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The Moss *Physcomitrella patens*, a Model System with Potential for the Study of Plant Reproduction

David J. Cove[1] and Celia D. Knight

Department of Genetics, University of Leeds, Leeds LS2 9JT, United Kingdom

INTRODUCTION

The moss *Physcomitrella patens* has been established as a model system for the study of plant development using a combination of physiological, genetic, and molecular techniques and has been shown to be particularly suitable for the study of morphogenesis at the cellular level. Genetic and molecular techniques devised to study development include mutant isolation and analysis, somatic hybridization of sexually sterile strains, and genetic transformation. Developmental studies of *Physcomitrella* have so far concentrated on the early stages of gametophyte growth following spore germination or tissue regeneration. Almost no work has been done in this species on the processes involved in sexual reproduction, but it is likely that these processes would be amenable to study and that the techniques that have been devised for studying other developmental processes will also be applicable to the study of sexual reproduction. In this article, we briefly review the current state of knowledge of how development is regulated in this species, the techniques available for developmental genetic studies, and the limited amount of work that has already been done that is relevant to sexual reproduction. A recent review containing background material is given by Cove (1992).

NORMAL DEVELOPMENT

The life cycle of *Physcomitrella* is typical of a moss, comprising alternating haploid gametophyte and diploid sporophyte generations, although the high capacity of somatic tissue for regeneration (see below) allows the prolonged culture of gametophyte tissue. Figure 1 summarizes the developmental events involved in the life cycle together with some of the environmental signals involved in controlling these steps.

The gametophyte of *Physcomitrella* has two major developmental stages. The initial stage, generated by spore germination, is a branching system of cell filaments, or protonema, as shown in Figure 2A. The second stage of gametophyte development comprises gametophores, the leafy shoots that are the more familiar part of most moss plants (Figure 2B). Gametophores arise from protonema and are the site of gamete production. *Physcomitrella* is monoecious; male gametes, or antherozoids, are produced within antheridia, and female gametes, or oogonia, within archegonia on the same gametophore (Figure 2C). Antherozoids affect fertilization by swimming through a surface water film and down the neck of the archegonia. The zygote develops into a diploid sporophyte that in *Physcomitrella* is small, consisting of a short stalk a few millimeters long that bears a spore capsule ~2 mm in diameter. Spore capsules have no specialized structures for dehiscence and, when mature, contain ~5000 haploid spores. The life cycle can be completed in ~3 months in culture.

Wild-type strains are normally self-fertile. However, self-sterility is a pleiotropic effect of some mutations to auxotrophy. Strains carrying mutant alleles leading to a requirement for *p*-amino benzoic acid, nicotinic acid, or thiamine are all self-sterile when grown on medium containing the required supplement at a level sufficient for normal vegetative growth. When crossed to other auxotrophic strains, they are as fertile as either the male or female parent, but only if the mutant alleles of the genes in the two strains complement one another (Engel, 1968; Ashton and Cove, 1977). It is likely that this phenomenon results from the at least partial metabolic isolation of the sporophyte from the gametophyte. Self-fertility of an auxotrophic strain can be restored by greatly increasing the concentration of the relevant growth supplement (Courtice et al., 1978; Grimsley, 1978). This suggests that a level of supplementation sufficient for gametophyte growth does not allow sufficient supplement to be transmitted from the gametophyte to the sporophyte to allow the latter to develop. The fertility of crosses between complementing strains is consistent with this hypothesis, because complementation will render the diploid sporophyte resulting from such crosses metabolically independent of the gametophyte.

Almost all cells, whether gametophytic or sporophytic, are capable of regeneration following tissue damage. Regeneration proceeds by a developmental pathway similar to spore germination, with protonemal tissue being generated first, followed by gametophores. It is therefore possible to obtain diploid protonema and gametophores by regenerating sporophyte tissue (aposporous regeneration). This early finding (see von

[1] To whom correspondence should be addressed.

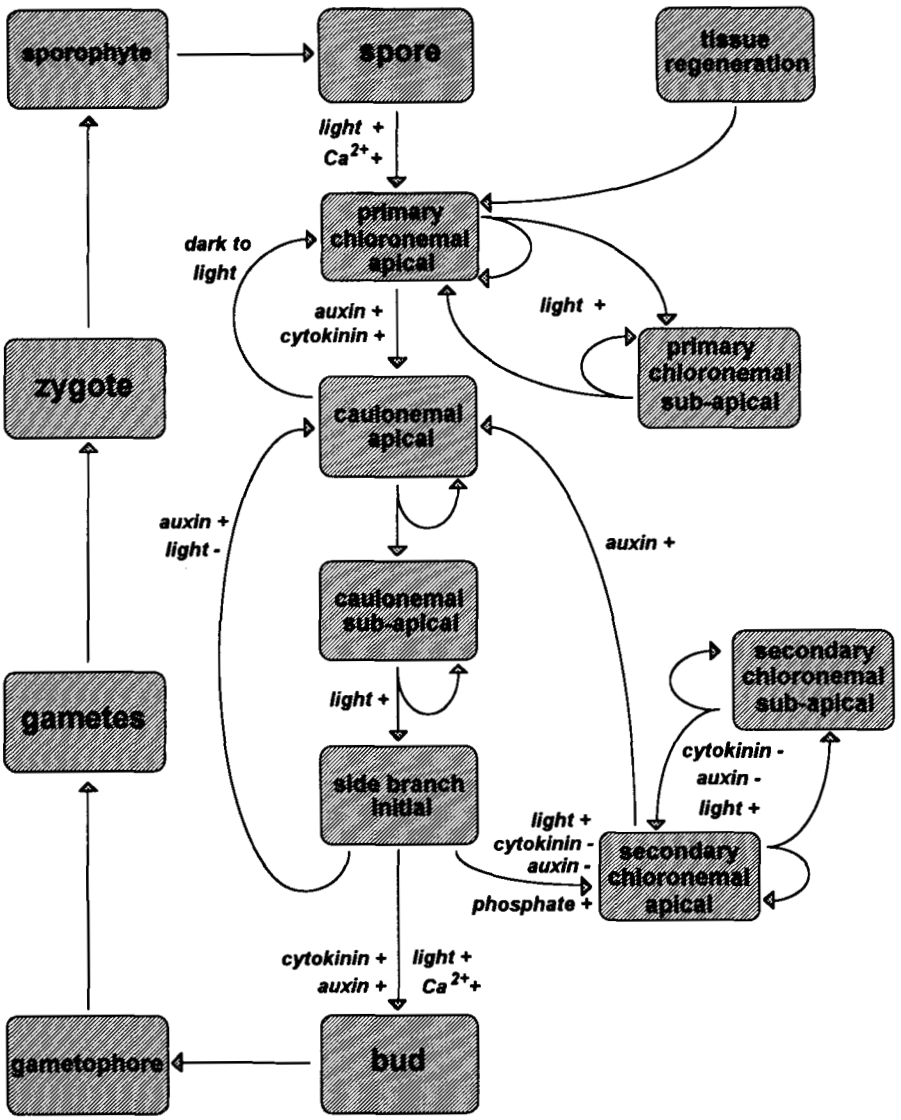

Figure 1. Life Cycle of *Physcomitrella*.

Diagram of the life cycle of *Physcomitrella* showing cell lineages and stages affected by phytohormones, light, and other environmental factors. +, the developmental step requires or is enhanced by the treatment; −, the developmental step is blocked or inhibited by the treatment.

Wettstein, 1932) established that ploidy is not responsible for the differences between gametophyte and sporophyte development. Diploid gametophytes produced aposporously can undergo sexual reproduction, although the speed of development is slower and mature sporophytes take at least 1 year to develop.

Two pathways of sporophyte production from diploid gametophytes can be distinguished (Cove, 1983). In one, diploid gametes are produced that fuse to form a tetraploid sporophyte. This then undergoes tetraploid meiosis to produce diploid spores. Alternatively, a diploid oogonium can develop parthenogenetically to produce a diploid sporophyte that will undergo normal diploid meiosis to produce haploid spores. These two alternative origins of sporophytes can be distinguished because the segregation ratios resulting from tetraploid and diploid meiosis are different (Cove, 1983). Attempts to influence the relative frequency of these alternative origins have so far been unsuccessful (D.J. Cove, unpublished data). There is a further pathway of sexual reproduction shown by diploid gametophyte cultures that is rarely observed in wild-type cultures but can be common in some mutant strains (D.J. Cove, unpublished data), in which sporophytes are produced

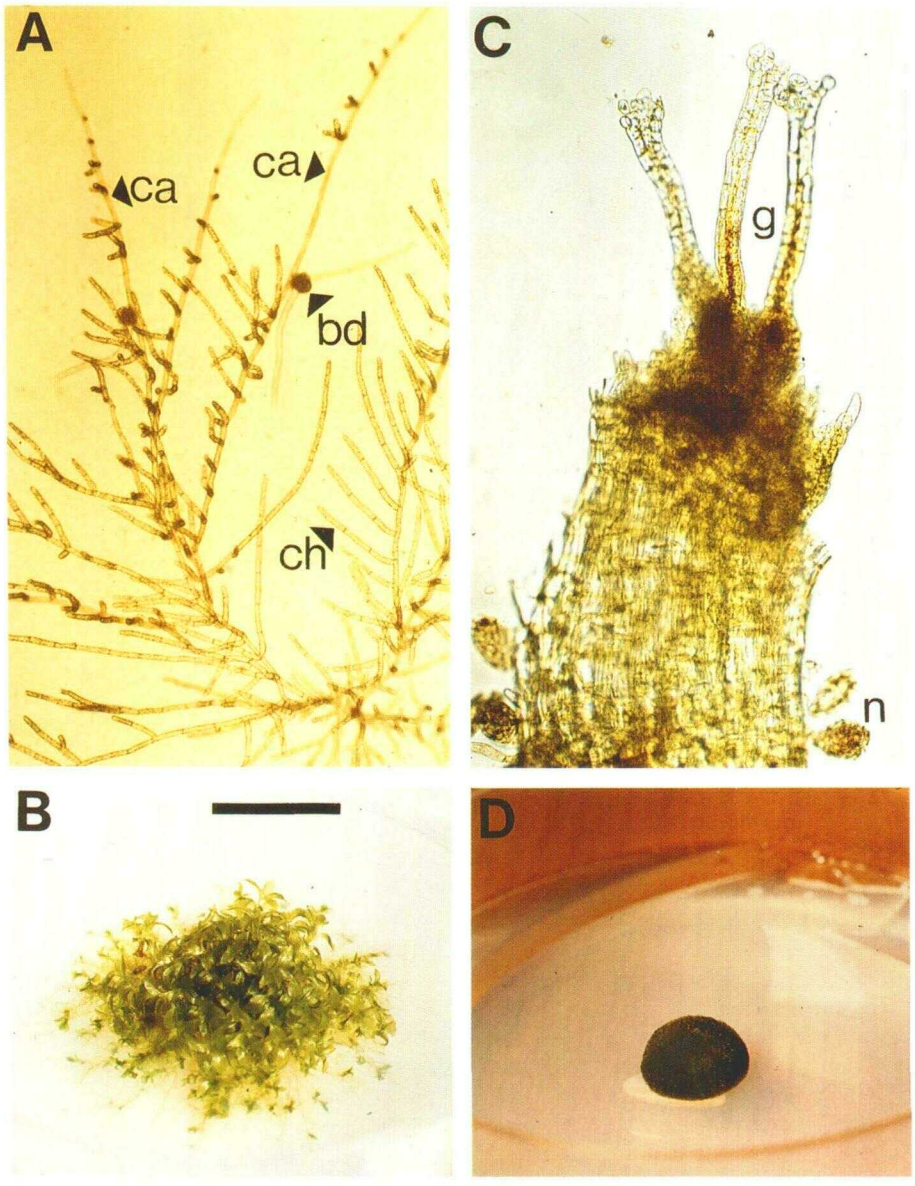

Figure 2. *Physcomitrella* at Several Stages of the Life Cycle.

(A) Part of a wild-type sporeling 14 days after spore germination. The spore was toward the bottom right, and the long axial filaments radiating from it are caulonema (ca). The majority of branches from the caulonemal filaments are of chloronema (ch), but several have developed into either young buds (bd) or further caulonemal filaments. Bar in **(B)** = 500 μm in **(A)**.

(B) A wild-type culture grown on defined medium for 28 days in continuous light at 25°C. Bar = 10 mm.

(C) Apical part of a wild-type gametophore. The leaves have been removed to show flask-shaped archegonia (g), borne terminally, and antheridia (n), borne in the leaf axils. Bar in **(B)** = 250 μm in **(C)**.

(D) Culture of cal-91, a mutant blocked in the transition of chloronema to caulonema. Details are as in **(B)**. Bar in **(B)** = 10 mm in **(D)**.

apogamously directly from protonema. The relationships between these various reproductive pathways are summarized in Figure 3.

Developmental genetic studies of *Physcomitrella* have concentrated on the growth of protonema and on gametophore formation. Protonemal development, whether from germinating spores or from tissue regeneration, begins with the production of branching filaments of chloronemal cells. Chloronemal cells are densely packed with large chloroplasts and have dividing walls that are perpendicular to the filament axis.

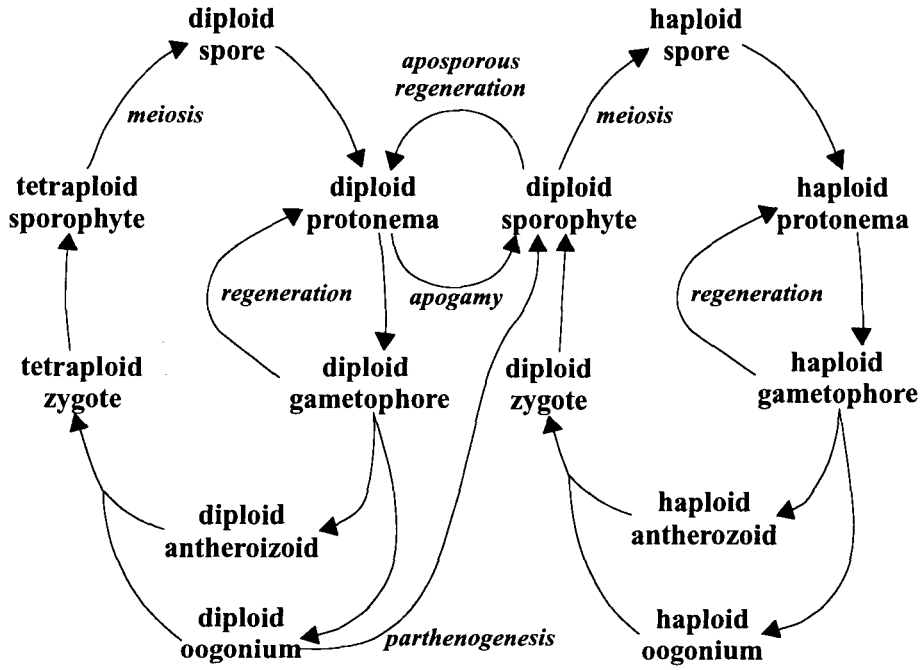

Figure 3. Pathways of Sexual Reproduction in *Physcomitrella*.

Chloronemal filaments grow by extension of the tip of the apical cell at a mean rate of 2 μm/hr, although individual apical cells may show marked variation in their rate of growth (A. Russell, unpublished data). The subapical cells of chloronemal filaments may divide several times to produce side branches of further filaments.

Chloronemal apical cells have a cell cycle of ~24 hr and may develop at any time into a second cell type, caulonema, which has a higher growth rate (30 to 40 μm/hr) and a shorter cell cycle time (6 hr). Caulonemal cells are morphologically distinct from chloronema, having fewer and less well developed chloroplasts and having cross walls between adjacent cells that are oblique to the filament axis. The transition from chloronema to caulonema may be completed within a single cell cycle, but more commonly it takes several cycles and may sometimes be so prolonged that a whole filament containing cells with a morphology intermediate between chloronema and caulonema may be formed (A. Russell, unpublished data).

Caulonemal apical cells also grow by extension of the cell tip. The subapical cells of caulonemal filaments divide to produce single-celled side branch initials. Under the conditions used routinely for culture, the probability of a caulonemal subapical cell producing a first side branch initial is near 100% and a second, ~40%; fewer than 3% of subapical cells produce a third side branch (A. Russell, unpublished data). Side branch initials can have one of four developmental fates. Under standard culture conditions, most develop into filaments of chloronema cells morphologically similar to those produced by germinating spores or by regenerating tissue. Some side branch initials (<5%) develop into buds, which subsequently develop into gametophores. A similar proportion of side branch initials develop into caulonemal filaments, and a few (<3%) remain undivided.

The relative proportions of side branch initials adopting these alternative developmental fates are influenced strongly by such environmental factors as the intensity and quality of light. Although at present it is only possible to establish the probability that a particular cell will divide or undergo a particular developmental transition, the reproducibility of these probabilities provides clear evidence for developmental programming. The establishment of these probabilities allows a more precise analysis of the effects of environmental manipulation and of mutation. This detailed approach to developmental analysis in *Physcomitrella* has so far been applied only to the early stages of gametophyte development, but it is likely that a similar probabilistic approach to the analysis of the cell lineages involved in gametophore development will be rewarding.

The stages of the life cycle following bud formation have not been studied extensively at the developmental genetic level, although there has been some work on mutants with altered leaf shape (Courtice and Cove, 1983). Gametogenesis requires a lower temperature (<18°) than that which is optimal for vegetative growth (25°C) and which is used routinely. No detailed study has been made of this requirement, and cultures are normally incubated at low temperature for several weeks to induce sporophyte formation. It should, however, be straightforward to establish more precisely the environmental triggers required to induce sexual reproduction and to determine, for

example, whether male and female gametogenesis respond similarly to these triggers.

The control of the early stages of gametophyte development has been investigated by both the manipulation of culture conditions and the use of mutants (Ashton et al., 1979). Results of these studies are summarized in Figure 1. These studies establish that the switch of chloronemal apical cells to caulonema requires both auxin and cytokinin. Mutants blocked at this stage (*cal*$^-$ mutants; Figure 2D) comprise a heterogeneous group. Some have phenotypes consistent with one or other of these hormones being absent or present at greatly reduced levels, whereas others have lost the ability to respond to hormone and are therefore likely to be blocked in signal transduction. The relative probabilities of the various developmental fates of side branch initials produced by caulonemal subapical cells are influenced by light quality and by both auxin and cytokinin. The nutrient status of the medium also affects the relative proportions of side branch fates. Phosphate limitation, for example, blocks almost all side branch initial development into chloronemal filaments but has little effect on the transitions to either buds or caulonema. The transition of side branch initials to buds requires light, auxin, and cytokinin, and some of the mutants blocked in this transition (*gam*$^-$ mutants), like *cal*$^-$ mutants, have altered hormone levels, whereas others are likely to result from abnormal signal transduction.

TECHNIQUES FOR THE ANALYSIS OF DEVELOPMENT

Physcomitrella is routinely cultured axenically in continuous illumination, on an agar medium containing inorganic salts but without phytohormones or a reduced source of carbon (Ashton and Cove, 1977). Conventional genetic analysis can be performed and crossing can be controlled by exploiting the self-sterility of auxotrophic strains. The segregation ratios of characters observed in the gametophyte, such as vitamin auxotrophies, conform to those expected for phenotypes observed in a haploid tissue (Engel, 1968; Ashton and Cove, 1977; Cove, 1983).

Because sterility is an inevitable consequence of mutants, such as *cal*$^-$ and *gam*$^-$, that are blocked in development prior to gametophore production, it has been necessary to devise a method of parasexual analysis. Somatic hybrids can be produced by the fusion of protoplasts, and by using two strains with complementing auxotrophies, somatic hybrids can be selected by plating an appropriately treated mixture of protoplasts on regeneration medium upon which neither individual component strain can grow (Grimsley et al., 1977a, 1977b). Somatic hybrids can be used directly for dominance and complementation studies (Featherstone et al., 1990). They can also be used for further genetic analysis in the same way as diploid strains produced by aposporous regeneration of sporophyte tissue (see above). Genetic analysis of progeny developing from spores produced by sporophytes that are parthenogenetic in origin is simpler, and such progeny, being haploid, allow the isolation of recombinant haploid strains (Knight et al., 1991). This route for genetic analysis is, however, slow and very difficult to control because no method is yet available to regulate parthenogenesis (Cove, 1983). If it should prove possible to isolate mutants affected in gamete production, in fertilization, or in sporophyte production, these would presumably be sexually sterile, but they could be analyzed using the techniques of parasexual genetic analysis.

Techniques for molecular genetic analysis have only recently begun to be developed. Transformation by the direct uptake of plasmid DNA into protoplasts (Schaefer et al., 1991) yields approximately two transformants per μg of DNA. Approximately 40% of these express the plasmid encoded genes only transiently, and the majority of the rest constitute a class of transformant referred to as unstable. Where antibiotic resistance is the transformed phenotype, these grow slowly on the selective medium, and if selection is relaxed, the transformed phenotype is lost in the course of ~14 days. Unstable transformants transmit plasmid DNA rarely if ever through meiosis (D. Schaefer and J.-P. Zryd, unpublished data), and using transformants for the *GUS* gene it has been shown that unstable transformants distribute plasmid DNA inefficiently in somatic tissue (W. Sawahel, C.D. Knight, and D.J. Cove, unpublished data).

The third class of transformants, which comprises ~5% of regenerants selected following transformation, retains the transformed phenotype even in the absence of selection, and plasmid DNA is transmitted in a Mendelian manner through meiosis. DNA gel blot analysis shows that stable transformants have a number of copies of the plasmid inserted in a random array at a single site in the moss genome. The frequency of transformation has recently been increased considerably by the biolistic delivery of DNA (Sawahel et al., 1992; W. Sawahel, C.D. Knight, and D.J. Cove, unpublished data), and a number of treatments following DNA uptake, including UV irradiation, increase transformation further (W. Sawahel, C.D. Knight, and D.J. Cove, unpublished data). The present best rate of transformation is ~400 per μg of DNA, although these treatments do not appear to increase the rate of stable transformation significantly.

The isolation of cDNAs corresponding to *Physcomitrella* genes by the use of heterologous probes presents no unexpected problems (Leech et al. 1993). The moss has a genome size of 6×10^8 bp (J.-P. Zryd and N. Grimsley, unpublished data). Attempts to tag genes using the maize transposon *Ac* have so far been unsuccessful (W. Kammerer and D.J. Cove, unpublished data), but transformation frequencies are now sufficiently high that they require little further improvement before it will be worthwhile to attempt to isolate genes by the direct complementation of mutant phenotypes. Such a procedure will be facilitated by the finding that it is possible to reisolate plasmid DNA from moss transformants by the selection of *Escherichia coli* transformants following treatment with DNA from unstable *Physcomitrella* transformants (W. Sawahel, C.D. Knight, and D.J. Cove, unpublished data). The isolation of the sequence

responsible for complementing the mutant phenotype should therefore be possible.

STUDIES OF *PHYSCOMITRELLA* DIRECTLY RELEVANT TO SEXUAL REPRODUCTION

The only study of fertility in *Physcomitrella* is preliminary. Courtice (1979) conducted a mutant screen for strains that were self-sterile but otherwise normal. Of 1895 strains that developed after treatment of spores with nitrosoguanidine, 125 were initially classified as self-sterile but morphologically and nutritionally normal. Sixteen of these were investigated in more detail. Nine were found to produce sporophytes upon retesting although at very reduced levels. The remaining seven were tested for cross-fertility, but none was found to be able to act as either a male or female parent. Thus, this study failed to identify strains that were either male or female sterile, but it must be emphasized that this was not an extensive survey. There are no reports of mutants abnormal in sporophyte development.

FUTURE PROSPECTS

The study of sexual reproduction in *Physcomitrella* is a wide open field with techniques already available. Physiological studies need to be performed to establish more precisely the temperature requirements for the induction of gametogenesis. It is likely that the failure to isolate mutants specifically abnormal in either male or female gametogenesis is due only to the small number of strains that have been screened, and a much larger screen should yield such mutants. Genetic variation affecting the apogamous production of sporophytes has already been observed, but, again, the studies are preliminary. A particularly interesting observation relating to apogamy is that in certain mutant strains, ploidy can be shown to have an effect on gametophyte development, thus contradicting to some extent the classic conclusion that ploidy is not responsible for the different development of gametophyte and sporophyte (see above). The evidence is that certain mutant strains as haploids have a phenotype of gametophore overproduction. As diploids obtained by somatic hybridization, however, they produce sporophytes apogamously, directly from caulonemal side branch initials instead of from gametophores (D.J. Cove, unpublished data).

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