *RHD Six-Like1*) are required for the expression of the genes that control root hair development (Heim et al., 2003; Masucci and Schiefelbein, 1994; Menand et al., 2007). *AtRHD6* and *AtRSL1* act downstream of transcription factors

Box 3. Gene targeting in *P. patens*

A Knockout strategy: replace the coding sequence of the gene with selection cassette

Wild-type locus

Knockout construct   Selection cassette

Altered locus   1   2   3   4

1 kb

B Knock-in strategy: GFP translational fusion at the C terminus

Wild-type locus

Knock-in construct   GFP   Selection cassette

Altered locus   1   2   3   4

1 kb

Homologous recombination is a powerful tool for specifically altering a genomic locus. In the accompanying figure, we show two approaches routinely used in moss: (A) knockout of the coding sequence of a gene; and (B) knock-in of GFP (green box) to translationally fuse GFP to the 3′ end. A hypothetical gene is depicted, showing the 5′ untranslated region (UTR, blue box), coding sequence (grey boxes), introns (black lines) and the 3′ UTR (red box).

To generate these genomic alterations, linear DNA molecules are introduced into moss protoplasts using polyethylene glycol (PEG). One-week-old protonemata are treated with cell wall-digesting enzymes, generating a suspension of single cells, which take up DNA fragments (for efficient targeting, 1000 bp of targeting sequences should be used) in the presence of PEG. Protoplasts regenerate without selection for 4-7 days. Selection identifies plants containing the DNA. Four antibiotics are commonly used: hygromycin B, Geneticin, Zeocin and blasticidin S. After 1 week, selection is relaxed. Plants containing the DNA, but that have not integrated it into the genome, rapidly lose the DNA without selection. Relaxation is maintained for 7-10 days. A second selection follows, and surviving plants are screened for proper integration, which can be verified by Southern blot analysis or PCR. For PCR, amplification with primers 1+2 and 3+4 ensures accurate integration at the 5′ and 3′ ends, respectively. Amplification with primers 1+4 diagnoses the number of copies integrated into the locus. From transformation to identification of stably transformed and properly integrated lines takes as little as 6 weeks.

Using homologous recombination, overexpression constructs can be introduced to a desired genomic site, reducing variable levels of expression that can occur because of the site of integration. A frequently used site is in an intergenic region that, when altered, does not produce any observable phenotypic consequences (Schaefer and Zryd, 1997).

functions similar to the root hair in *Arabidopsis*. Most interestingly, constitutive expression of *PpRSL1* in the *Atrhd6* mutant results in normal root hairs (Menand et al., 2007). Thus, the molecular function of *PpRSL1* and *AtRHD6* arose before the divergence of bryophytes and seed plants, and has been conserved in both lineages. This study elegantly demonstrated that closely related transcription factors control the development of root hairs in seed plants and of caulonemata and rhizoids in mosses. These two tip-growing cell types are non-homologous, because in seed plants they are found in the sporophyte and in the moss they are in the gametophyte. These data have led to a model of land plant evolution, whereby, in addition to traits arising de novo in the diploid generation (Floyd and Bowman, 2007; Maizel et al., 2005; Sakakibara et al., 2008), some traits in the sporophyte-dominant land plants developed morphological diversity by recruiting gene modules used for patterning their gametophyte-dominant ancestors (Menand et al., 2007).

Evolution of hormone-signaling pathways

Vascular plants have developed multiple features in order to retain water, including water-transporting vessels, stomata and a cuticle (see Glossary, Box 1). By contrast, nonvascular plants are in equilibrium with the surrounding air and thus not surprisingly, nonvascular plants have a robust tolerance to desiccation. This is exemplified by the bryophyte *Tortula ruralis* (Oliver et al., 2005), which can recover from extreme dehydration, after losing as much or more than 80% of its original fresh weight (Bewley, 1972).

The phytohormone ABA is crucial for various growth, developmental and stress-response pathways in plants. The transcription factor ABI3 activates a cadre of genes in response to ABA. In seed plants, ABI3 plays a crucial role in seed maturation, including imparting desiccation tolerance to the seed (Finkelstein et al., 2008). ABA functions through similar signaling networks in seed plants and bryophytes, because a wheat ABA-responsive promoter is activated by ABA in moss (Knight et al., 1995). Additionally, one of the three moss ABI3 homologs partially rescues the seed maturation defect in the *abi3* mutant in *Arabidopsis* (Marella et al., 2006). Most recently, deletion of the three *ABI3* genes in moss demonstrates that ABI3 is required for desiccation tolerance mediated by ABA (Khandelwal et al., 2010). Wild-type moss plants can survive complete desiccation only if pretreated with ABA. However, plants lacking ABI3 are unable to survive, demonstrating that ABI3 is responsible for gene expression required for ABA-mediated desiccation tolerance (Khandelwal et al., 2010). These studies have lead to the hypothesis that ABA gene networks in bryophytes evolved to protect plants from water shortages; in seed plants, the gene networks are thought to be necessary for desiccation tolerance in addition to seed maturation (Khandelwal et al., 2010).

Epigenetic regulation of phase transitions

The development of a vascular system was accompanied by the expansion of the sporophyte and by the reduction of the gametophyte during land plant evolution. Not only have transcription factor networks played a key role in the expansion of sporophyte diversification, but the epigenetic control of gene expression, specifically histone methylation, may have also helped to coordinate genome-wide changes in gene expression associated with developmental transitions, such as the vegetative-to-reproductive or gametophyte-to-sporophyte phase transitions. Using reverse genetics, recent studies have shown that the
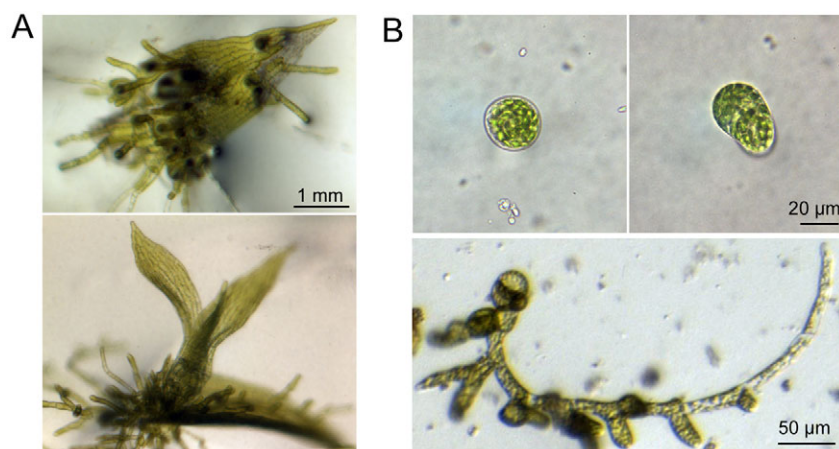
involved in epidermal pattern formation, but upstream of root hair development. Plants lacking these bHLH transcription factors do not form root hairs. Interestingly, these transcription factors affect only the development of root hairs, because pollen tubes, the other tip-growing cell in seed plants, are not affected (Menand et al., 2007).

Phylogenetic analyses showed that two out of the seven *RHD Six-Like* genes in moss (*PpRSL1* and *PpRSL2*) form a monophyletic clade with *AtRHD6* and *AtRSL1*. Moss plants lacking both *PpRSL1* and *PpRSL2* do not form caulonemata or rhizoids (see Glossary, Box 1); both these cell types are involved in rooting

Fig. 5. Vegetative propagation of *P. patens*.
(**A**) A *P. patens* gametophore leaflet picked off a shoot and placed on nutrient agar changes into protonema. Within 1 week, protonemal filaments grow out of damaged regions of the leaflet (top). Within 3 weeks, a gametophore grows out of the protonemal mesh that has regenerated from the leaflet (bottom). (**B**) Protoplast 24 hours after isolation (left). Within 48 hours, the first cell division occurs, and the emerging protonemal cell is seen as an emerging protuberance (right). Within 7 days, a small plant composed of ~20 cells is growing via tip growth (bottom).

polycomb repressive complex 2 (PRC2), which regulates gene expression by modifying the methylation of histone H3, is required to repress sporophyte development in the gametophyte stem cell (Mosquna et al., 2009; Okano et al., 2009). In the sporophyte, PRC2 expression is detected once the apical cell stops proliferating and the reproductive sporangium begins to form, implying a role for PRC2 in the transition from the vegetative to the reproductive form of the sporophyte (Okano et al., 2009).

In flowering plants, PRCs are involved in several phase transitions, including from gametophyte to endosperm (Goodrich, 1998; Guitton et al., 2004; Kohler et al., 2003; Ohad et al., 1996; Ohad et al., 1998) and gametophyte to sporophyte (Chaudhury et al., 1997; Guitton and Berger, 2005). Importantly, PRC2 complex function has been conserved throughout evolution, as partial complementation has been observed in *Arabidopsis* mutants expressing moss PRC2 complex members, and vice versa (Mosquna et al., 2009). In moss, the absence of PRC2 results in the formation of a sporophyte-like body that emerges from the gametophyte and grows indeterminately from an apical cell (Mosquna et al., 2009; Okano et al., 2009). If PRC2 expression is restored in the sporophyte-like body, then a sporangium-like body develops (Okano et al., 2009), further implicating PRC-mediated epigenetic changes during phase transitions. Interestingly, an indeterminately growing sporophyte-like body has been observed to branch (Okano et al., 2009). This is extremely rare among extant bryophytes. Yet, a branching sporophyte coupled with indeterminate growth is a hallmark of innovations accumulated in the dominant sporophyte body plans found in seed plants.

Limitations and future directions
Given the large evolutionary distance between vascular plants and mosses, many pathways are present in vascular plants that have no obvious counterparts in moss (Hirano et al., 2007; Rensing et al., 2008; Yasumura et al., 2007). This inherent limitation does not allow molecular characterization of these pathways in mosses. However, some insights can be gained from studying potential evolutionary roots in mosses, as has been the case for ABA signaling and transcription control.

P. patens studies would benefit from having more selectable markers and tightly controlled inducible promoters. One of the most successful promoters used for inducible expression to date has been the heat-shock promoter (Finka et al., 2007; Okano et al., 2009; Saidi et al., 2005). This promoter is tightly controlled, but using heat as the inducer presents inherent limitations, particularly if studying a pathway that is sensitive to temperature. Thus, the development of additional inducible promoters with tight control and/or tissue-specific promoters would enable future molecular manipulations of this plant at key developmental transitions.

Much of the work in *P. patens* has focused on reverse genetic approaches owing to the ease of transformation and vegetative propagation of transformed lines. Forward genetic approaches pose a potential drawback, which is the failure to identify mutations in key pathways because of the genetic redundancy inherent in a predominately haploid organism. However, a predominately haploid life cycle enables the rapid identification of dominant and recessive mutations that affect moss development. A number of mutants have been isolated that affect key hormone pathways, as well as responses to polarity cues such as gravity (Abel et al., 1989; Ashton and Cove, 1977; Ashton et al., 1979a; Ashton et al., 1979b; Engel, 1968; Jenkins et al., 1986). It has been challenging to identify the molecular lesions in these mutants. However, recently, a genetic linkage map has been generated by crossing a French ecotype to a British ecotype (Kamisugi et al., 2008). Thus, the tools to map the location of a particular mutant are actively under development. Furthermore, the corresponding genes could be identified using a candidate-gene approach (Brucker et al., 2005; Prigge et al., 2010) or by using next-generation sequencing technologies.

Conclusions
With a completed genome sequence and many emerging tools, *P. patens* is emerging as the yeast equivalent of plant research. *P. patens* is rapidly propagated, easily transformed and relatively simple in development and morphology compared with most vascular plants. Although its genome is not necessarily a 'stripped-down' version of vascular plant genomes, the ease with which genetic manipulations can be performed in this plant has, in a short time, enabled several key findings to be made, ranging from plant evolution to silencing pathways to the molecular basis of cell growth.

Competing interests statement
The authors declare no competing financial interests.

DEVELOPMENT

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