

## The Moss *Physcomitrella patens*, Now and Then

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Mosses (Musci, Bryophytaea) are one of the oldest groups of land plants present among the earth's flora. They originated 500 million years ago (for a recent discussion of the time scale involved, see Heckman et al., 2001) and are currently represented by approximately 10,000 species that colonize diverse habitats that range from high mountains to deep forests and from Antarctica to deserts. Evolutionary studies support the monophyletic origin of land plants and indicate that bryophytes may form a sister clade with tracheophytes, although the exact relationship between bryophytes (hornworts, liverworts, and mosses) and vascular plants is still a matter of debate (Kenrick and Crane, 1997; Nickrent et al., 2000). This nevertheless places mosses at an evolutionary position that is ideal for comparative studies of the evolution of biological processes in land plants. Their life cycle is dominated by a photoautotrophic haploid gametophytic generation that supports a relatively simple and mainly heterotrophic diploid sporophyte. The haploid gametophyte itself is characterized by two distinct developmental stages: the protonema, a filamentous network of chloronemal and caulonemal cells, which develop by apical growth and cell division of apical and subapical cells; and the gametophore or leafy shoot, which differentiates by caulinary growth from a simple apical meristem (the bud). The latter is made up of a photosynthetic non-vascularized stem, which carries the leaves and the reproductive organs and of filamentous rhizoids that arise from the base of the stem (for review, see Reski, 1998).

The potential of mosses as model systems to study plant biological processes was already recognized in the forties and reflects their relatively simple developmental pattern, their suitability for cell lineage analysis, their similar responses to plant growth factors and environmental stimuli as those observed in other land plants, and the facilitated genetic approaches resulting from the dominance of the gametophyte in their life cycle (Cove et al., 1997). Plant physiologists have focused their studies on species like *Funaria hygrometrica*, *Ceratodon purpureus*, and *Physcomitrella patens*, but it was the possibility to realize crosses in vitro that led the latter to be chosen for genetic approaches. *P. patens* is a monoecious

moss (i.e. both sex organs are present on the same individual) that requires very simple growth conditions for the completion of its life cycle (Fig. 1A; Cove, 1992). *P. patens* is the first moss to be successfully transformed (Schaefer et al., 1991) and has recently been singled-out as the first land-plant, and perhaps more interestingly the first multicellular eukaryote, in which gene targeting occurs with an efficiency similar to that observed in the yeast *Saccharomyces cerevisiae* (Schaefer and Zryd, 1997; Schaefer, 2001).

### THE EARLY STORY (THE LAST 25 YEARS OF THE TWENTIETH CENTURY) OF *P. PATENS*

The modern history of *P. patens* goes back to a seminal paper (Engel, 1968) describing the successful isolation and genetic analysis of auxotrophic mutants for thiamine, nicotinic acid, and para-aminobenzoate. This work prompted the groups of David Cove in Cambridge and later in Leeds and of Wolfgang Abel at the University of Hamburg to further develop *P. patens* as a model genetic system. Their work led to the isolation and biological characterization of several biochemical and developmental mutants generated by chemical mutagenesis (Ashton and Cove, 1977; Ashton et al., 1979; Abel et al., 1989; Cove et al., 1997). Techniques for genetic analysis were also developed; *P. patens* is self fertile and test crosses were found to be facilitated by the use of self-sterile but cross-fertile auxotrophic mutants (Courtice et al., 1978). One critical step was achieved with the successful isolation and efficient regeneration of protoplasts from young chloronemal filaments (Grimsley et al., 1977). The simultaneous development of polyethylene-glycol-mediated protoplast fusion made genetic dominance studies and complementation analysis of sterile mutants possible (Grimsley et al., 1977). These initial studies indicated that *P. patens* could be a useful model system for studying developmental genetics in plants (Cove, 1992).

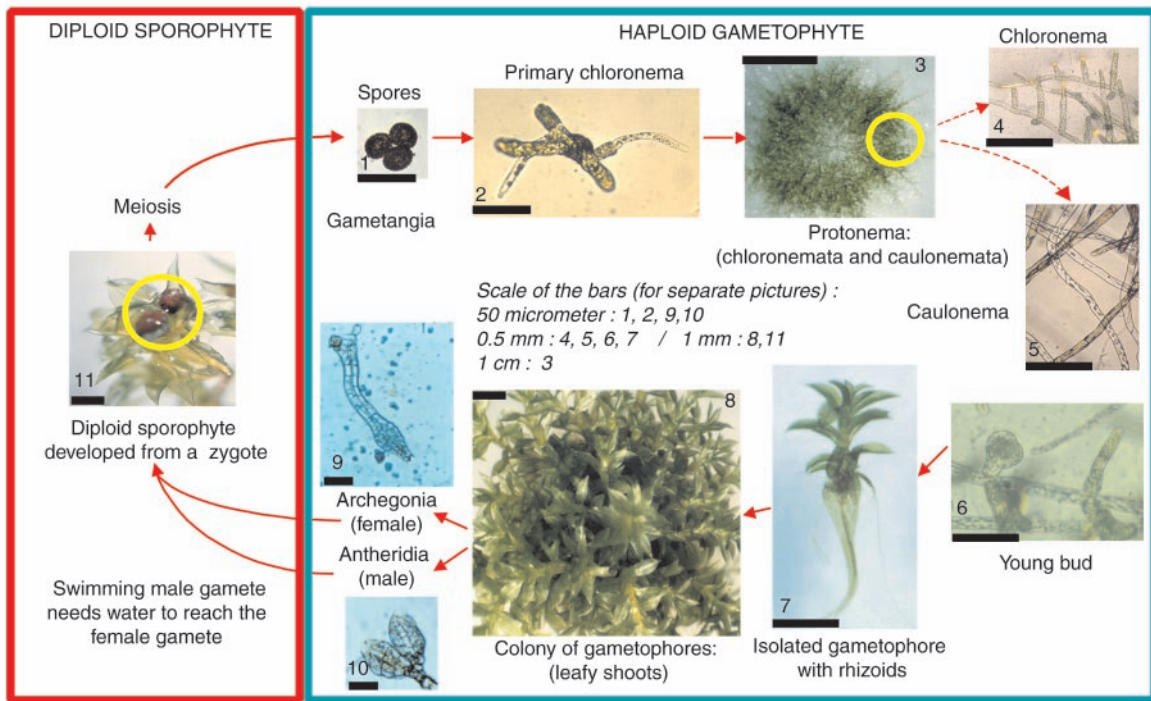
In the early eighties the major focus in plant research was the development of plant transformation methods using either *Agrobacterium tumefaciens* or polyethylene glycol (PEG)-mediated direct DNA transfer to protoplasts. These methods coupled with the newly discovered 35S promoter allowed a variety of selectable expression cassettes to be introduced into plants. The huge potential of these approaches also attracted a handful of scientists who realized their potential for studies on *P. patens*. As

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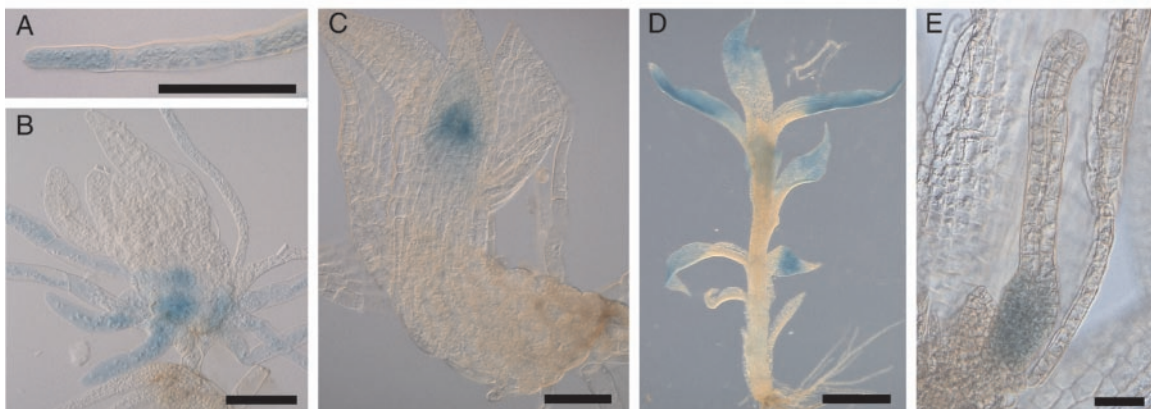
www.plantphysiol.org/cgi/doi/10.1104/pp.010786.

1A

The life cycle of *Physcomitrella patens*



1B



**Figure 1.** A, The life cycle of *P. patens*. (1) Spores, (2) light-dependent spore germination generating primary chloronemata, (3) 15-d-old protonemal colony, (4) branching chloronema, (5) caulonema cells characterized by an oblique cell wall and a small number of chloroplasts, (6) filamentous two-dimensional structure switches to three dimensions with the appearance of young bud, (7) young bud developing to form the leafy shoot of the gametophore, (8) a fully developed moss leafy gametophyte (4 weeks old), (9) an archegonium (female structure) dissected out from a gametophore. *P. patens* being a monoecious species both structures are present on the same plant and appears after a cold treatment (15°C for 3 weeks), (11) after fertilization by swimming spermatozoids (under water), the egg cell develops into a small diploid sporophyte and within its capsule meiosis occurs leading to spore formation (approximately 5,000 spores per capsule). The whole cycle can be achieved under optimal conditions in less than 12 weeks. B, Developmentally regulated expression of the GUS gene in gene- and enhancer-trap lines of *P. patens* (by courtesy of Yuji Hiwatashi and Mitsuyatsu Hasebe). A, Chloronema cells of a gene-trap line YH261. B, A gametophore of a gene-trap line YH206 showing the rhizoid cells predominantly stained. C, A gametophore of an enhancer-trap line ET41. The apex of the gametophore is stained. D, A gametophore of an enhancer-trap line ET326. Leaf blades are stained. E, An archegonium of a gene-trap line YH126; the ventral cell and egg cell are stained. The bars in A, B, C, and E = 50 μm; in D = 500 μm.

mosses do not interact with *A. tumefaciens*, the main focus was on direct transformation of protoplasts through various methods with an emphasis on PEG-

mediated transformation. The first successful transformation was achieved by PEG-mediated DNA transfer into protoplasts only 10 years later using

35S-driven plasmids carrying antibiotic resistance markers (Schaefer et al., 1991). Biolistic delivery of genes has since been used with partial success (Knight et al., 1995).

When sequential transformation of *P. patens* was attempted (for example by retransforming a transgenic strain already resistant to kanamycin with the same transformation plasmid containing instead the hygromycin resistance marker) it was observed that the resulting transformed plants almost always displayed a very close genetic linkage of the two resistance transgenes. These genetic data suggested that the second plasmid integrated at the previously generated artificial locus by homologous recombination, providing the first evidence for efficient gene targeting in *P. patens* (Schaefer, 1994; Kammerer and Cove 1996). Transformation experiments using cloned *P. patens* genomic sequences confirmed this hypothesis leading to the conclusion that, contrary to other plants studied to-date, the integration of foreign DNA sequences into the genome occurs predominantly at targeted locations by homologous recombination (Schaefer and Zryd, 1997). These studies opened the door for high efficiency targeted mutagenesis in a plant species (see Schaefer [2001] for a detailed account of gene targeting in *P. patens* and other eukaryotes).

Targeted mutagenesis by gene targeting is the ultimate method for studying gene function in biological systems as it enables the direct generation of loss-of-function and point mutations in the gene under study. It is used as a routine method for functional genomic studies in bacteria and yeast since transfected DNA integrates essentially at targeted locations by homologous recombination. Yet, in multicellular eukaryotes, this methodology is not accessible since integration of foreign DNA sequences occurs at random locations in the genome by illegitimate recombination with a frequency that is orders of magnitude higher than that observed for homologous recombination. The only notable exception is in mice where embryonic stem cells transformation is used to generate predetermined mutations in the mouse genome (Müller, 1999). In the whole plant kingdom, gene targeting is still unfeasible as a routine procedure (Vergunst and Hooykaas, 1999) with the sole exception of *P. patens*.

The potential of *P. patens* was soon demonstrated by the publication of several key papers characterizing specific gene disruptions. Strepp and coworkers (1998) disrupted the *ftsZ1* gene, a moss homolog of a bacterial protein that shares structural features with tubulin and is an essential component of the prokaryotic cell division machinery. Cells of *ftsZ1* knock-out *P. patens* strains are characterized by the presence of a single huge chloroplast per cell instead of the approximately 50 chloroplasts found in normal cells. This phenotype resulted from dysfunc-

tional chloroplast division and provided functional evidence for the involvement of FtsZ protein in the process. Another group interested in the metabolism of unsaturated fatty acids was able to show that disruption of a  $\Delta$ -6 desaturase gene was responsible for a severe alteration of the lipid profile of *P. patens* (Girke et al., 1998). The exquisite specificity of gene targeting was assessed in a successful experiment designed to disrupt one specific member of the highly conserved chlorophyll *a/b*-binding protein (Cab) multigene family (Hofmann et al., 1999). In a study of the proteasome-ubiquitin-mediated proteolytic pathway of *P. patens*, Girod and coworkers (1999) successfully knocked-out the *mcb1* gene. The Mcb1 protein is a component of the 19S regulatory complex of the highly conserved 26S proteasome present in all eukaryotes and its function remains to be elucidated. *Mcb1* knock-out performed in yeast did not reveal any strong phenotype, except an increased sensitivity to amino acid analogs, whereas it led to embryonic lethality in mouse (Kawahara et al., 2000). Remarkably, the *P. patens* knockout displayed a developmental phenotype characterized by impaired bud differentiation. This study illustrates out how critical it is that different multicellular organisms are used to study the biological functions of proteins involved in complex regulatory pathways.

The success and the potential of gene targeting in *P. patens* has also prompted heavy private investment from the German agrochemical company BASF, which has developed a large expressed sequence tag (EST) database (more than 110,000 entries to date representing more than 20,000 genes). In the public domain, the EST program involving the University of Leeds (UK) in collaboration with Washington University (St. Louis, MO) has totaled 14,000 entries to-date (Quatrano et al., 1999). Other groups, notably in Japan, have recently reported their commitment to develop new EST databases. The genome size of *P. patens* is estimated to be around 460 Mb distributed among 27 chromosomes, which corresponds to the size of the rice genome (Reski, 1999), and preliminary analyses of ESTs and of genomic sequences clearly indicate that *P. patens* and other land plant genes are highly similar, at the level of both intron-exon structure and codon usage. We have mentioned only the first published papers from a selection of the 160 to 170 papers dedicated to *P. patens* until today (a list is available at <http://www.unil.ch/lpc/docs/physcol.html>). Recently, there has been a rapid growth of *P. patens* research as was clearly demonstrated during the last international MOSS meetings held in Switzerland (MOSS 2000 Abstracts can be found at <http://www.unil.ch/lpc/docs/moss2000.pdf>) and in Japan (MOSS 2001 Abstracts can be found at <http://www.nibb.ac.jp/%7Emhasebe/MOSS2001/index.html>).



## FUNCTIONAL GENOMICS FROM YEAR 2000 ONWARD: OF MICE, MEN AND MOSSES

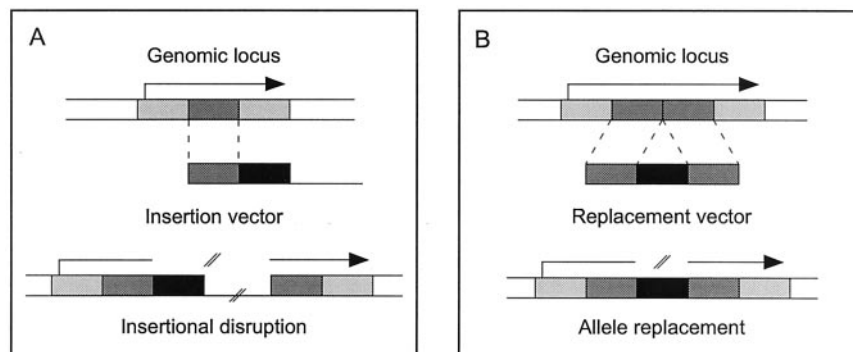
The complete sequence of the Arabidopsis genome (The Arabidopsis Genome Initiative, 2000) and the million of sequences from other plants deposited in the databases provides ample scope for functional analysis of plant genes. Sophisticated molecular genetic tools that have been developed in model plant systems, such as gene tagging and gene trapping approaches or collections of insertional mutants (for a discussion, see Bouchez and Hofte, 1998), provide an extremely valuable set of methods for deciphering plant gene functions. What is the additional potential of the *P. patens* model system and how can it be used by the biological community for functional genomic studies?

Sequencing full or partial genomes is only the first part of a greater challenge confronting biology today. The next step is to decipher the function of genes and to unravel the complex interactions governing genetic networks. Global approaches aimed at describing the expression levels of sets of genes under specific experimental conditions (transcriptomic), and the protein patterns that follow (proteomic) provide extremely valuable information on the genetic networks controlling specific biological processes. Yet a precise understanding of how these proteins function and interact with each other in a cellular context also requires the ability to introduce precise alterations within specific components of these networks. In this respect *P. patens* is poised to fill a gap in the tools that are presently available for studying the function of proteins in vivo.

Highly precise and specific predetermined modifications of any sequence of the genome of *P. patens* are now possible by targeted transgenesis. Knocking-out specific genes is straightforward with both types of targeting vectors (Fig. 2, A and B). Point mutagenesis by gene conversion (i.e. the replacement of a chromosomal sequence by a single copy of an in vitro mutated one, Fig. 2B) is efficient since it accounts for approxi-

mately 25% of targeted integration events observed with replacement vectors. Subsequent excision of undesired integrated sequences (such as plasmid repeats or expression cassettes) using the site-specific recombination Cre/lox system (Sauer, 1998) can be achieved upon transient expression of the Cre recombinase (Chakhparonian, 2001). This enables the recycling of selectable markers for sequential mutagenesis and guarantees that the observed phenotype exclusively results from the introduced mutation. Promoters and reporter genes currently used to transform higher plants display similar biological features and can be used with the same efficiency in *P. patens* (Knight et al., 1995; Chakhparonian, 2001). Gene targeting also enables comparative over-expression studies to be conducted at predetermined locations of the genome. This eliminates the stochastic position effects observed upon illegitimate transgenesis. Finally, studying the spatial and temporal expression of any gene in vivo can be easily achieved following transformation with replacement vectors carrying transcriptional and/or translational fusion of moss sequences with cytological marker genes such as *uidA* ( $\beta$ -glucuronidase [GUS]) or green fluorescent protein (GFP).

Sophisticated tools applied so far only to microbiological systems have been used recently in *P. patens* by the group of Hasebe in Okasaki to identify developmentally related genes (Nishiyama et al., 2000; Hiwatashi et al., 2001). Shuttle mutagenesis (Ross MacDonald et al., 1997) was used to generate libraries of moss genomic sequences mutagenized by the insertion of a bacterial transposon carrying a gene- or enhancer-trap GUS reporter cassette. Detectable phenotypes were obtained at frequencies of 4% and 30% for *P. patens* transformed with gene- and enhancer-trap libraries, respectively. This is 10 times higher than frequencies of trapping observed in Arabidopsis by illegitimate transgenesis. Figure 1B shows five different lines, resulting



**Figure 2.** Typical vector design used in *P. patens* transformation. A, The insertion vector carries a genomic fragment (dark gray) beside a selectable marker (black). Targeted integration is characterized by the insertion of one or several copies of the vector through homologous recombination with the genomic sequences. The resulting mutation is a gene inactivation by insertion. B, A replacement vector carries a selectable marker (black) inserted between two genomic sequences. Integration occurs through two homologous recombination events. The result is a loss-of-gene function through allele replacement. Gene conversion is the result of such a strategy and could be designed to produce subtle point mutations.

from such an experiment, each histochemically stained for the expression of the GUS reporter gene. Expression of tagged lines at different developmental stages (from primary chloronema to archegonium) illustrates that the whole life cycle of *P. patens* is accessible with this high through-put methodology.

With these tools in hand it is now possible to use *P. patens* to answer biological questions that cannot easily be studied using other plants including *Arabidopsis*. Table I compares the current situation of both systems for the fine analysis of protein function in vivo and in a wild-type background. Point mutation (affecting, for example, a phosphorylation site or a critical protein domain) and promoter replacement at the native locus, relocation of a gene to a new locus, or improving the function of plant genes through "protein design" are examples of what can only be done in *P. patens*. Contemporary biomedical research relies heavily on the development of large collections of knock-out mice; if we compare the difficulties involved and the time required to obtain a transgenic mouse with the ease of use and the short time needed to get a targeted transformant in *P. patens*, plant biologists using *P. patens* are at the forefront of functional genomic studies.

#### PHYSCOMITRELLA EVOLUTIONARY-DEVELOPMENTAL GENETICS

How do bryophytes and angiosperms relate to each other and how far have these groups diverged? Understanding the molecular mechanisms underlying the diversity of biological processes in various organisms is one of the basic issues in biology; in this respect, understanding the relation between function and structure of related genes is crucial. Evolutionary develop-

mental genetics (or "evo-devo") is a field that could benefit greatly from more extensive studies on *P. patens* developmental biology and from the establishment of large EST databases. Multigene families are thought to evolve by gene duplications, followed by a diversification correlated with changes in the whole organization of the plant; it is therefore of prime importance to study such gene families in plant groups other than angiosperms and to correlate molecular findings with developmental patterns. We will illustrate the problem with two examples.

The first example involves *c-MYB* oncogene-like genes, a family of DNA binding proteins present in plants and animals. Animal MYB proteins are characterized by the presence of three helix-turn-helix domains (R1R2R3-MYB genes) whereas plant MYB proteins usually have only two domains (R2R3-MYB genes). Recently, R1R2R3-MYB genes have been isolated from *P. patens* and *Arabidopsis* (Kranz et al., 2000). The amino acid sequences of their DNA-binding domains show a surprisingly high similarity to those of animal MYB factors and less similarity to the well-known R2R3-MYB proteins from plants. This suggests that DNA-binding proteins containing three MYB repeats existed before the divergence of the animal and plant lineages; R1R2R3-MYB genes may have a conserved function in eukaryotes, whereas the plant related R2R3-MYB genes might predominantly regulate plant-specific processes, which evolved during plant speciation. Both R1R2R3-MYB and R2R3-MYB genes have been identified in *P. patens* and functional studies by targeted mutagenesis could shed light on their respective roles in plant development.

The second example deals with proteins involved in the establishment of the architecture of the plant. Many genes that control vegetative and reproductive development belong to the MADS-box and homeobox genes families and *P. patens* homologs have been isolated and compared with the higher plant genes (Krogan and Ashton, 2000; Champagne and Ashton, 2001; Sakakibara et al., 2001). From these data, it seems that the diversification of *KNOX* genes (a family of homeobox containing genes) occurred before the separation of the bryophyte lineage from the lineage leading to vascular plants. On the other hand, the extensive diversification of MADS-box genes observed in angiosperms may have occurred after the divergence of both lineages, since MADS-box genes identified so far in *P. patens* display limited diversification. Determining the function of the MADS-box or homeobox genes in *P. patens* may lead to the identification of the moss developmental stages that correspond to the ancestral shoot apical meristem or inflorescence meristem that are essential in the development of vascular plants. Furthermore, comparing this information with that available from flower plants may help define a model that can account for the evolution of development in land plants (Thiessen et al., 2001).

**Table I.** Comparison of genome structure and availability to functional genomic approaches in *Arabidopsis* (At) and *P. patens* (Pp)

+, Feasible in the natural chromosomal environment of the studied gene; -, not feasible in the natural chromosomal environment of the studied gene; +/-, feasible at ectopic locations or with low efficiencies.

<i>P. patens</i> versus <i>Arabidopsis</i>	Pp	At
Genome structure		
Genome size	460 Mb	120 Mb
Chromosome number	27	5
Available sequence information	1%	Complete
Estimated number of genes	>20,000	25,498
Transgenic functional genomic tools		
Knocking out genes	+	+/-
Fine functional analysis by point mutation	+	-
Altering gene expression pattern by promoter replacement	+	-
Generating a non random collection of allelic mutants	+	-
Improving plant gene products by protein design	+	+/-
Functional studies of non coding chromosomal sequences	+	-

In both cases reported here, we await the results of analyses of the expression pattern and of the knockout phenotype of the corresponding genes. In the future studies, the ability in *P. patens* to introduce specific point mutations in these genes will allow us to monitor precisely the effect of subtle modifications of specific protein domains. In more general terms, we can expect that comparing the functional properties of moss genes governing protein-protein and protein-DNA interaction or developmental processes with those of the corresponding angiosperm genes will be highly informative for plant biologists.

Finally, we are left with another major question of interest related to the evolution of land plants: the shift of the alternation of generations. Land plants are very peculiar in this respect in that the extremes of the evolutionary group are characterized on the one hand by bryophytes with a dominant haploid gametophytic generation and on the other hand by modern angiosperms, which display typically a dominant diploid sporophytic generation. At first glance it is not clear what are the evolutionary driving forces behind the progressive appearance of a predominant diploid sporophytic phase in plants. Organisms with diploid genomes face well-known problems: diploidy provides redundancy in the genome, which protects the organism from the immediate consequence of detrimental mutations, but it also masks the accumulation over time of detrimental mutated alleles, and this might decrease the long-term fitness of populations. Comparison of the rates of mutation and recombination of specific sets of genes in bryophytes and angiosperms should help shed some light on this question.

Directly related to the last question is the status of homologous recombination mechanisms and the nature of the respective regulation of non-homologous versus homologous DNA repair mechanisms. Three hypotheses have been formulated to explain the largely dominant homologous recombination process observed upon transformation of *P. patens* (for further discussion, see Schaefer, 2001): the first is related to the transformation process per se; the second is related to possible specific properties of the physiology of the haploid gametophyte stage itself; and the third deals with cell-cycle distribution in protoplasts. The large amounts of naked DNA that are delivered to protoplasts in the presence of high PEG concentrations might be an important factor influencing the balance between homologous and illegitimate recombination-mediated integration events, but there is yet no evidence available that would correlate transformation methods with gene targeting efficiencies. The demonstration that the haploid gametophyte shows a preference for homologous recombination-mediated DNA integration compared with the sporophyte has yet to be shown in mosses and flowering plants. Unfortunately, access to the angiosperm gametophyte is difficult; transformation of pollen (before the deposit of exine or during pollen tube germination) would be one possibility, but

possible negative results will not provide conclusive evidence concerning this question. Nevertheless this approach is worth trying as we can expect that in the diploid phase some mechanisms might exist that repress somatic homologous recombination that would otherwise lead to genetic exchanges between chromosome homologs. On the other hand, in a haploid phase such mechanisms are not necessary whereas homologous recombination-dependent DNA repair would be a critical requirement for maintaining the integrity of the genome. Finally, the third hypothesis results from the observation that *P. patens* protoplasts are highly synchronized cells arrested at the G2/M boundary during the transformation process; whether this is affecting gene targeting efficiency is not known and deserves further investigations (Reski, 1999).

Favored experimental approaches involve the characterization of the enzymes and proteins involved in double-strand break repair and homologous recombination which have been conserved through evolution. We already know a great deal from prokaryote and yeast studies and we can expect that testing mutations affecting homologous recombination positively or negatively in *Physcomitrella* may shed light on this question. Experiments that identify genes responsible for efficient gene targeting will be indispensable for designing effective strategies for gene targeting in other Eukaryotes.

#### PHYSCOMITRELLA ENVIRONMENTAL PHYSIOLOGY AND BIOCHEMISTRY

We will not here describe the whole corpus of knowledge that has accumulated over the years on this subject (for further references, see Cove, 1992, 1997; Reski, 1998), but we will instead concentrate on some more recent developments. *P. patens*, like *Arabidopsis*, is a short lived (3-month life cycle) opportunist living in an open disturbed habitat. Like other mosses, it is heavily dependent on water (flooding) for its reproduction but can survive some desiccation. Spores of *P. patens* have an absolute requirement for light for their germination, whereas the protonema and the leafy gametophore are responsive to light (both quality and periodicity), to gravity, and to mechanical, cold, salt, or drought stress.

Spectral responses to red or blue light have been extensively studied in moss and fern gametophytes. *P. patens* phytochrome (Schneider-Poetsch et al., 1994) and cryptochrome genes (Imaizumi et al., 1999) have been characterized. Besides the well-known effect of increasing photon flux and directional growth responses to light (phototropism), mosses and ferns display a photopolarotropic response; in this case, tip growth localization, orientation of the plane of cell division as well as organelle movement can be modulated by the orientation of the plane of the electrical vector of linearly polarized light (Jenkins and Cove, 1983; Kadota et al., 2000). Using the photopolarotropic response it is pos-



sible to analyze the relative spatial orientation of the photoreceptor and to study the rearrangement of the cell structures. Moss protoplasts and protonemal filaments provide excellent systems for studying all aspects of cell polarity, including modification of cell shape, reorientation of apical growth, or reorganization of cytoskeletal structure (Cove et al., 1996). For example, the actin network has been visualized in vivo through the use of GFP-talin transgenic plants (Kost et al., 1998), which allows the cortical actin cables to be brightly labeled with GFP (Fig. 3). In this figure, the apical-basal distribution of microfilaments that supports the asymmetry of cell polar growth can be clearly discerned.

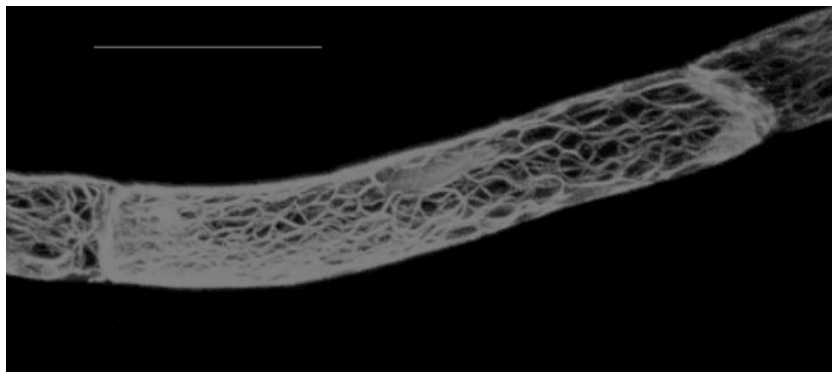
Many hormonal factors are common between bryophytes and angiosperms (Schumaker and Dietrich, 1997). Auxins, cytokinins, and abscisic acid among others have been studied in *P. patens* by classical means including mutant isolation (Cove, 1992). *P. patens* shows a dramatic switch in development upon differentiation from a two dimensional structure displaying apical growth (the protonema) to a three dimensional structure (the bud), which will further differentiate into the leafy shoot by caulinary growth. This is one of the main transitions that can be manipulated by hormones, and nutritional factors ( $\text{NO}_3^-$  versus  $\text{NH}_4^+$  nitrogen source,  $\text{Ca}^{2+}$  availability, etc.). Calcium fluxes have been shown to play a critical role in the establishment of cell polarity and consequently in the branching pattern and fate of the protonema cells (Ermolayeva et al., 1997). The transition to three-dimensional growth occurs in a side-branch initial cell derived from a subapical caulonema cell; when cytokinins are added to the medium the transition that normally occurs in approximately 5% of the side-branch initials occurs in 100% of those cells (Reski and Abel, 1985). This morphogenetic process provides a unique experimental tool to study the mode of action of cytokinins (Reutter et al., 1998). In this respect, *P. patens* has a tremendous advantage over angiosperms in that cell lineage can be easily followed from the spore through the protonema stage and all the way to the more complex leafy shoot and reproductive organs. This property is rare among model systems in

developmental biology and accounts for the success of the worm *Caenorhabditis elegans* as model organism in animal biology.

Filamentous rhizoids differentiate from the basal part of the gametophore; these are non-photosynthetic organs that can be considered as the functional equivalent of the root system (they are also positively gravitropic). The leaf blades are disposed on a phyllotactic spiral along the stem axis. Under proper conditions (an induction temperature of  $15^\circ\text{C}$ ), gametophores will undergo differentiation, leading to the formation in the apical part of the shoot of the reproductive organs (the male antheridia and the female archegonia). When water is added, the antherozoids from the antheridia swim to the archegonia and fertilize the single egg cell within. Each zygote gives rise to a "parasitic" sporophyte that will produce through meiosis about 5000 spores contained within a capsule. It is interesting that gametangia differentiation and/or development of the sporophyte seems to be short-day dependent.

Mosses are known to interact with mycorrhizal fungus and other soil organisms (Richardson, 1981; During and Vantoreen, 1990). Nevertheless, little has been reported about the responses of *P. patens* to pathogenic microorganisms. This situation, which is not very different from the situation of *Arabidopsis* 15 years ago, reflects the lack of research in bryophyte pathology and also the difficulty of observing the plant in its natural habitat. A number of strategies might be used to overcome this knowledge-gap; among them, challenging the moss with highly virulent or broad host-range pathogens or using higher plant elicitors or defense signals to induce specific pathogen-related responses. It is interesting that several homologs of plant resistance genes (*R*-genes) can be found in the EST database.

Moss biochemistry lags well behind molecular and genetic approaches but new information is accumulating at a rapid pace. Some sugar and lipid metabolism genes have been isolated and characterized (Girke et al., 1998) and EST collections contain a large number of sequences related to primary metabolism. From these data we can already conclude that *P. patens* biochemis-



**Figure 3.** An example of polarity study in vivo in the early filamentous growth of a moss plant; subapical chloronemal cell of a GFP-talin *P. patens* transgenic plant showing the typical polar distribution of actin cortical cables (bar is  $25\ \mu\text{m}$ ).

try appears to be very similar to the biochemistry of other land plants. We can predict that deciphering the regulation of critical pathways in photosynthetic organisms can be speeded up in many cases by using high efficiency targeted mutagenesis in *P. patens*.

## CONCLUSIONS

*P. patens* is an easy plant to work with and requires neither expensive maintenance facilities nor large laboratory space. Most of the basic tools for high precision mutagenesis have been tested on this plant, are found to work, and are easily available. Highly efficient gene targeting in *P. patens* allows the precise mutagenesis of specific sequences in their proper original chromosomal location in a plant wild-type background. Mutagenesis can be applied to coding sequences, promoters, and other regulating elements as well as to non-coding sequences that flank genes of interest. Gene targeting also enables the direct tagging or trapping of genes to monitor their function in vivo. As illustrated by the examples of yeast and mouse embryonic stem cells, efficient gene targeting is essential if an organism is to be developed as a general model system. In yeast, the combination of efficient gene targeting coupled with a fully sequenced and well-annotated genome has allowed researchers to perform true and systematic functional genomics (as opposed to simple gene expression studies). *P. patens* presents with the same opportunity to address gene functions in plants. Efficient gene targeting in *P. patens* provides a fundamentally new tool for plant research and we can expect that it will be used increasingly during the forthcoming years. For this reason, it is highly desirable that an extensive genome sequencing project be developed for *P. patens*; given its genome size of 460 Mb, contemporary sequencing technologies and adequate funding, the whole genome could be captured in silico in less than 2 years. Since we are presented only rarely with the chance to develop and utilize a fundamentally new approach to studying biological problems, we should not neglect the opportunity provided by *P. patens*.

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## LITERATURE CITED

Abel WO, Knebel W, Koop H-U, Marienfeld JR, Quader H, Reski R, Schnepf E, Spörlein B (1989) A cytokinin-

- sensitive mutant of the moss, *Physcomitrella patens*, defective in chloroplast division. *Protoplasma* **152**: 1–13
- Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**: 796–815
- Ashton NW, Cove DJ (1977) The isolation and preliminary characterization of auxotrophic and analogue resistant mutants in the moss *Physcomitrella patens*. *Mol Gen Genet* **154**: 87–95
- Ashton NW, Grimsley NH, Cove DJ (1979) Analysis of gametophytic development in the moss *Physcomitrella patens*, using auxin and cytokinin resistant mutants. *Planta* **144**: 427–435
- Bouchez D, Hofte H (1998) Functional genomics in plants. *Plant Physiol* **118**: 725–732
- Chakhparonian M (2001) Développement d'outils de la mutagenèse ciblée par recombinaison homologue chez *Physcomitrella patens*. Thèse de Doctorat ès Sciences. Université de Lausanne, Lausanne, Switzerland. <http://www.unil.ch/lpg/docs/theses/MCthesis.pdf>
- Champagne CEM, Ashton NW (2001) Ancestry of KNOX genes revealed by bryophyte (*Physcomitrella patens*) homologs. *New Phytol* **150**: 23–36
- Courtice GRM, Ashton NW, Cove DJ (1978) Evidence for the restricted passage of metabolites into the sporophyte of the moss *Physcomitrella patens* (Hedw.). *Br Eur J Bryol* **10**: 191–198
- Cove DJ (1992) Regulation of development in the moss, *Physcomitrella patens*. In S Brody, DJ Cove, S Ottolenghi, VEA Russo, eds, *Developmental Biology. A Molecular Genetic Approach*. Springer Verlag, Heidelberg, pp 179–193
- Cove DJ, Quatrano RS, Hartmann E (1996) The alignment of the axis of asymmetry in regenerating protoplasts of the moss, *Ceratodon purpureus*, is determined independently of axis polarity development. *Development* **122**: 371–379
- Cove DJ, Knight CD, Lamparter T (1997) Mosses as model systems [review]. *Trends Plant Sci* **2**: 99–105
- During HJ, Vantoreen BF (1990) Bryophyte interactions with other plants. *Bot J Linn Soc* **104**: 79–98
- Engel PP (1968) The induction of biochemical and morphological mutants in the moss *Physcomitrella patens*. *Am J Bot* **55**: 438–446
- Ermolayeva E, Sanders D, Johannes E (1997) Ionic mechanism and role of phytochrome-mediated membrane depolarisation in caulonemal side branch initial formation in the moss *Physcomitrella patens*. *Planta* **201**: 109–118
- Girke T, Schmidt H, Zahringer U, Reski R, Heinz E (1998) Identification of a novel delta 6-acyl-group desaturase by targeted gene disruption in *Physcomitrella patens*. *Plant J* **15**: 39–48
- Girod PA, Fu H, Zrýd JP, Vierstra RD (1999) Multiubiquitin chain binding subunit MCB1 (RPN10) of the 26S proteasome is essential for developmental progression in *Physcomitrella patens*. *Plant Cell* **11**: 1457–1472
- Grimsley NH, Ashton NW, Cove DJ (1977) Complementation analysis of auxotrophic mutants of the moss, *Physcomitrella patens*, using protoplast fusion. *Mol Gen Genet* **155**: 103–107
- Heckman DS, Geiser DM, Eidell BR, Stauffer RL, Kardos NL, Hedges SB (2001) Molecular evidence for the early



- colonization of land by fungi and plants. *Science* **293**: 1129–1133
- Hiwatashi Y, Nishiyama T, Tomomichi F, Hasebe M** (2001) Establishment of gene-trap and enhancer-trap in the moss *Physcomitrella patens*. *Plant J* **28**: 105–116
- Hofmann A, Codon A, Ivascu C, Russo V, Knight C, Cove D, Schaefer D, Chakhparonian M, Zrýd J** (1999) A specific member of the Cab multigene family can be efficiently targeted and disrupted in the moss *Physcomitrella patens*. *Mol Gen Genet* **261**: 92–99
- Imaizumi T, Kiyosue T, Kanegae T, Wada M** (1999) Cloning of the cDNA encoding the blue-light photoreceptor (cryptochrome) from the moss *Physcomitrella patens* (accession no. AB027528) (PGR 99–110). *Plant Physiol* **120**: 1205
- Jenkins GI, Cove DJ** (1983) Phototropism and polarotropism of primary chloronemata of the moss *Physcomitrella patens*: responses of the wild-type. *Planta* **158**: 357–364
- Kadota A, Sato Y, Wada M** (2000) Intracellular chloroplast photorelocation in the moss *Physcomitrella patens* is mediated by phytochrome as well as by a blue-light receptor. *Planta* **210**: 932–937
- Kammerer W, Cove DJ** (1996) Genetic analysis of the result of re-transformation of transgenic lines of the moss, *Physcomitrella patens*. *Mol Gen Genet* **250**: 380–382
- Kawahara H, Kasahara M, Nishiyama A, Ohsumi K, Goto T, Kishimoto T, Saeki Y, Yokosawa H, Shimbara N, Murata S et al.** (2000) Developmentally regulated, alternative splicing of the Rpn10 gene generates multiple forms of 26S proteasomes. *EMBO J* **19**: 4144–4153
- Kenrick P, Crane P** (1997) The origin and early evolution of plants on land [review]. *Nature* **389**: 33–39
- Knight CD, Sehgal A, Atwal K, Wallace JC, Cove DJ, Coates D, Quatrano RS, Bahadur S, Stockley PG, Cuming AC** (1995) Molecular responses to abscisic acid and stress are conserved between moss and cereals. *Plant Cell* **7**: 499–506
- Kost B, Spielhofer P, Chua N-H** (1998) A GFP-mouse talin fusion protein labels plant actin filaments in vivo and visualises the actin cytoskeleton in growing pollen tubes. *Plant J* **16**: 393–401
- Kranz H, Scholz K, Weisshaar B** (2000) c-MYB oncogene-like genes encoding three MYB repeats occur in all major plant lineages. *Plant J* **21**: 231–235
- Krogan NT, Ashton NW** (2000) Ancestry of plant MADS box genes revealed by bryophyte (*Physcomitrella patens*) homologues. *New Phytol* **147**: 505–517
- Müller U** (1999) Ten years of gene targeting: targeted mouse mutants, from vector design to phenotype analysis. *Mech Dev* **82**: 3–21
- Nickrent DL, Parkinson CL, Palmer JD, Duff RJ** (2000) Multigene phylogeny of land plants with special reference to bryophytes and the earliest land plants. *Mol Biol Evol* **17**: 1885–1895
- Nishiyama T, Hiwatashi Y, Sakakibara I, Kato M, Hasebe M** (2000) Tagged mutagenesis and gene-trap in the moss, *Physcomitrella patens* by shuttle mutagenesis. *DNA Res* **7**: 9–17
- Quatrano R, Bashiardes S, Cove D, Cuming A, Knight C, Clifton S, Marra M, Hillier L, Pape D, Martin J et al.** (1999) Leeds/Wash U Moss EST Project, <http://www.ncbi.nlm.nih.gov> or <http://www.moss.leeds.ac.uk>
- Reski R** (1998) Development, genetics and molecular biology of mosses. *Bot Acta* **111**: 1–15
- Reski R** (1999) Molecular genetics of *Physcomitrella* [review]. *Planta* **208**: 301–309
- Reski R, Abel WO** (1985) Induction of budding on chloronemata and caulonemata of the moss *Physcomitrella patens* using isopenentenyladenine. *Planta* **165**: 354–358
- Reutter K, Atzorn R, Haderler B, Schmülling T, Reski R** (1998) Expression of the bacterial ipt gene in *Physcomitrella* rescues mutations in budding and plastid division. *Planta* **206**: 196–203
- Richardson DHS** (1981) *The Biology of Mosses*. Blackwell, Oxford
- Ross MacDonald P, Sheehan A, Roeder GS, Snyder M** (1997) A multipurpose transposon system for analyzing protein production, localization, and function in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **94**: 190–195
- Sakakibara K, Nishiyama T, Kato M, Hasebe M** (2001) Isolation of homeodomain-leucine zipper genes from the moss *Physcomitrella patens* and the evolution of homeodomain-leucine zipper genes in land plants. *Mol Biol Evol* **18**: 491–502
- Sauer B** (1998) Inducible gene targeting in mice using the Cre/lox system. [review]. *Methods* **14**: 381–392
- Schaefer DG** (1994) Molecular genetic approaches to the biology of the moss *Physcomitrella patens*. PhD thesis. University of Lausanne, Switzerland. <http://www.unil.ch/lpc/docs/DSThesis.htm>
- Schaefer DG** (2001) Gene targeting in *Physcomitrella patens* [review]. *Curr Opin Plant Biol* **4**: 143–150
- Schaefer DG, Zrýd J-P** (1997) Efficient gene targeting in the moss *Physcomitrella patens*. *Plant J* **11**: 1195–1206
- Schaefer DG, Zrýd J, Knight CD, Cove DJ** (1991) Stable transformation of the moss *Physcomitrella patens*. *Mol Gen Genet* **226**: 418–424
- Schneider-Poetsch HAW, Marx S, Kolukisaoglu HU, Hanelt S, Braun B** (1994) Phytochrome evolution: phytochrome genes in ferns and mosses. *Physiol Plant* **91**: 241–250
- Schumaker KS, Dietrich MA** (1997) Programmed changes in form during moss development. *Plant Cell* **9**: 1099–1107
- Strepp R, Scholz S, Kruse S, Speth V, Reski R** (1998) Plant nuclear gene knockout reveals a role in plastid division for the homolog of the bacterial cell division protein Ftsz, an ancestral tubulin. *Proc Natl Acad Sci USA* **95**: 4368–4373
- Thiessen G, Munster T, Henschel K** (2001) Why don't mosses flower? *New Phytol* **150**: 1–5
- Vergunst AC, Hooykaas PJJ** (1999) Recombination in the plant genome and its application in biotechnology [review]. *Crit Rev Plant Sci* **18**: 1–31