

Mosses as model systems

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Mosses hold many attractions as model organisms for research in plant science. Their position as the simplest of land plants makes them central to the study of plant evolution, particularly in shedding light on how their aquatic predecessors evolved to survive on land. The use of mosses for developmental studies hinges on the ability to observe development in living material at the level of the individual cell. However, more recently techniques for the molecular analysis of mosses have provided tools for new approaches for determining the mechanisms controlling plant development, incorporating both cell and molecular biology.

The use of mosses as model systems for the study of plant genetics and development is not new. The research of von Wettstein and his co-workers on mosses, from 1920–1945, was at the forefront of genetic research at that time. These studies, which put mosses alongside *Drosophila* and maize as important model systems for genetic studies, contributed to our understanding of tetrad formation and both X-ray and chemical mutagenesis. The potential of mosses for the study of plant development, using a combination of genetic and physiological procedures, was already recognized over 70 years ago¹, and was beginning to be realized by von Wettstein's research group by 1940 (Ref. 2). However, von Wettstein's death in 1945 led to a hiatus in moss research, and only in recent years has their use as model organisms, particularly for the study of plant development, become reestablished. Although there is an extensive literature on the developmental physiology of many moss species³, this review will concentrate principally on three species – *Ceratodon purpureus*, *Funaria hygrometrica* and *Physcomitrella patens* – for which there are also recent genetic studies. These species may be cultured without difficulty under controlled conditions, using microbiological techniques including axenic culture – not only in petri dishes, but also, for example, in fermenters⁴ (see Fig. 1).

Moss development

The moss life cycle comprises a free-living haploid gametophyte stage and a diploid sporophyte stage, with the sporophyte partly dependent for nutrition and support on the gametophyte. Gametes are produced by the gametophyte via mitosis, and fuse to produce zygotes that develop into the sporophyte. The sporophyte produces spores by meiosis that germinate to produce further gametophytes.

Most developmental studies, and certainly those employing genetic analysis, have concentrated on the gametophyte stage. Its haploid condition alone gives it an advantage, allowing the effects of recessive mutations to be observed directly. Spore germination in almost all moss species results in the production of a filamentous stage, the protonema. This stage is short-lived in some species; however, in other species, including the three considered here, the protonemal stage can be long-lived, especially under lab culture. The protonemal stage of *Funaria* comprises branching cell filaments extending by the serial division of the filament apical cell, with subapical cells dividing to produce side filaments. Protonemata contain

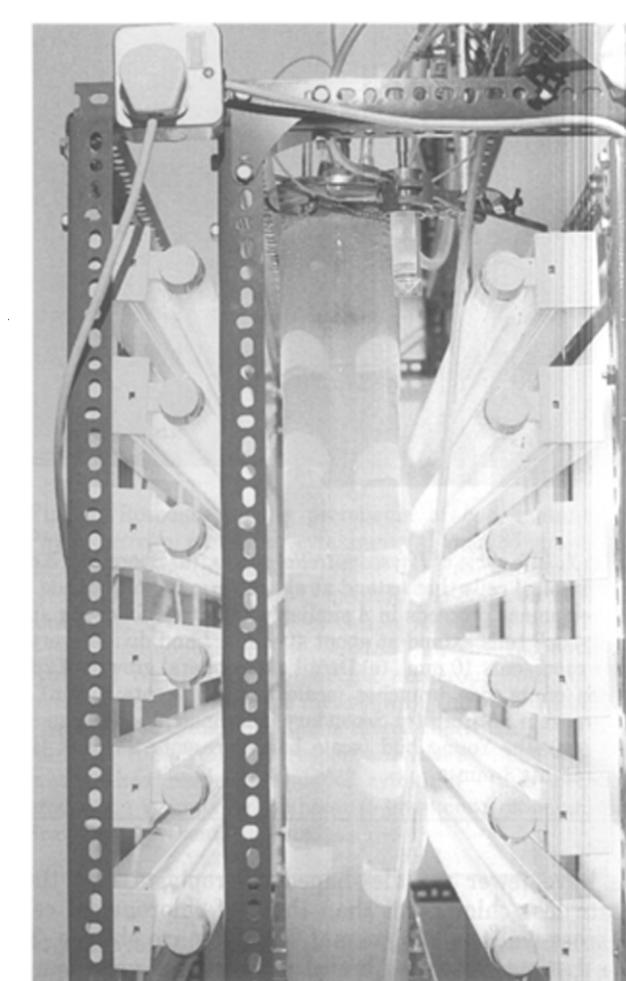


Fig. 1. Air-lift fermenter culture of *Physcomitrella patens*. The green moss cells in suspension in the central cylinder are illuminated by banks of fluorescent tubes on either side. Protonemal tissue, cultured in a 6 l air-lift fermenter incorporating a tissue chopper, grows exponentially with a doubling time of approximately 26 h to give yields in excess of 1 g (dry weight) l⁻¹.

two distinct cell types, chloronemata and caulinemata. Chloronemal filaments have cells densely packed, with large chloroplasts and cross walls between adjacent cells that are perpendicular to the filament axis. Caulinemal

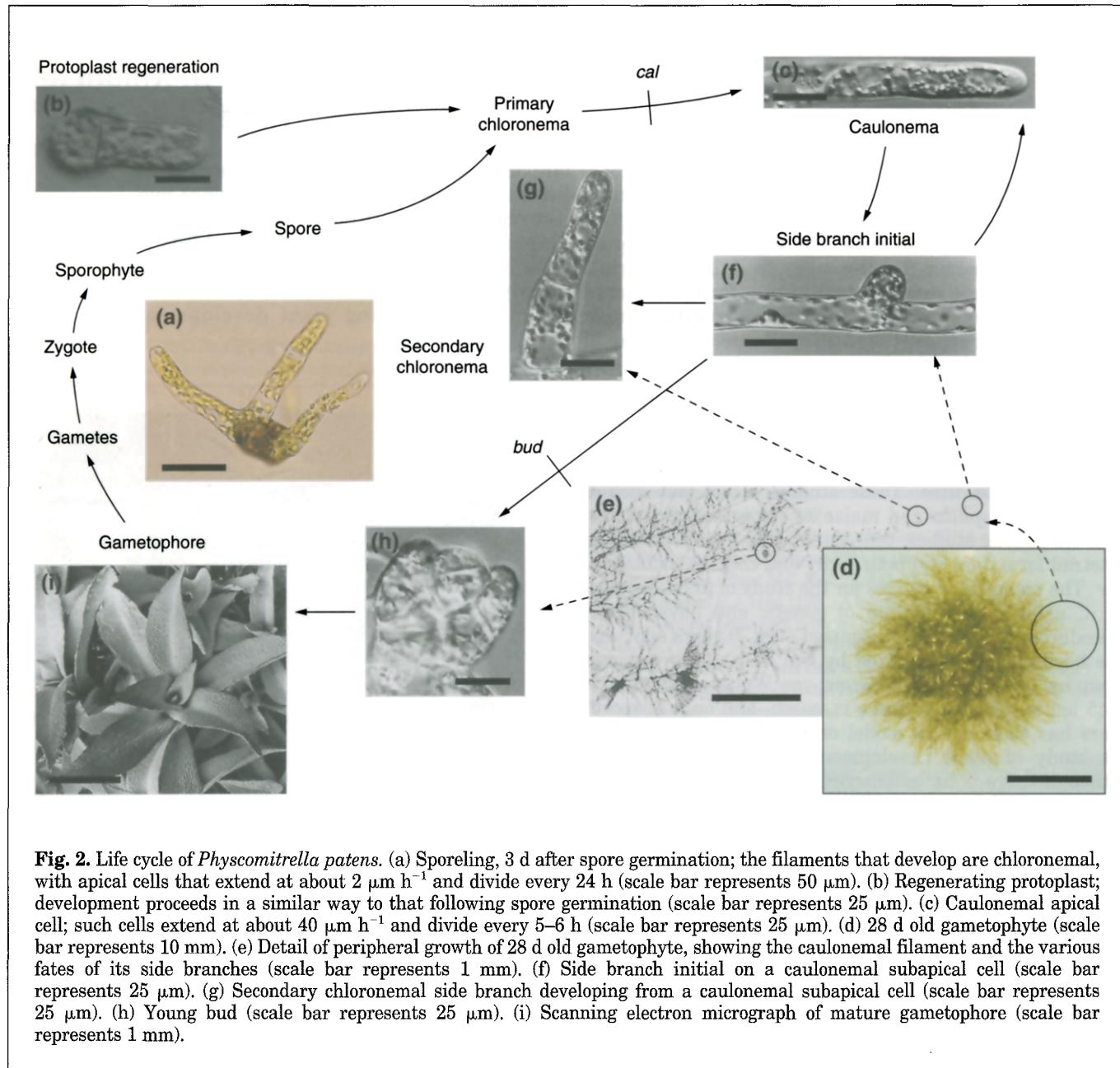


Fig. 2. Life cycle of *Physcomitrella patens*. (a) Sporeling, 3 d after spore germination; the filaments that develop are chloronemal, with apical cells that extend at about $2 \mu\text{m h}^{-1}$ and divide every 24 h (scale bar represents 50 μm). (b) Regenerating protoplast; development proceeds in a similar way to that following spore germination (scale bar represents 25 μm). (c) Caulonemal apical cell; such cells extend at about $40 \mu\text{m h}^{-1}$ and divide every 5–6 h (scale bar represents 25 μm). (d) 28 d old gametophyte (scale bar represents 10 mm). (e) Detail of peripheral growth of 28 d old gametophyte, showing the caulonemal filament and the various fates of its side branches (scale bar represents 1 mm). (f) Side branch initial on a caulonemal subapical cell (scale bar represents 25 μm). (g) Secondary chloronemal side branch developing from a caulonemal subapical cell (scale bar represents 25 μm). (h) Young bud (scale bar represents 25 μm). (i) Scanning electron micrograph of mature gametophore (scale bar represents 1 mm).

cells have fewer spindle-shaped chloroplasts, and these contain less chlorophyll than those of chloronemal cells. The cross walls in caulonemal filaments are oblique. The same distinct cell types are also present in protonemata of *Physcomitrella* and *Ceratodon*, but the morphological differences are less distinct. Chloronemal filaments are produced directly following spore germination or tissue regeneration, and caulinemata are produced subsequently from the apical cells of some chloronemal filaments. Protoplast regeneration also results in the direct production of chloronemal filaments, with no intervening callus stage, making moss protoplast regeneration an excellent model for the study of cell polarity⁵. In all three species, buds develop as side branches from caulinemal filaments and these develop into gametophores, the leaf-bearing shoots that produce gametes. In *Funaria* and *Physcomitrella*, female and male gametes are produced by the same plant, but in *Ceratodon* there are separate

sexes. Figure 2 summarizes the moss life cycle and illustrates some of the main stages in *Physcomitrella*.

Genetics and morphology of developmentally abnormal mutants

Morphologically abnormal mutants of mosses were first isolated in *Physcomitrium piriforme* following exposure of spores to either polonium chloride or X-ray irradiation². The mutants included some showing abnormal protonemal development, but the majority of mutations affected gametophore development, including some with variant numbers of cells in the leaf and others affecting leaf cell size. However, genetic analysis of such mutants was very limited, and with the demise of von Wettstein's group no further research emerged for a considerable period. The isolation of auxotrophic (vitamin-requiring) mutants of *Physcomitrella patens*⁶ reopened interest in the use of mosses for genetic and developmental studies. Since then,

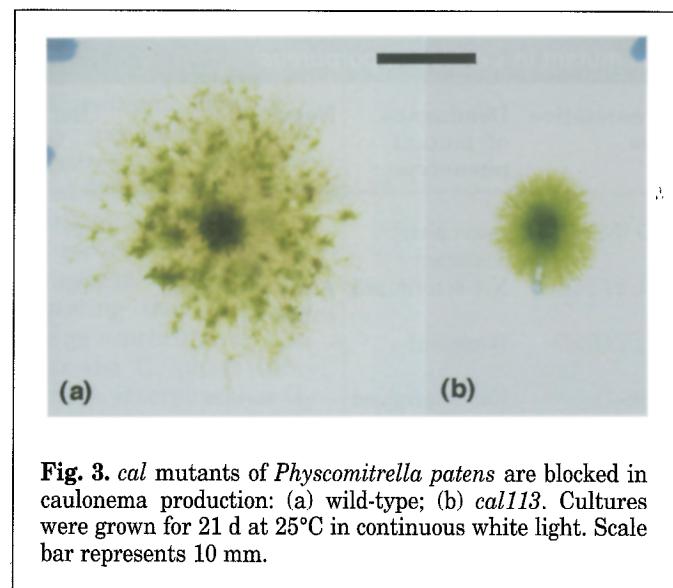


Fig. 3. *cal* mutants of *Physcomitrella patens* are blocked in caulinema production: (a) wild-type; (b) *cal113*. Cultures were grown for 21 d at 25°C in continuous white light. Scale bar represents 10 mm.

many more mutants have been isolated in this species, and a more restricted number of mutants have also now been obtained in *Funaria* and *Ceratodon*.

Table 1 gives details of developmentally abnormal mutants that have been characterized, and Fig. 2 indicates the stages of development affected. Because of the regenerative capacity of moss tissue, even mutants blocked early in development, at the chloronema-to-caulinema transition step (Fig. 3), may be maintained in culture indefinitely. Mutant studies in both *Physcomitrella* and *Funaria* have revealed developmental steps at which auxins or cytokinins are required, and analysis of mutant phenotypes has divided mutants affecting these stages into those in which hormone levels are reduced and those in which there is a defect in the hormone response pathway (Fig. 4). Conventional genetic analysis of such mutants is impossible, because they are blocked in development before gamete production. However, it is possible to produce somatic diploid strains by fusing protoplasts from two different strains using as selective markers either complementary auxotrophies or, more recently, transgenic antibiotic resistance. Such hybrids allow dominance tests to be carried out and, where mutant phenotypes are recessive, complementation analysis is possible (e.g. see Ref. 7).

Mutants have also been obtained that are abnormal in their responses to light and gravity (Table 1). Mutant analysis has already added to our knowledge of the role of the phytochrome photoreceptor in mosses. The phototropic (response to unidirectional light) and polarotropic (response to polarized light) responses are phytochrome-mediated, and aphototropic mutants are also apolarotropic. One class of aphototropic mutant in *Ceratodon* is impaired in the synthesis of the phytochrome chromophore⁸, and shows pleiotropic abnormalities in other phytochrome-mediated processes, including chlorophyll synthesis. The tropic response of these mutants can be restored by the inclusion of biliverdin in the growth medium, which is a precursor of the phytochrome chromophore (see Fig. 5). Other aphototropic mutants of *Ceratodon* and *Physcomitrella* show no other abnormalities in developmental processes involving phytochrome, and so are likely to be impaired in the specific signalling pathway required for the phytochrome-mediated tropic response to

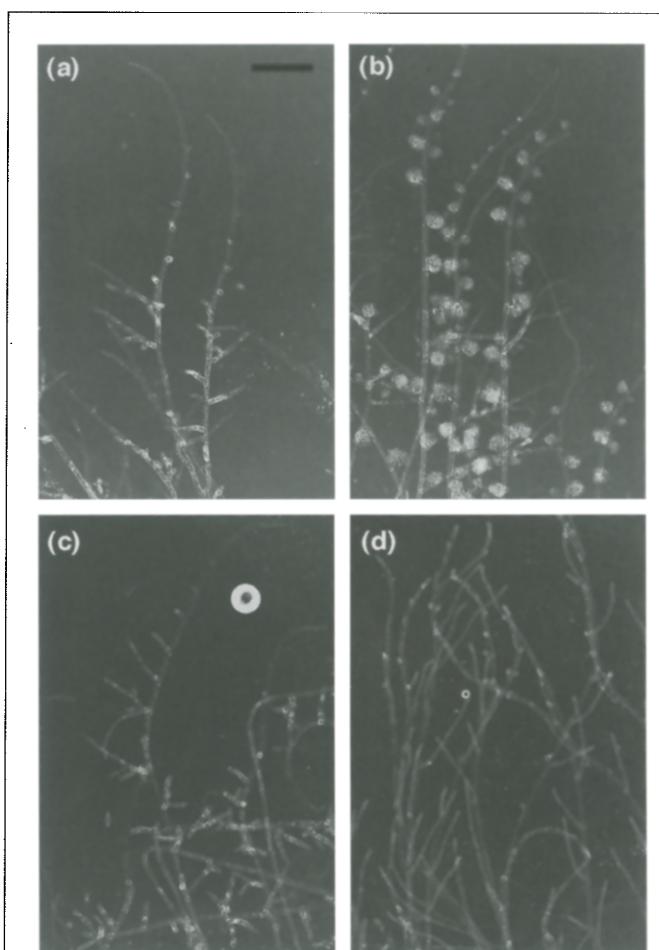


Fig. 4. Response of the protonema of *bud* mutants of *Physcomitrella patens* to cytokinin: (a) *bud185* grown on basal medium; (b) *bud185* grown on medium containing cytokinin; (c) *bud1* on basal medium; (d) *bud1* on medium containing cytokinin. The cytokinin treatment was 1 µM 6-benzylaminopurine. The *bud185* mutant responds to cytokinin treatment by producing numerous buds, in the same way as the wild-type (not shown). The *bud1* mutant is insensitive to cytokinins, and is therefore thought to be deficient in the cytokinin response pathway. The *bud185* mutant has reduced levels of cytokinin either because production is impaired or because breakdown is enhanced. Scale bar represents 400 µm.

light. Analysis of these mutants has also shown that the gravitropic response is actively turned off in the light, via phytochrome⁶. Mutants of *Physcomitrella* altered in the gravitropic response include those in which the response is impaired, and mutants in which the polarity of the response is reversed (i.e. gravitropism is positive rather than negative^{9,10}). Detailed analysis of a *Ceratodon* mutant, in which the polarity of the gravitropic response is similarly reversed, shows that the kinetics of the response itself is unchanged and that all aspects of the response show a reversal of polarity – this indicates that the gene that has mutated [*wwr* ('wrong way response')] is likely to be directly involved in determining polarity¹¹.

Although genetic studies have largely concentrated on the protonemal stage of gametophyte development, many of the mutants isolated are affected in gametophore development

Table 1. The main classes of developmental mutant in *Ceratodon purpureus*^a

| Developmental stage affected | Phenotype | Species | Representative alleles | Dominance of mutant phenotype | Notes | Ref. |
|------------------------------------|--|----------------|---|-------------------------------|--|------|
| Chloronema to caulonema transition | Blocked, but partially restored by auxins. | P ^b | <i>cal113</i> (<i>NAR113</i>) | Incompletely dominant | — | 35 |
| | Blocked, but partially restored by auxins. | F ^c | (87.13, 87.25) | Not determined | Auxin catabolism enhanced. | 36 |
| | Blocked; no response to auxins. | P | <i>cal91</i> (<i>NAR91</i>) | Dominant | — | 35 |
| | | F | <i>cal1</i> (86.1) | Not determined | — | 37 |
| Caulonemal apical cell tropism | Aphototropic. | P | <i>ptrA1</i> , <i>ptrB2</i> | Recessive | Gametophores also aphototropic; other phytochrome-mediated steps unaffected. | 38 |
| | Aphototropic; phototropism restored by biliverdin. | C | <i>ptr1</i> , <i>ptr116</i> | Not determined | Other phytochrome-mediated events impaired. | 8 |
| | Aphototropic; phototropism not restored by biliverdin. | C | <i>ptr103</i> | Not determined | Other phytochrome mediated events unimpaired. | 8 |
| | Gravitropism impaired. | P | <i>gtrA12</i> , <i>gtrB1</i> | Recessive | <i>gtrA</i> also has an abnormal leaf cell shape. | 38 |
| Bud formation | Gravitropic polarity reversed. | P | <i>gtrC13</i> | Recessive | — | 38 |
| | C | <i>wur1</i> | — | — | — | 11 |
| | Blocked, but restored by cytokinins. | P | <i>bud87</i> (<i>NAR87</i>) | Incompletely dominant | — | 35 |
| | Blocked; no response to cytokinins. | P | <i>bud1</i> (<i>BAR1</i>) | Dominant | — | 35 |
| Gametophore morphology | Enhanced production of buds. | P | <i>oveA78</i> , <i>oveB100</i> , <i>oveC200</i> | Recessive | Cytokinin levels higher than wild type. | 7 |
| | Bud development ceases at an early stage. | P | <i>gad139</i> | Not determined | — | 4 |
| | Narrow leaves. | P | <i>gadA33</i> , <i>gadB74</i> | Recessive | — | 39 |

^aC represents *Ceratodon purpureus*; ^bP represents *Physcomitrella patens*; ^cF represents *Funaria hygrometrica*; ^dD.J. Cove, unpublished.

(see Fig. 6). No genetic or detailed phenotypic analysis has yet been carried out on these mutants, which, together with those showing altered protonemal development, provide a valuable resource for the future analysis of pattern development.

Analysis by cell biology

The growth and development of moss protonemata offer an excellent opportunity to observe developmental processes at the level of the single cell. The establishment of polarity in single cells has been the subject of recent studies^{5,12}. Cellular morphogenesis (e.g. in response to hormones during the development of buds or during tropisms in response to light⁸ and gravity^{9–11,13,14}) can be observed microscopically with the minimum disruption to cellular integrity. Furthermore, since gametophores exhibit tropic responses as well as protonemata, the same developmental response can be observed in mosses at single and multicellular levels.

The cytoskeleton has been observed in tip-growing cells by immunofluorescent labelling for microtubules and rhodamine-phalloidin labelling for microfilaments^{13,15,16}. The cytoskeleton has been implicated in redirecting cell polarity during a protonemal phototropic response^{16,17}. Changes in the distribution of microtubules have been observed in caulonemal apical cells following their reorientation with respect to the gravity vector¹⁸.

Genome analysis

The possession of a small genome and relatively few chromosomes has helped to facilitate the establishment of genetic maps for other model species, such as *Arabidopsis* and *Drosophila*. In general, members of the Funariales (including *Physcomitrella* and *Funaria*) have larger genomes than might be expected given their taxonomic position, and they contain a relatively large number of small chromosomes. Consequently, no genetic map has been generated for these mosses to date. Nevertheless,

some important features of genome analysis have been reported recently for *Physcomitrella*. Flow cytometry has been used to estimate a haploid genome size of 600 Mb (Ref. 18). A surprising aspect noted in this report is that only a single peak was obtained, suggesting that the cells were synchronized in either the G₁ phase (pre-synthetic interphase) or G₂ phase (post-synthetic interphase) of the cell cycle. Recent data indicate that the cells were indeed in G₂ phase, indicating a haploid (1C) genome size for *Physcomitrella* of 300 Mb, which is about three times the size of the *Arabidopsis* genome (R. Reski, pers. commun.). Reski *et al.*¹⁸ also report, based on visual detection by chromosome staining, that *Physcomitrella* has 27 chromosomes per haploid cell. There has been much discussion over the years on the chromosome number of mosses^{18,19}. One suggestion is that *Physcomitrella* is polyploid, and perhaps a near-complete tetraploid derivative of an ancestral species that had seven chromosomes. However, no evidence of polyploidy is provided by the analysis of segregants in crosses – the observed ratios are consistent with the parental strains being haploid¹⁹.

Plant transformation

The capacity to transform a species is a prerequisite for molecular genetic analysis. The ease with which *Physcomitrella* and *Ceratodon* protoplasts can be isolated and regenerated (there is no callus stage or any requirement for the addition of hormone) made PEG-mediated uptake of plasmid DNA by protoplasts an obvious route for transformation. This method has proved to be successful, and has led to the production of stable transformants²⁰. The routine method of subculturing moss as a protonemal lawn on cellophane overlaying a solid medium also made DNA transfer by microprojectile bombardment a suitable alternative, and this technique has also proved successful²¹. Electroporation and microinjection are further successes, although not using *Agrobacterium tumefaciens*, which does not appear to infect moss (C.D. Knight, unpublished). Both PEG-mediated uptake and microprojectile bombardment generate a low percentage of stable transformants, but a high percentage of unstable transformants, which maintain the plasmid (probably extrachromosomally) under continuous selection. The fate of the transforming DNA is still not fully understood, and the mechanisms controlling whether plasmid molecules integrate stably within the genome, or exist extrachromosomally as monomers or multimeric concatamers, have been discussed^{22,23} but remain to be determined.

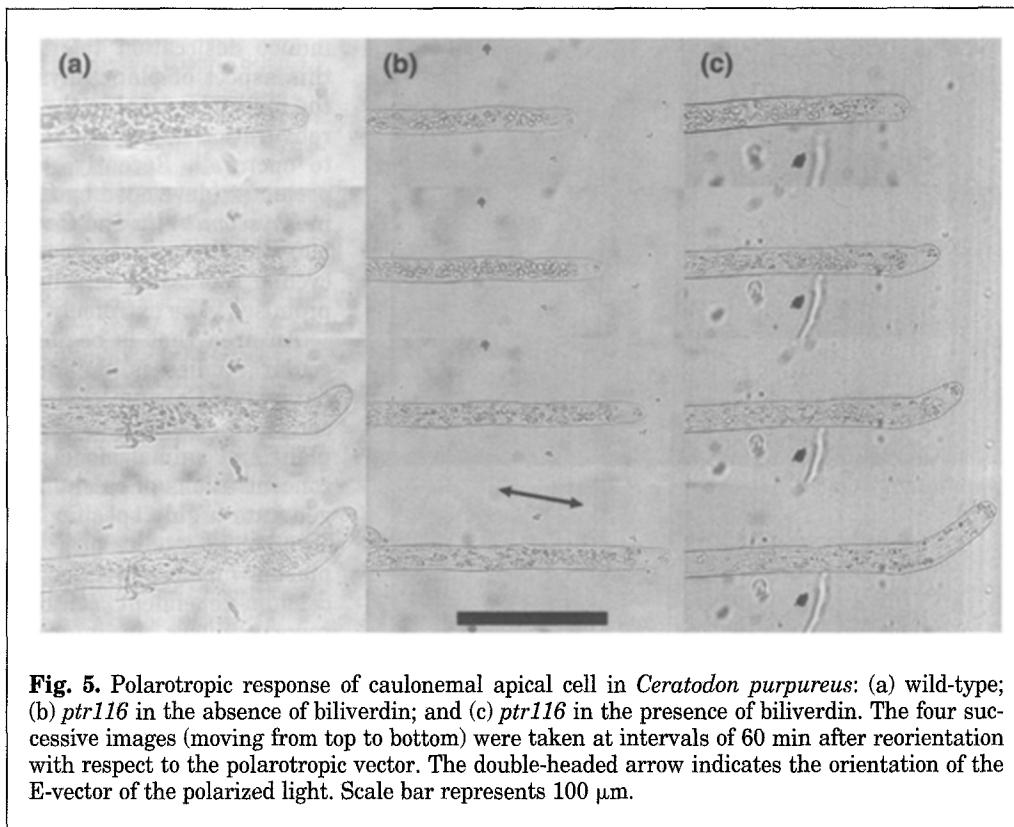


Fig. 5. Polarotropic response of caulinemal apical cell in *Ceratodon purpureus*: (a) wild-type; (b) *ptr116* in the absence of biliverdin; and (c) *ptr116* in the presence of biliverdin. The four successive images (moving from top to bottom) were taken at intervals of 60 min after reorientation with respect to the polarotropic vector. The double-headed arrow indicates the orientation of the E-vector of the polarized light. Scale bar represents 100 μ m.

Molecular genetic analysis

In comparison with higher plants, the number of moss genes analyzed at the molecular level is still low. Sequencing of ribosomal RNA has resolved land plants as a monophyletic group with liverworts and mosses as sister taxa distinct from other land plants^{24,25}. Cloning of moss nuclear *cab*, *myb* and phytochrome genes, in addition to mitochondrial and plastid genes, has revealed high sequence similarities with the higher plant homologues (see also Ref. 22). However, a phytochrome gene from *Ceratodon* differs from other known phytochrome sequences in that its C-terminus has high homology to protein kinases^{26,27}.

Significant advances for molecular approaches are being made in *Physcomitrella* by gene knockouts and gene targeting, techniques that are not yet possible in higher plants. Genetic analysis has shown that retransformation of transgenic lines of *Physcomitrella* results in the retransforming plasmid molecules being integrated at, or near to, the chromosomal location of the original plasmid integrated²⁸. The inclusion of moss DNA in a transforming plasmid increases the frequency of stable transformation tenfold²³. Molecular analysis has shown that there is efficient recombination between randomly chosen moss genomic sequences, cloned into vectors used in transformation, and the corresponding sequences in the moss genome (D. Schaefer, pers. commun.). Recently, a cDNA into which an antibiotic-resistance gene cassette had been inserted has been used successfully to target the corresponding genomic sequence, and phenotypic and molecular analysis has demonstrated that gene knockout by allele replacement has occurred (D. Schaefer *et al.*, pers. commun.). This finding confirms the potential of detecting gene knockouts directly in the primary transformants.

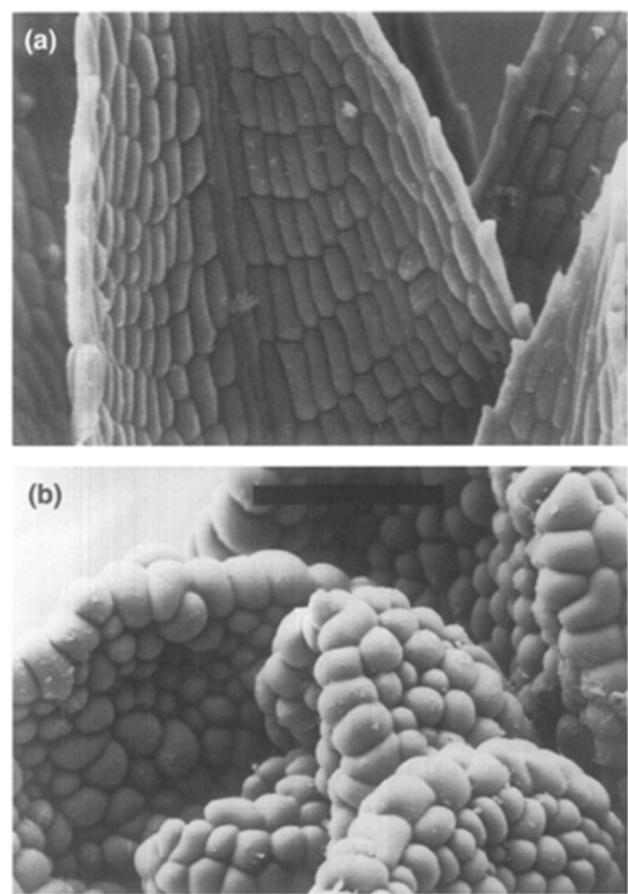


Fig. 6. Leaf cell morphology in wild-type (a) and the *gtrA10* mutant (b) of *Physcomitrella patens*. The *gtrA* mutants have characteristically rounded leaf cells as well as showing a pleiotropic impairment in the gravitational response of caulinemal apical cells. Scale bar represents 100 μm .

The significance of the gene targeting data, and its implications for higher plant research, rest with the analysis of gene function. Reverse genetics depends on analyzing the role of a cloned gene during development, either by loss of function (achieved by gene disruption or antisense) and/or the temporal and spatial control of gene expression in the transgenic plant. Success in mosses will depend on loss of gene function or reporter gene expression being easy to screen for and detect, and in this respect the simplicity of the system has much to offer. The moss plant has few cell types (see Fig. 2) and both single and multicellular structures respond to hormones and environmental cues, thus offering the potential to observe positive or negative gene expression in single cells with the minimum disturbance to cellular integrity.

The *35S* and *nos* constitutive promoters function adequately in moss, although stronger expression is achieved from the rice *Act1* promoter²⁹. The use of inducible promoters in *Physcomitrella* will be helpful for the analysis of essential genes whose null phenotypes are lethal. The wheat *Em* promoter in moss is induced 30-fold by abscisic acid (ABA) and sixfold by osmotic stress³⁰, with the ABA-induced levels being over 100 times those of the *35S* constitutive promoter. The activity levels in the absence of added ABA are probably caused by endogenous

ABA or cellular stress. Both ABA and osmotic stress induce desiccation tolerance within higher plants, and this aspect of plant physiology has been investigated at the molecular level in the moss *Tortula ruralis*, in which a rehydration-dependent repair mechanism has been shown to operate³¹. Recently, the tetracycline-inducible *Top10* promoter (developed by C. Gatz) has been adapted for use in *Physcomitrella* and *Ceratodon*²⁹ and shown to mediate a 500-fold increase in reporter gene expression when tetracycline repression is relieved²⁹ – this promoter therefore promises to be extremely useful in future research.

An area that is beginning to bear fruit in moss molecular studies is the dissection of signal transduction pathways. Calcium clearly plays a role as a second messenger in moss development. However, as with higher plant and animal models, the precise way in which local concentrations of calcium are detected and transduced to generate highly specific effects are yet to be established. Two recent studies on *Physcomitrella patens* have linked plasma membrane-associated G proteins³² and light³³ to calcium-dependent cellular events. The first of these reports shows an interaction between G proteins and dihydropyridine binding to calcium channel receptors, and the second demonstrates calcium-dependent plasma membrane depolarization by light, and suggests that this effect is phytochrome-mediated. In addition to the identification of signal transduction components immediately upstream and downstream of calcium, it will clearly be useful to map the intracellular location of calcium. To this end, transgenic lines of *Physcomitrella* containing the apoaequorin gene have been used to report changes in intracellular calcium in response to cold shock, mechanical perturbation and pH (Ref. 34). These transgenic plants will be useful in identifying the range of stimuli that trigger transient changes in calcium concentration, and advances in cellular imaging techniques may soon result in the ability to detect small local changes in intracellular calcium concentrations.

The role of the cytoskeleton in the phototropic response of *Ceratodon* has already been noted, and another recent study has investigated the interaction between calcium (stained using chlortetracycline) and the cytoskeleton in this phytochrome-mediated response¹⁷. Now that a gamma-tubulin cDNA from *Physcomitrella* has been isolated (T.A. Wagner and F.D. Sack, pers. commun.) it will be possible to analyze the cytoskeleton at the molecular level and determine how the reorganization of cytoskeletal elements is regulated.

Conclusion

With some moss species established as model organisms, it now is possible to investigate how genes from higher plants function within a much simplified cellular organization. Similarities between mosses and higher plants will be exploited to study processes such as the acquisition of polarity, and differences will make a valuable contribution to our understanding of molecular phylogeny and the evolution of land plants.

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