

Mosses as Model Systems for the Study of Metabolism and Development

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Abstract

The haploid gametophyte stage of the moss life cycle is amenable to genetic and biochemical studies. Many species can be cultured on simple defined media, where growth is rapid, making them ideal material for metabolic studies. Developmental responses to hormones and to environmental inputs can be studied both at the level of individual cells and in multicellular tissues. The protonemal stage of gametophyte development comprises cell filaments that extend by the serial division of their apical cells, allowing the investigation of the generation and modification of cell polarity and the role of the cytoskeleton in these processes. Molecular techniques including gene inactivation by targeted gene replacement or by RNA interference, together with the nearly completed sequencing of the *Physcomitrella patens* genome, open the way for detailed study of the functions of genes involved in both development and metabolism.

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INTRODUCTION

This review, a companion to one published earlier this year (27), which concentrated on the genetics of *Physcomitrella patens*, does not deal with genetic topics in detail, but concentrates on metabolic and developmental studies, many of which have exploited genetic techniques. Most such studies have been carried out on a limited number of species, principally *Funaria hygrometrica*, *P. patens*, and *Ceratodon purpureus*.

The potential utility of mosses for developmental studies was recognized more than 50 years ago (6, 7, 117). In addition to their simplicity for genetic studies, the attractiveness of mosses for studying metabolism lies in their ease of culture under defined and controlled conditions, and the simplicity of their tissues, particularly in the gametophyte stage, makes developmental studies especially attractive.

Mosses, like most plants, show an alternation of generations. The haploid gametophyte stage is dominant, and constitutes most of the familiar moss plant. Gametes are produced on gametophores, shoots bearing leaf-like structures. Different species show a range of sexual morphology, some producing male and female gametes on the same shoot, some on different shoots but on the same plant, and some having distinct and separate male and female plants. This makes mosses attractive for evolutionary studies.

Life Cycle

Cove's work (27) contains details of the life cycle of *P. patens*. **Figure 1** illustrates the life cycle diagrammatically and **Figure 2** shows images of different stages. A detailed description of the moss life cycle is not given here, but reference is made to aspects of the life cycle in the relevant sections of this review. Like many moss species, including *F. hygrometrica* and *C. purpureus*, *P. patens* has a vigorous and persistent filamentous stage, the protonema. In *F. hygrometrica*, two types of protonemal cell are clearly differentiated, chloronema and caulonema, the former having an assimilatory and the latter an adventitious role. This distinction is less marked in *P. patens* and is much less apparent in *C. purpureus*. The protonemal stage in some species (e.g., *Pottia intermedia*) is transitory and is absent in a few (e.g., *Sagnum*). Gemmae, specialized cells with a role in propagation, are produced by protonema of many moss species. Duckett et al. (40) gives a review of protonemal morphogenesis in more than 300 moss species.

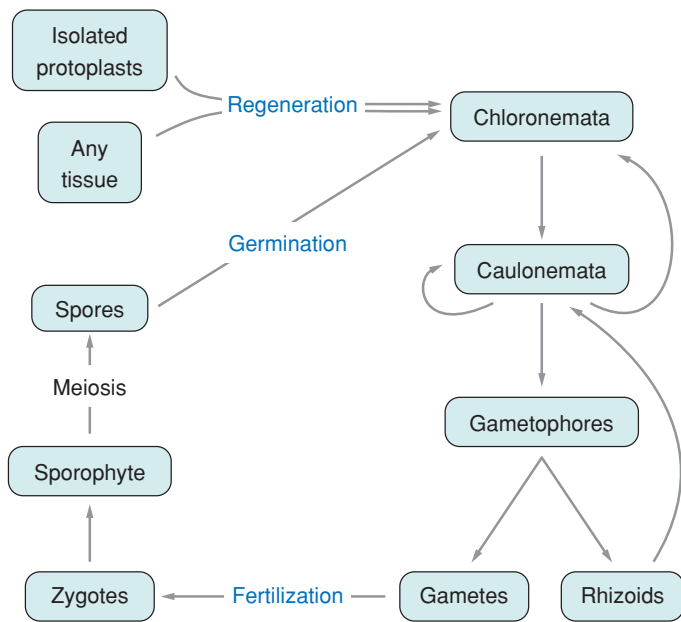


Figure 1

Life cycle of *P. patens*. All stages are haploid except for zygotes and sporophytes.

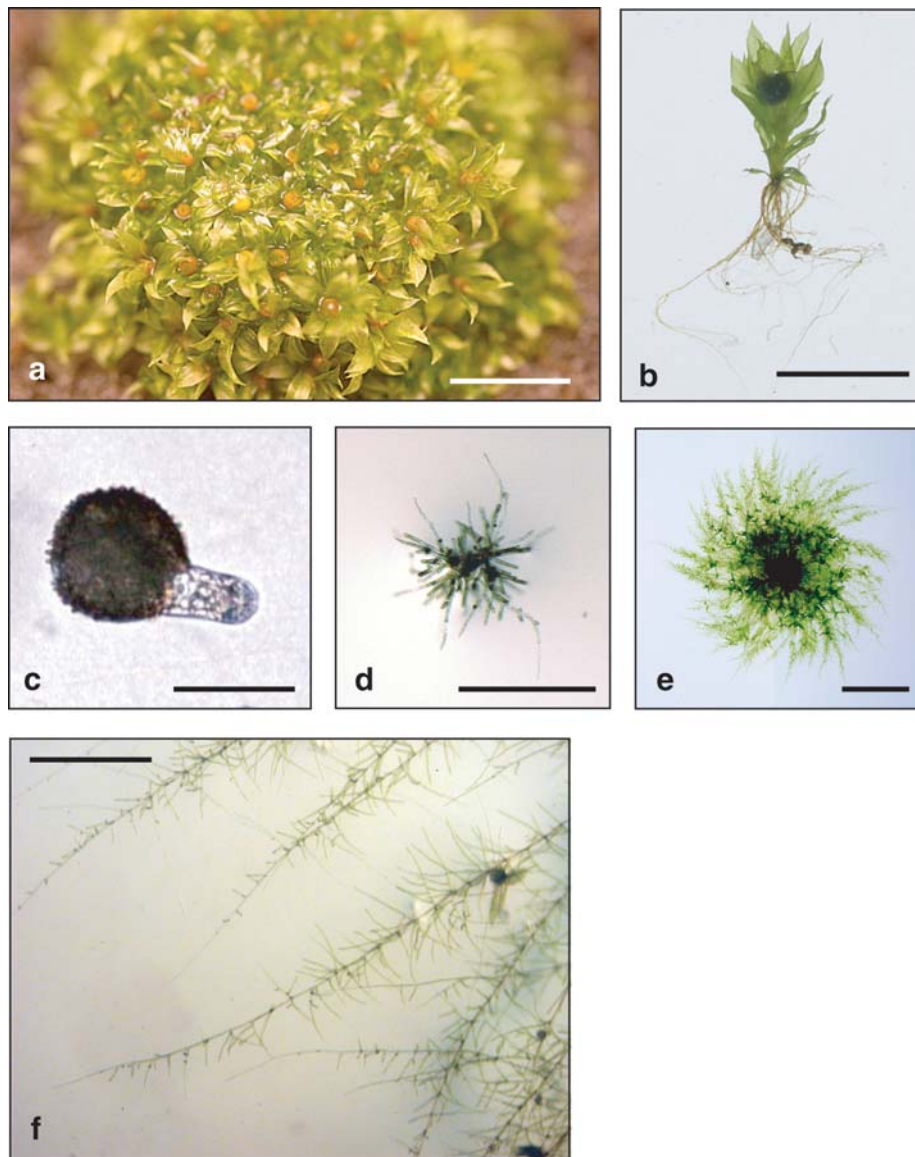


Figure 2

Stages in the life cycle of *P. patens*. (a) Gametophyte with abundant sporophytes. Scale bar: 10 mm. (b) Dissected gametophore with a sporophyte in its apex. The spore capsule is borne on a very short seta, and is therefore loosely surrounded by gametophore tissue. Abundant pigmented rhizoids originate from the base of the gametophore. Scale bar: 5 mm. (c) Germinating spore. Scale bar: 50 μm . (d) Seven-day-old sporeling. The first rapidly growing caulonemal filaments can be seen emerging from the central chloronemal tissue. Scale bar: 500 μm . (e) Twenty-two-day-old culture. Scale bar: 10 mm. (f) Detail of 22-day-old culture. The main axes are caulonemal filaments, extending by the division of their apical cells. Most subapical cells have divided to produce one or two side branches, the majority of which have developed into chloronemal filaments, but a few of which are developing into gametophores. Scale bar: 1 mm. All photographs except (a) are of cultures growing on basal medium solidified with agar. The culture in (a) is growing on a commercial compost.

Gametophyte: the haploid stage of the life cycle, comprising protonema and gametophores, upon which gametes are produced

Gametophores: leafy shoots, arising as side branches of caulonemal filaments, upon which archegonia and antheridia develop

Antheridia: male gametangia, in which motile male gametes (spermatozoids) are produced

Archegonia: female gametangia, in which female gametes are produced

Caulonema(ta): rapidly growing filament containing cells with few poorly developed chloroplasts, the adventitious component of protonemal tissue (q.v.)

Chloronema(ta): slow-growing filament containing cells with many large chloroplasts, the assimilatory component of protonemal tissue (q.v.)

METABOLIC STUDIES

Most bryophytes can be cultured on simple defined media (38), and it is possible to grow some species in continuous culture (17, 33, 57). Bryophytes are a rich source of metabolites (8, 58). The availability of both forward and reverse genetic techniques makes bryophytes superb potential material for metabolic studies. This potential is now beginning to be exploited, but metabolic studies are still not extensive.

Engel (41) isolated the first auxotrophic mutants of mosses in *P. patens*. These included mutants requiring vitamins for growth. Subsequent studies using *P. patens* have added further auxotrophs, mutants requiring sucrose, and mutants unable to utilize nitrate as a nitrogen source (73). In no case has the gene associated with the mutant phenotype been isolated.

Studies using reverse genetic approaches are now increasing. This review does not attempt to catalogue such studies but illustrates their potential by focusing on two examples.

The first example is the study of sulfur assimilation. Plants, like fungi and bacteria, acquire sulfur by the reduction of sulfate ions, by way of sulfite, to sulfide and thence to the sulfur-containing amino acids. The reduction of sulfate to sulfite may proceed by two possible routes. The formation of adenosine 5'-phosphosulfate (APS) from ATP and sulfate is catalyzed by the enzyme ATP sulfurylase. APS may then be reduced to sulfite by APS reductase, or alternatively, be phosphorylated by APS kinase to form phosphoadenosine 5'-phosphosulfate (PAPS), which is then reduced to sulfite by PAPS reductase. To investigate the route used by *P. patens*, the APS reductase gene, which is present in the genome in a single copy, was disrupted by targeted gene replacement (77), exploiting the very high rate at which recombination occurs in *P. patens* between transforming DNA and homologous genomic sequences (27). Although this resulted in the complete absence of APS reductase activity, and of the APS reductase gene

transcript, knockout plants were still able to utilize sulfate as their sole source of sulfur (77), and sulfate assimilation was only reduced by about 50%, showing that a second route for sulfate assimilation must exist in *P. patens*. A putative *P. patens* gene for PAPS reductase has been identified and inactivation of this gene by homologous recombination should establish if this gene is required for the alternative route.

The next step in sulfate assimilation is converting sulfite to sulfide by sulfite reductase. This enzyme is also coded for by a single-copy gene in *P. patens*, and has been inactivated by homologous recombination (G. Wiedemann, S. Kopriva & R. Reski, unpublished data). Although knockout plants show some retardation of growth, they can still use sulfate as the sole source of sulfur, and it has been proposed that this may be because nitrite reductase, an enzyme of nitrate assimilation, also has sulfite reductase activity (G. Wiedemann, S. Kopriva & R. Reski, unpublished data). These studies have already advanced knowledge of the pathways of sulfate assimilation in plants and further studies utilizing homologous recombination to inactivate other genes should soon characterize the metabolic network responsible.

The role of sugar metabolism in developmental signaling provides a second example of a metabolic study in *P. patens*. The gene coding for the major hexokinase, Hxk1, has been disrupted (92). Hxk1 knockout mutants have only about 20% of the wild-type glucose phosphorylating activity, indicating that this gene codes for the major *P. patens* hexokinase. Consistent with this, growth in darkness on medium containing glucose is much reduced in the mutant compared to the wild type (92). Another effect of the inactivation of the gene is to eliminate the effect of glucose on the induction of caulonemal filaments.

In *Saccharomyces cerevisiae*, the product of the Hxk2 gene, the major hexokinase, also plays a role in glucose repression. A protein kinase encoded by Snf1 forms part of the chain leading to the transcriptional shut down of

carbon catabolism in the presence of glucose. The Hxk2 hexokinase is needed for inhibiting Snf1 by glucose, but details of its involvement are not clear. There is some evidence that similar systems may exist in plants and *P. patens* provides excellent material to advance these studies (113). Two Snf-related genes, Snf1a and Snf1b, have been cloned from *P. patens*, and the genes have been inactivated, both singly and in combination. Strains in which only one of the genes was inactivated have no obvious phenotype, but the double knockout strain, in which no Snf1-like kinase activity can be detected, has a number of developmental abnormalities, including an overproduction of caulonemata, which are opposite to the effects shown by the Hxk1 knockout strain. This is consistent with energy supply being involved in the control of the balance between chloronemata and caulonemata, and with both the Hxk1 gene and the Snf1a and Snf1b genes playing a role in the control and monitoring of energy supply (114). Another unexpected pleiotropic effect of the *snf1a snf1b* double mutant is its inability to grow in a light-dark cycle, conditions under which wild-type strains of *P. patens* thrive (113), suggesting that these gene products are necessary for the metabolic adjustments required for survival in darkness. These studies should go on to elucidate whether or not the link between hexokinase and the snf1 gene products is direct.

DEVELOPMENTAL STUDIES

Hormone Synthesis and Action

Auxin. In mosses, exogenous auxin induces chloronemal to caulonemal differentiation and inhibits chloronemal branching (4, 26, 49, 109, 114). The mosses *F. hygrometrica* and *P. patens* both contain at least one native auxin, indole-3-acetic acid (IAA) in gametophytic tissue (5, 60). The use of a β -glucuronidase (GUS) reporter gene driven by the auxin inducible promoters GH3 and DH5 has been used to determine auxin levels in different *P.*

patens tissues (15). GUS staining reveals that all cell types are capable of responding to auxin, although the strongest GUS expression was found in young, actively growing cells of protonemal and gametophore tissue.

The isolation of *P. patens* mutants resistant to the auxin analog, 1-naphthalene acetic acid (NAR mutants) (4) reveals a connection between auxin sensitivity and cytokinin production. When category 2 NAR mutants are treated with auxin, they show increased chloronemal production in comparison to wild type and produce no gametophores. These mutants are restored to wild-type morphology by cytokinin treatment, indicating that category 2 mutants are defective in cytokinin production. Likewise, mutants resistant to the cytokinin analog, 6-benzyl aminopurine (BAR mutants) can be rescued by exogenous auxin and are thus likely defective in auxin biosynthesis. Dependency of cytokinin-induced bud formation upon auxin concentration was first noted in the moss *Aoecetangium thomsonii* (22) and has been interpreted as a necessity for auxin to create target cells competent for cytokinin response (79). Experiments in which the medium supply is continuously replaced confirm that in *P. patens* and *Physcomitrium sphaericum* auxin is required for caulonemata production, and that cytokinin does not induce bud formation in the absence of auxin (26). Although more remains to be learned about the connection between these two hormones, these mutants demonstrate that sensitivity of *P. patens* to either auxin or cytokinin requires the presence of both hormones.

Relatively little is known regarding auxin-signaling pathways in moss. Work in *P. patens*, however, has begun to reveal the complex and diverse signaling cascades in which auxin plays a role. The auxin antagonist, naphthylphthalamic acid (NPA), was used to demonstrate that auxin efflux into the media is required for protoplasts to establish cellular polarity necessary for asymmetric division (13). This auxin efflux precedes and seems to be required for a polar redistribution of calcium channels. It

GUS:
 β -glucuronidase

was recently shown that chloronemal tip cells of *F. hygrometrica* respond to auxin treatment with a rapid influx of calcium ions (Ca^{2+}) into the cell, followed by establishment of a tip to base Ca^{2+} gradient (14). A similar response was also noted in *F. hygrometrica* treated with cytokinin (53).

Evidence integrating auxin and light-receptor signaling was provided by the disruption of cryptochrome genes (blue-light receptors), which increases sensitivity of *P. patens* to exogenous auxin and suppresses auxin-induced gene expression (59). It is proposed that blue light acts to suppress auxin sensitivity by downregulating the expression of auxin-induced genes. In other experiments, auxin induced the expression of the homeodomain gene *Ppbb7*, which had been implicated in rhizoid differentiation (100). The establishment of these links between auxin and major developmental regulators such as light signaling and homeodomain genes emphasizes the critical and complex role that auxin and other hormones play in plant growth and development.

Cytokinin. Treating mosses with cytokinins has long been known to induce bud formation (51). The strength of the cytokinin response in mosses has made them attractive plant models for studying cytokinin perception and signaling. The enhancement of bud production in response to cytokinin has been demonstrated in more than 20 species of moss (28) including *P. patens* (4). Cytokinin treatment of *P. patens* induces a large increase in gametophore production resulting from the formation of buds on caulonemal filaments, and also in some cases chloronemal filaments (4, 97).

The haploidy of moss gametophyte tissue makes mutant isolation relatively straightforward. By screening a mutagenized population of *P. patens*, 25 gametophore overproducing (OVE) mutants have been identified (3), one class of which cross feeds adjacent wild-type cultures, to overproduce gametophores, suggesting that these mutants

might be cytokinin overproducers. This was confirmed by demonstrating that these mutants produce greatly elevated levels of the cytokinins isopentenyladenine and zeatin in their growth media (120, 122). Complementation analysis between 15 OVE mutants showed that 14 are recessive, and are thus likely loss-of-function mutants. Seven of these mutants were grouped into three complementation groups (44). The identities of these genes remain to be elucidated. Isolating a temperature-sensitive OVE mutant from *P. patens* that strongly overproduces cytokinin at 25°C (approximately 260 times wild-type levels) represents a valuable tool for further characterizing cytokinin response and synthesis in moss (104).

Biochemical techniques have also been used to identify proteins involved in the moss cytokinin response. Using radio labeling, coupled with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, cytokinins induce expression of a 14-kDa extracellular protein in *P. patens* (91). Additionally, photo-affinity labeling with a cytokinin agonist identified a protein potentially involved in cytokinin signaling (47). Although the function of these proteins remains unknown in mosses, the identification of proteins involved with cytokinin signaling and response is critical to achieving an understanding of cytokinin-related processes.

A proteomic approach has identified eight cytokinin upregulated proteins that comprise three key energy-conversion enzymes (67). This reflects the strong energy requirement associated with the morphological changes induced by cytokinin. Additionally, recent work with phospholipase C mutants implicated the phosphoinositide (PI)-signaling pathway in the cytokinin response (96). In *P. patens*, phospholipase C mutants show reduced gametophore formation and a loss of sensitivity to exogenous cytokinin.

The mode of cytokinin synthesis is still unclear. Because tRNA hydrolysis provides a possible source of cytokinins (21), research has attempted to distinguish this possibility

from de novo cytokinin synthesis. When *P. patens* OVE mutants are fed radio-labeled adenine, it is rapidly converted to isopentenyladenine, which in turn is excreted into the culture medium (121), providing strong evidence for de novo synthesis of cytokinin in *P. patens* and arguing against tRNA degradation as the route of cytokinin production. Further support for de novo synthesis comes from the finding that tRNA levels in OVE mutants are comparable to wild type (93). Additionally, ribose to base conversion, the last step of isopentenyladenine formation, is upregulated in OVE mutants (104).

To date, relatively little work has focused on cytokinin metabolism in mosses. Following feeding *P. patens* with labeled cytokinins, the major metabolites are adenine and adenine nucleotides (123). The recent cloning of an adenosine kinase from *P. patens* and the demonstration of its ability to phosphorylate cytokinin in vivo provides evidence that moss can indeed metabolize cytokinin to its nucleotide in a single step (116).

Abscisic acid. In angiosperms, abscisic acid (ABA) plays a key role in seed development, including the control of the synthesis of nutrient reserves, desiccation tolerance, the acquisition of dormancy, as well as the inhibition of the developmental transition from embryonic to vegetative growth. ABA also mediates, at least in part, adaptation to stresses during vegetative growth, e.g., temperature extremes, wounding, and drought.

ABA is present in bryophytes and evidence for a signaling pathway has been clearly established (see below), but its role(s) has not been elucidated. Bryophytes represent examples of the first land plants, and as such had to adjust to environmental stresses not found in water. Consequently, mechanisms have evolved to cope with drought and other stresses that may represent the earliest form of a stress-signaling pathway. Most research on ABA in bryophytes has concentrated on the role of ABA in acquiring tolerance to environmental stresses.

Less than 10% of cells of *P. patens* can survive a slow drop in temperature to 4°C, but pretreatment with ABA significantly increases the survival rate (86) and increases the expression of 14 ABA-responsive genes within 24 hours, indicating not only that an ABA response pathway is operative, but that the resulting enhancement of freezing tolerance is associated with an increase in specific gene expression. Treatment with sodium chloride and mannitol also enhances freezing tolerance and increases the expression of 11 of the 14 ABA-responsive genes. These results suggest that at least these 11 genes are likely responsive to multiple stresses. It was recently shown that freezing injury in *P. patens* results in ultrastructural changes that do not occur when ABA is added prior to the cold treatment (90). The ABA treatment also increases the osmotic concentration of the protonemal cells by accumulating free soluble sugars, which may mitigate the freezing-induced structural changes and lead to freezing tolerance. Freezing tolerance of protonemal cells is greatly increased by incubating at 0°C for several days (87). This treatment results in the accumulation of transcripts of Late Embryo Accumulating (LEA) and “boiling soluble” proteins. Both of these classes of proteins play a major role in protecting cells during water stress in desiccating seeds and vegetative tissues of angiosperms.

Protonema of *F. hygrometrica* tolerate slow desiccation but not rapid dehydration. Application of ABA enables protonemal tissue to survive rapid drying and is correlated with increases in endogenous ABA during drying (124). ABA exerts its influence in this system through specific proteins that are synthesized during drying, some of which resemble dehydrins (16, 103). It has also been reported that short, thick-walled desiccation-tolerant protonemal cells form under the influence of desiccation or ABA in *Aloina aloides* (50) and *Diphyscium foliosum* (39).

In *Atrichum androgynum*, ABA treatment or partial dehydration increases resistance to desiccation-induced cation leakage (9). Pretreatment of *Atrichum undulatum* with

ABA: abscisic acid

ABA also increases desiccation tolerance (11). More specifically, ABA treatment improves the tolerance of photosystem II (PSII) to water stress by allowing PSII to recover faster. Other parameters of photosynthesis that were measured also indicate that ABA allows recovery of photosynthetic damage during desiccation much faster than untreated tissue. This protective effect of ABA on photosynthesis might explain, in part, how ABA has a more general protective effect on many different stresses. It was later shown that pretreatment with ABA induces the synthesis of proteins that enhance desiccation tolerance in *Atrichum* (10). Interestingly, the ABA-induced desiccation tolerance is much less when plants are pretreated with ABA in the dark. Red light could not be substituted for white light, suggesting that ABA action for protection does not require phytochrome. Like the results above in *Atrichum* and *F. hygrometrica*, Machuka et al. (80) showed that treatment of seven-day-old *P. patens* protonemal tissue with ABA led to upregulation of genes that could be identified as responders to oxidative and chemical stresses, as well as those associated with protection of cells during desiccation and extreme temperature changes.

Investigation of the desiccation/rehydration cycle in *Atrichum* after pretreatment with ABA shows that pretreatment increases the rate of recovery of PSII activity, increases the concentration of soluble sugars, and also doubles the amount of nonphotochemical quenching (84). An increase of nonphotochemical quenching reduces reactive oxygen species and may explain how ABA enhances pathways that protect moss during desiccation. Finally, the effects of ABA pretreatment on changes in lipid composition during desiccation and rehydration have been studied and reveal that ABA pretreatment reduces the overall extent of changes in lipid/membrane components, thereby reducing membrane damage during desiccation (52). The increase in freezing and drought tolerance induced by ABA treatment is likely the result of increased expression of genes

that participate in protecting protonemal cells during stress.

Although exogenous ABA clearly enhances tolerance to these stresses, it is not clear whether endogenous ABA or the ABA-signaling pathway is required for such an effect. In fact, ABA has not been detected in one of the most desiccation-tolerant species of moss, *Tortula ruralis*, nor does this moss synthesize detectable proteins when ABA is added exogenously (12, 94). Additionally, transcripts that specifically accumulate during drying in *T. ruralis* (125) are not associated with an increased accumulation of endogenous ABA. The molecular techniques now available for *P. patens* offer unique opportunities to identify genes that participate in the acquisition of stress tolerance.

The involvement of abscisic acid in regulating gene expression and in signaling pathways has also been studied. In *F. hygrometrica*, cytokinin-stimulated bud formation is inhibited by ABA in a concentration-dependent manner (23), providing a potentially specific and quantitative bioassay for ABA. Experiments in which protonema were transferred between cytokinin and cytokinin plus ABA show that ABA does not interfere with the initial perception of cytokinin. Supporting this result, it was shown that ABA is not a competitive inhibitor of cytokinin. Through these studies, a new regulatory step that involves ABA in the developmental process of bud formation in mosses is proposed. Although this study did not identify ABA-induced transcripts, a separate study (32) reported that ABA treatment of protonemal cells results in a rapid increase in the activity of a 38-kDa protein kinase. The transcription of this gene was also enhanced by NaCl, and when added together with ABA, an additive response is observed, suggesting that both ABA and NaCl act via independent and parallel pathways (32).

More recently, it was demonstrated that *P. patens* is tolerant to drought, salt, and osmotic stress and that specific transcripts are induced under these conditions (46). Using a cDNA

macro array composed of 45 putative stress-associated cDNAs from *P. patens*, many cDNAs were identified that were induced under the stresses imposed, and by exogenous application of ABA. Both overlapping as well as unique expression patterns were observed, indicating that there are both ABA-dependent and -independent stress-responsive pathways.

The presence of an ABA-responsive pathway in *P. patens* was first demonstrated by transforming moss with a wheat promoter from the ABA-responsive Em gene linked to the GUS reporter gene (76). Following transformation, adding ABA to moss protonema results in GUS expression. Mutational analysis of the promoter indicated that the same nucleotides that are responsible for expression in angiosperms are also responsible for expression in moss. This indicates that at least at the transcription level the controls for expression from the Em promoter appear to be conserved between bryophytes and seed plants. Furthermore, gel retardation and DNAase footprint analyses demonstrate that the transcription factors in moss that react with the Em promoter display the same footprint as proteins from seed plants that interact with the Em promoter. The synthesis of several stress-related polypeptides in response to ABA was also reported in this study. These results clearly point the way toward comparative studies to identify conserved signaling intermediates, promoter elements, and genes that will help to elucidate the evolution of the ABA-signaling pathway.

An example of such an approach comes from results recently obtained (H. Marella, Y. Sakata & R. Quatrano, unpublished data). The transcriptional regulator ABI3 from *Arabidopsis* and VP1 from maize are part of the transcriptional regulatory complex that controls a set of genes required for seeds to survive desiccation (45). An ABI3-like gene from *P. patens* (PpABI3) was recently identified that can function similarly to ABI3 and VP1 in enhancing the ABA responsiveness of reporter genes in both *P. patens* and aleurone cells of barley. Furthermore, PpABI3 can

partially complement the *abi3-6* mutant allele from *Arabidopsis*. By comparing sequences between PpABI3 and ABI3/VP1, it is possible to start to decipher the domains that have been conserved along with specific functions, and understand how regulation of ABA-induced gene expression has evolved from drought/desiccation tolerance in the vegetative tissues of moss to the seeds of angiosperms.

Organelle Structure and Function

Organelle studies in mosses have so far concentrated almost exclusively on chloroplasts, and most have been carried out using *P. patens*. Under normal growth conditions, *P. patens* chloronemal cells contain approximately 50 chloroplasts randomly distributed in the cortex of the cell (68). Sequencing the chloroplast genome revealed some of the evolutionary history of *P. patens* (112). The genome is 122,890 bp and encodes for 83 proteins, 31 tRNAs, 4 rRNAs, and 1 pseudo gene. Although the overall structure of the chloroplast genome resembles the liverwort *M. polymorpha*, some key differences indicate that *P. patens* diverged from hepatic bryophytes and is more closely related to vascular plants. One unique finding from the sequence is that mosses transferred a critical gene from the chloroplast to the nucleus. The alpha subunit of the RNA polymerase (*rpoA*), used to transcribe genes in the chloroplast, has been lost from the chloroplast genome and is instead encoded in the nucleus. Using green fluorescent protein (GFP) fusions of the nuclear *rpoA* gene, it was shown that its gene product is imported into the chloroplast (112).

P. patens chloroplasts are particularly amenable for studying photosynthesis. A recent study shows that, like in cyanobacteria, it is possible to measure fluorescent parameters in *P. patens* tissue in vivo (115). This is in contrast to vascular plants, where these measurements have only been performed on isolated thylakoid membranes. In addition, this study showed that *C. purpureus* could be easily

GFP: green fluorescent protein

mutagenized to produce photosynthetic mutants (115).

Chloroplast import pathways appear to be conserved between *P. patens* and vascular plants. Sequence information from expressed sequence tag (EST) databases shows that *P. patens* contains genes involved in all known chloroplast import pathways, including thylakoid membrane transport (56). By performing protein import assays on isolated chloroplasts, all major protein-targeting pathways in the chloroplasts operated similarly between *P. patens* and vascular plants (56). This conservation of pathways coupled with the near completion of the *P. patens* genome validate the use of *P. patens* as a model system for studying plant gene function, particularly in the chloroplast.

Gene targeting has also been used to disrupt a chloroplast gene that codes for a transfer RNA (trnR-CCG) (111). Lines were generated that contained plastids lacking the trnR-CCG gene, which, when selfed, retained this genotype but were not impaired in growth. This study confirms that this tRNA is not essential for chloroplast function (111) and lays the foundation for a detailed analysis of the function of individual chloroplast genes using gene-disruption techniques in *P. patens*.

Similar to other plant systems, *P. patens* chloroplasts use RNA editing to regulate gene expression in the chloroplast (89). The rps14 transcript is modified by RNA editing in a unique way that is not observed in any other plant system (89). This modification, originally detected at very low efficiency, is a C to U transition that creates a translation initiation codon AUG (89). A recent study demonstrated that the modification is regulated in a tissue- and stage-specific manner, indicating that RNA editing in chloroplasts is a regulated process required to precisely control chloroplast gene expression (88).

Because many plastid and mitochondrial proteins are encoded in the nucleus, N-terminal transit peptides are used to target these proteins into plastids and mitochondria. *P. patens* employs translation initiation

as a mechanism to differentially target a single gene to two different subcellular compartments (71, 98). In the cases studied, the initial translation initiation site targets the chloroplast. For an *rpo* gene, the second initiation site targets the mitochondria (98), and for FtsZ1-2 the second site targets the cytoplasm (71).

FtsZ2-1 encodes one of four FtsZ isoforms in *P. patens* (71). FtsZ is the bacterial ancestor of tubulin and is essential for cell division in bacteria (42). It is present in the chloroplasts of plants and by inference was thought to be involved in chloroplast division, which has been shown for vascular plants (42) and for *P. patens* (110). Deletion of FtsZ2-1 blocks chloroplast division, resulting in one macrochloroplast per cell (110).

Localization of the FtsZ isoforms in *P. patens* has revealed novel structures within the chloroplast that resemble cytoskeletal networks. Functional GFP fusions of FtsZ2-1 and FtsZ2-2 localize to the chloroplast, forming filamentous structures (70). In contrast to bacterial FtsZ, that only forms a ring at the time of cell division, the *P. patens* FtsZ isoforms form networks of filaments at all times. Additionally, FtsZ2-2 appears to form a ring during chloroplast division (70). It was also demonstrated that the novel FtsZ1-2 isoform can be targeted to both the chloroplast and the cytoplasm via regulation of translation initiation (see above). This isoform also localizes to rings in the cytoplasm and is hypothesized to link cell division to chloroplast division (71).

In addition to mutations in the FtsZ gene, chloroplast division can also be blocked by treating *P. patens* protonema with antibiotics that inhibit the bacterial peptidoglycan synthesis pathway. The β -lactam antibiotics form covalent complexes with penicillin-binding proteins of bacteria. This is a lethal complex, because it interferes with the bacteria's ability to synthesize a cell wall. After two days of treatment with β -lactam-type antibiotics, chloroplast division is blocked, as evidenced by a decreasing number of chloroplasts per cell (68). However, cell division occurs

normally and results in chloroplast separation. The sensitivity of *P. patens* to β -lactam antibiotics indicates that *P. patens* chloroplast division has conserved enzymes derived from the bacterial peptidoglycan synthesis pathway. It is pertinent to note that not all antibiotics result in the same severity of phenotype, possibly indicating that the plant enzymes have diverged sufficiently from their bacterial homologs to confer a certain amount of resistance. Alternatively, it is possible that not all the antibiotics have the same permeability.

P. patens chloroplasts alter their position in the cell in response to the quality and fluency of light. *P. patens* chloroplasts accumulate in low-fluency red and blue light and avoid high-fluency red and blue light (66). This response is nullified for red light by simultaneously illuminating with far-red light, suggesting that both dichroic phytochrome and a dichroic blue-light receptor are involved in chloroplast photorelocation (66). The cytoskeletal networks driving chloroplast movement are discussed below.

Studies of mitochondrion structure and function in mosses are much more limited. Mitochondrial DNA has been isolated from *P. patens* (83), but no complete sequence is yet available. The mitochondrion-encoded cytochrome oxidase III gene (*cox3*) from *P. patens* has been sequenced and contains no introns (82). Its protein-encoding sequence showed a strong similarity (about 72%) to *cox3* sequences from seed plants (82). RNA editing of the *cox3* gene is observed in the mosses *Tetraphis pellucida* and *C. purpureus*, indicating that RNA editing in the mitochondria predates the evolution of the tracheophytes (81).

Morphogenesis

Moss development provides outstanding opportunities for studying the control of pattern formation and cell polarity, shape, and division—all fundamental processes in the regulation of morphogenesis.

Polar axis determination. Spore germination and the regeneration of isolated protoplasts involve polar outgrowth from apparently unpolarized structures to generate protonemal filaments. These processes therefore provide material for studying polar axis establishment. Spores of *F. hygrometrica* and *C. purpureus* do not require light for germination, but spores of *P. patens* only germinate in light, with a peak activity at wavelengths around 660 nm (30). The point of outgrowth cannot be aligned by either gravity or light (D. J. Cove, unpublished data).

Protoplasts of *P. patens* undergo cell wall synthesis in darkness and cell division in low-light intensities, but require high intensities of light to form a polar axis (18, 64). Photon fluence rates of about $7 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$ for either red (665 nm) or blue (442 nm) light are required to achieve polar outgrowth from 50% of protoplasts (64). The effects of light direction on polar axis orientation can therefore only be studied in high-light intensities. To regenerate protoplasts in unidirectional light, the light source is usually placed so that light falls on the side of the Petri dish containing the regenerating protoplasts, which are embedded in soft agar (64). Because the Petri dish and agar attenuate the light intensity, it is difficult to standardize light treatments. Burgess & Linstead (18) reported that light intensity fell more than threefold across a 50-mm Petri dish, and thus regeneration occurred in a range of intensities. They provided unidirectional white light by a mirror, and the light was therefore partially polarized. Response to polarized light is generally taken as evidence that the photoreceptors detecting the light direction are held in the cell, at or near the plasma membrane, in a fixed array (62). In their studies, Burgess & Linstead (18) showed that 90% of regeneration axes of protoplasts in the part of the dish nearest to the light source were away from the direction of light. They later showed that regeneration axes can also be aligned by an electrical field (19). In a similar experimental setup, but not using reflected light, regeneration axes in

high-intensity ($75 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) white light were not strongly aligned but tended to be oriented at right angles to the light direction (D.J. Cove, unpublished data). The problem of standardizing light intensities is much reduced if polarized light is used, as this can be shone directly at the protoplasts. Studies using polarized light are now in hand (54; D.J. Cove, unpublished data).

C. purpureus protoplasts regenerate in darkness, making it possible to study the effect of a wider range of light conditions on the formation of a polar axis in this species. Measuring the orientation of the polar regeneration axes of large populations of protoplasts shows that, in darkness, gravity is not an important input, the distribution being only slightly biased upward (31). When regeneration occurs in unidirectional light, the axis distributions vary with different wavelengths (31). In red light, regeneration axes are well aligned to the direction of light, although about 25% of protoplasts orient their outgrowth away from the light source. In blue light, most protoplast regeneration axes are oriented toward the light source, but are much more poorly aligned to it (31). *C. purpureus* protoplasts do not regenerate synchronously and this allows the kinetics of axis determination to be investigated (31). When protoplasts, regenerating in unidirectional light, are reoriented, the cohort of protoplasts regenerating over the next 8 to 9 hours align their regeneration axes to the original light direction, indicating that the alignment of the regeneration axis is fixed well in advance of polar outgrowth. The protoplasts that align their axes to the new light direction are evenly divided between those growing toward and those growing away from the light source, showing that although alignment to the new direction can be established, the correct orientation, i.e., toward or away from the light source, is established much more slowly (31).

Modification of polar axes. Although the direction or plane of polarization of light does not influence the point of outgrowth of ger-

minating *P. patens* spores, the apical cells of the chloronemal filaments they produce respond to either the direction or plane of polarization of light (62). Chloronemal apical cells show alternative responses depending on the wavelength and intensity of light. The low-intensity response is growth toward a light source, or perpendicular to the E-vector of polarized light, whereas the high-level response is perpendicular to the direction of the light source or parallel to the E-vector. The change between the low- and high-intensity responses can only be observed at some wavelengths (480–500 nm and 630–690 nm), and then the switch occurs over a narrow range of fluence rates. At other wavelengths, only either the high-intensity response (<480 nm) or the low-intensity response (500–630 nm and >690 nm) is observed.

In contrast to chloronemal filaments, which do not grow in darkness and show no response to gravity in light, the apical cells of caulonemal filaments (or of older protonemal filaments in species where the morphological distinction between chloronemata and caulonemata is not apparent) of a number of moss species grow well in darkness if supplied with a reduced carbon source, and respond to gravity by growing upward (20, 63, 108, 126). The response to gravity in *P. patens* and *C. purpureus* only occurs in darkness, but the apical cells of protonemal filaments of *C. purpureus* mutants, deficient in the synthesis of the phytochrome chromophore, continue to respond to gravity in light (78). The response to gravity is therefore actively switched off by light, by way of phytochrome.

The kinetics of the gravitropic response was studied in *P. patens* (72, 74) and *C. purpureus* (126), using time-lapse microscopy. In *C. purpureus*, plastids sediment upon reorientation, in a zone within the apical cell, and it is proposed that these plastids have a gravity perception function (126). In *P. patens*, *C. purpureus*, and *F. hygrometrica* (108), the immediate response to 90° reorientation is a brief period of up to 30 minutes of downward growth. Thereafter, filaments begin to

respond by growing upward, but this response is interrupted and even reversed each time the apical cell divides. It has been proposed that the correlation of cell division with the reversal of the gravitropic response arises because the reorganization of the microtubule cytoskeleton, required during the division of the nucleus and the formation of a new cell wall, disturbs the mechanisms for the perception of gravity (74). In dark-grown protonemata of *C. purpureus*, microtubules are mostly axially oriented throughout the entire apical cell and are closely associated with plastids. Cells that were gravistimulated for more than 20 minutes show an accumulation of microtubules proximal to the sedimented plastids and near the part of the tip that elongates more to produce curvature. Inhibitors of the microtubule cytoskeleton disrupt the gravitropic response, but do not inhibit plastid sedimentation (107). In fact, plastid sedimentation is increased upon treatment with the inhibitor, suggesting that microtubules restrict the sedimentation of plastids along the length of the cell and are load-bearing for all plastids in the apical cell (106). In dark-grown caulonemata of *P. patens*, microtubules accumulate in the lower flank of the tip cell 30 minutes after reorientation (1). These studies demonstrate that microtubules are involved in sensing and responding to changes in the gravity vector.

Mutants affecting the gravitropic response of apical cells have been isolated in both *P. patens* (63, 75) and *C. purpureus* (118). These include not only mutants that are unable to align to the gravity vector (*P. patens gtrA*), but also mutants that align to gravity as well as the wild type, but orient their response downward rather than upward (*P. patens gtrC*), a further example of the independence of alignment and orientation to a directional input. Complementation analysis of *C. purpureus* mutants indicates that some agravitropic mutants are likely allelic with response-reversed mutants (D.J. Cove, unpublished data). The gametophores of *P. patens gtr* mutants show a wild-type upward-growth phenotype, and so the *gtrA* and C gene products are not required

for the gravitropic response of the multicellular gametophore axis.

Caulonemal apical cells also respond to a directional light input, or to the plane of polarized light. A number of lines of evidence identify phytochrome as the photoreceptor responsible for the photo- and polarotropic response (55, 78). The most direct evidence is provided by the aphototropic phenotype of *ptr* mutants of *C. purpureus* blocked in their synthesis of the phytochrome chromophore (43, 78). These mutants have an extended phenotype, being deficient in other phytochrome-mediated processes including chlorophyll synthesis. A further class of aphototropic mutants of *C. purpureus* is only impaired in the phototropic response, showing none of the pleiotropic traits associated with phytochrome function (78). These mutants are presumably downstream of the action of phytochrome in the perception of light direction. Similar mutants of *P. patens* have been isolated and complementation analysis shows that at least three genes (*ptrA*, B, and C) can mutate to give a similar phenotype (24, 30). These mutants also are impaired in the phototropic response of their gametophores. *P. patens ptr* mutants have a puzzling phenotype with respect to chloronemal phototropism, showing the same low- and high-intensity responses as the wild type, but the intensity at which the change from the low- to high-intensity response occurs is lower (61). The products of the three *ptr* genes must therefore be required for detecting light direction by caulonemal apical cells and gametophores, but not by chloronemal apical cells, where their role instead appears to be to detect light intensity.

The chloronemal filaments produced as side branches from caulonemal filaments (secondary chloronemata) differ from chloronemata arising from spores or regenerating tissue (primary chloronemata) in that their apical cells grow toward the light, even at high intensities (30). In high intensities of polarized white light, caulonemal apical cells show alternative alignments, aligning to the E-vector

$\pm 16^\circ$, and often switch between the two alignments after a few cell divisions (29). Secondary chloronemal side branches also show alternative alignments, but these depend on the alignment of the caulonemal filament from which they arise (D.J. Cove, unpublished data). The adoption by caulonemal apical cells of alternative alignments to polarized light is consistent with the relevant photoreceptors being arranged spirally around the cell, with the choice of alignment depending on whether photoreceptors are activated on the upper side or lower side of the cell.

The interaction between light and gravity on the orientation of caulonemal apical cells of *P. patens* has been studied by growing cultures in unidirectional monochromatic red light (660 nm) supplied at right angles to the gravity vector (30, 63). At intensities down to $200 \text{ nmol quanta m}^{-2} \text{ s}^{-1}$, growth is toward the light source. At $60 \text{ nmol quanta m}^{-2} \text{ s}^{-1}$, growth is aligned at 45° to both the light direction and gravity vector, but at intermediate intensities alignment switches between growth toward the light and upward growth (29).

In contrast to gravitropic responses that depend on the microtubule cytoskeleton, the phototropic response in *C. purpureus* depends on the actin cytoskeleton. Actin filaments are localized in the apical cell of dark-grown protonemata such that bundles of filaments run from subapical regions to the apex axially along the cell cortex where they converge toward a central area of the tip (85, 119). This produces a collar-like structure. Dark-grown protonemal tip cells irradiated with unilateral red light form a bulge preceding a light-directed outgrowth. During an irradiation, the actin filaments reorient toward the irradiated apical flank. The collar-like structure is essential for tubular outgrowth. This process can proceed in the absence of microtubules. In fact, unilateral red light suppresses morphological distortion induced by inhibitors of microtubules and restores the actin collar structure. However, without microtubules, the actin reorientation is no longer restricted to the apical region of the tip cell (85). The

actin collar-like structure has also been observed in *F. hygrometrica* during side-branch initiation (95).

Protonemal patterning. The protonemal stage of moss development is essentially two dimensional, comprising branching filaments that grow by extension at the tip of their apical cells. New filaments are produced as side branches from filament subapical cells. In *P. patens* and related species, spore germination gives rise to chloronemal filaments, but these also arise from tissue regeneration and as the most common fate of caulonemal side branches. Caulonemal filaments arise as a result of a transition of some chloronemal apical cells, but also occur as side branches from caulonemal filaments and, rarely, as a result of the transition of a rhizoid apical cell. In *P. patens*, the transition of a chloronemal to a caulonemal apical cell can be rapid and completed within a single cell cycle, but usually takes several cell divisions (99). The transition depends on auxin and the supply of exogenous auxin increases the production of caulonemal filaments (4). Some mutants unable to produce caulonemata are likely impaired in auxin synthesis, whereas others are blocked in their response to auxin (4).

The apical cells of the two filament types have contrasting growth patterns. Chloronemal apical cells extend slower and have a longer cell cycle than caulonemal apical cells (27). No studies have been carried out to investigate the basis of these differences.

The pattern of caulonemal development has been studied in *P. patens* using a combination of the analysis of large numbers of caulonemal filaments together with time-lapse video microscopy of living cultures (99). The pattern is affected by both genotype and the environment. For a given environment and genotype, pattern is not rigidly determined, but the probabilities of occurrence of each cell transition vary little, and so patterning is far from random. The alternative fates of caulonemal side-branch initials into chloronema, caulonema, or buds is influenced

not only by inputs such as the supply of exogenous hormones but also by the developmental fates of adjacent side branches. Thus, the probability of a side branch developing into a bud is greatly increased by the addition of cytokinins, but also by buds developing on side branches adjacent to it on the same filament. As a result, buds tend to be clustered (99).

The pattern of caulonemal side-branch fate in *P. patens* is strongly dependent on light intensity. In darkness, few side branches are produced. At low-light intensities, caulonemal side-branch development is favored, at intermediate intensities almost all side-branch initials remain undivided, and at high intensities most initials develop into secondary chloronemal filaments, with less than 5% becoming either caulonemal filaments or buds (D.J. Cove, unpublished data). Patterning is also influenced by the source of nitrogen in the medium. The basal medium used in most *P. patens* studies is a modified Knop's, and contains nitrate as the nitrogen source. Adding an ammonium salt leads to the culture producing more chloronemata, largely as a result of secondary chloronemal filaments being more branched and containing more cells. The effect of including a sugar in the medium depends on the concentration used. Using 0.15 M glucose in medium containing an ammonium salt leads to an increase in the production of caulonemal filaments (92), but no detailed analysis of patterning has yet been carried out. Including glucose concentrations higher than 0.2 M leads to a general inhibition of growth, and this is likely an osmotic effect, because the nonmetabolizable sugar, mannitol, has a similar effect at the same concentrations (92).

Gametophore and sporophyte development. The development of caulonemal side-branch initials into gametophores is characterized at its first cell division, which occurs diagonally, producing a more apical cell containing many chloroplasts, and a basal cell containing few chloroplasts (99). The apical cell divides further to produce the game-

tophore axis, and division of the basal cell produces most of the rhizoids (99). Treatment with auxin or cytokinin affects this pattern of development. Treatment with 1 μ M benzylaminopurine greatly increases bud production, but the buds produced have no rhizoids and do not develop into gametophores. Treatment with 1 μ M 1-naphthylene acetic acid leads to buds that do not form a gametophore axis, but have abundant rhizoids (4).

A detailed study of the role of auxin in rhizoid production has identified two types of rhizoid, one developing from the base of the gametophore and the other from specific cell lineages in the gametophore epidermis (100). Once an epidermal cell is committed to rhizoid development, expression of a homeodomain-leucine zipper I gene is induced, but knockout of this gene does not affect rhizoid production, suggesting that it functions downstream of the auxin input.

Mutants affected in gametophore development have been isolated following chemical mutagenesis (25) or gene disruption (105). Mutant phenotypes include alterations in leaf shape and leaf number. Gametophore leaves consist of a single cell layer. Although no detailed analysis of their programming has yet been undertaken, they should provide good material for morphogenetic study.

Variations in the distribution of gametangia on gametophores of *P. patens* have been reported, but these studies were carried out on a single strain and so the variations are not genetic (2). Sporophyte production by this line was low, probably as a result of it having been cultured vegetatively for many years (2). No detailed studies have been made of sporophyte development.

Role of the cytoskeleton. Both the microtubule- and actin-based cytoskeletons have been visualized in a number of moss species (34, 36, 48, 69, 85, 95, 119). The extensive gene database now available for *P. patens* contains a relatively large number of genes coding for actin (at least seven) and for beta tubulin (at least six) (65). Both

Sporophyte: the diploid phase of the life cycle, comprising the spore capsule, in which spores are produced by meiosis, borne on a short seta

cytoskeletons appear to be predominately associated with the cortex. Microtubules have been visualized in protonemal tissue by immunofluorescence and microfilaments have been visualized by immunofluorescence or with fluorescent phalloidin (35, 36, 48).

Using immunofluorescence, microtubules have been shown to be oriented along the long axis of the cell with an accumulation at the apex of the tip cell (36, 107). During mitosis, microtubules comprise the spindle that separates the chromosomes, and what appear to be “astral” microtubules may play a role in reorienting the spindle during anaphase. During cytokinesis, microtubules are incorporated into a phragmoplast similar to that observed in other plant cells (35). In the subapical cell, microtubules appear to be preferentially associated with the apical cross wall. It is from this cross wall that the nucleus migrates toward a central position in the subapical cell. When a subapical cell branches, the nucleus migrates toward the site of branch formation. Bundles of microtubules exist between the nucleus and the prospective division site. These become progressively thicker and shorter as the nucleus migrates. Treatment with Cremart, a microtubule destabilizing drug, inhibits nuclear migration and concomitant treatment with taxol, a microtubule stabilizer, relieves this inhibition (37), demonstrating a clear role for microtubules during nuclear migration. Microtubules also appear to play a role in polarizing cells. Treatment with concentrations of Cremart that disorganize the microtubule cytoskeleton can alter the direction of growth away from the apex of the cell. Concentrations of Cremart that completely destroy the microtubule cytoskeleton cause the apical region of the cell to swell, but do not entirely stop tip growth (36).

In contrast, actin filaments, which also appear to be axially oriented along the cortex and concentrated at the apex of the tip cell, are required for tip growth (36). Depolymerization of these apical actin filaments by cytochalasin D inhibits tip growth (36).

A recent study shows the importance of the actin cytoskeleton in tip growth by examining the role of the actin filament-nucleating Arp2/3 complex. This study employed RNA interference (RNAi) to reduce the function of the ARPC1 subunit of the Arp2/3 complex in *P. patens* (54). Deregulation of proper actin dynamics through perturbation of the Arp2/3 complex function has dramatic effects on tip growth, polar outgrowth, and cell differentiation. *P. patens* lines containing an RNAi construct targeting degradation of ARPC1 have multiple defects. During protoplast regeneration, *arp1-rnai* plants are unable to establish a polar axis, although they eventually begin to grow chloronemal filaments of abnormal size and shape from an unpolarized group of cells. These phenotypes can be mimicked by treatment with the actin depolymerization drug latrunculin B. The protonemal filaments that do grow remain chloronemal in character. There are no apparent caulonemal cells and thus no gametophore formation in *arp1-rnai* plants, even in response to cytokinin treatment (54).

Both the microtubule and actin cytoskeletons appear to play a role in chloroplast photorelocation (101). *P. patens* chloroplasts accumulate in low-fluence red and blue light, and avoid high-fluence red and blue light (66). Chloroplast photorelocation was studied in the presence/absence of drugs that affect the microtubule and actin cytoskeletons. The red-light response is mediated only by microtubules and not actin. However, the blue-light response is mediated by both microtubules and actin. The rate of microtubule-driven movement (around 2.5 $\mu\text{m}/\text{min}$), measured using the blue-light response and drugs that selectively inhibit one of the cytoskeletal networks, is five times faster than actin-driven movement (101).

In addition to movement in response to light signals, chloroplasts also move in response to mechanical perturbation of the cell, assembling at the pressure point. This movement depends on microtubules and calcium. This is in contrast to chloroplast

photorelocation, which does not depend on calcium (102). The mechanism behind chloroplast movement remains unclear, although initial studies indicate that the actin-based movements in photorelocation do not appear to depend on class VIII myosins (M.

Bezanilla & Y. Sato, unpublished data). Future studies, taking advantage of the tools available for studies in *P. patens*, should elucidate whether this type of motility is driven by cytoskeletal motors or by the dynamic character of actin and microtubules.

SUMMARY POINTS

1. The ability to grow on simple liquid or solid media allows metabolic studies to be carried out, similar to those used with micro-organisms.
2. Responsiveness to the plant hormones, auxin, cytokinin, and abscisic acid enables investigation of their synthesis and mode of action.
3. The accessibility of living cells to direct observation allows unrivaled opportunities for cell biological research.
4. The simple pattern of development, with few cell types, allows detailed study of morphogenesis.
5. The effects of environmental inputs on polarity can be studied in both single cells and multicellular structures.
6. The involvement of the actin and microtubule cytoskeleton in the programming of cell shape can be studied using gene knockout and RNAi technologies.

UNRESOLVED ISSUES AND FUTURE DIRECTIONS

1. The link between mutant phenotype and genes is still often obscure.
2. A publically available forward genetic resource of tagged lines is required to enable genes associated with specific phenotypes to be isolated.
3. An international stock center is now required.

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