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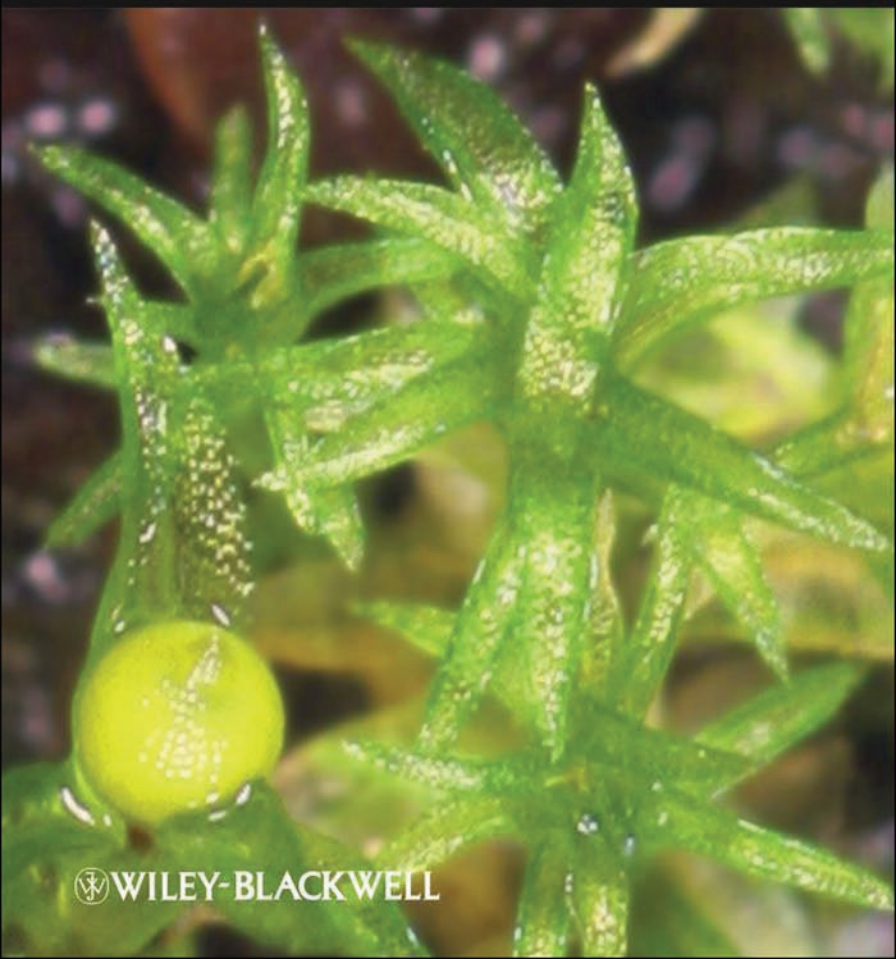
The Moss Physcomitrella

Edited by Celia Knight, Pierre-Francois Perroud and David Cove



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The Moss *Physcomitrella patens*

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PREFACE

When we received the invitation to write a book on *Physcomitrella patens*, while it was yet another call on our time, the decision was in fact easily made. All of us have been committed to showing the value of *P. patens* as a model organism for most of our scientific careers and for the three of us this covers over 70 years (one of us contributes 38 years to the total).

This is a timely publication. The selection of *P. patens* as the first non-angiosperm land plant to have its genome sequenced and the reporting of the sequence in Science in 2008 as part of an international collaboration are amongst the best of recent significant advances in biological research.

We are fortunate to know the moss research community and the selection of chapters and authors was relatively easy. Some of them come from a long history of moss research, others attracted to the system have learned of its utility by laboratories willingly sharing their expertise or by attending practical workshops such as those held in Leeds in the late 1990s. It was in 1999 that the decision was made to meet annually and conferences have since seen a steady increase in new interest and international collaborations.

The chapters included in this book cover a broad range, from those using *P. patens* as a marker against other species for evolutionary or ecological studies (Chapters 1, 2 and 3), to those investigating the unique features of *P. patens*, for example gene targeting (Chapter 4), to those using moss, either as a comparator for other organisms or because of the ease of study in moss, to investigate standard biological processes (Chapters 5–12).

The chapters speak for themselves; however, in bringing them together, there are some general comments to make. The first is one of nomenclature. As described in the first sentence of Chapter 1, *Physcomitrella patens* is sometimes known as *Aphanorrhegma patens.* The difference, according to Brent Mishler, is a matter of opinion as to whether *Physcomitrella* is considered a different genus to *Aphanorrhegma*, in which case *Physcomitrella* is the correct name, but if the two are considered as the same genus, then *Aphanorrhegma* is the correct name. Most taxonomists believe that they are different genera. Of course, much of the research referred to in this book may eventually be able to provide a definitive answer, but in the meantime, we will only refer to this moss as *Physcomitrella patens*.

The second general point is one of terminology and particularly whether mosses have leaves. The single-cell layer of cells with a primitive mid-rib is commonly called a leaf but is clearly not equivalent to an angiosperm leaf. Phyllode is sometimes used as an alternative for leaves but does not meet the exact needs of the bryophytes. The proper word to describe the leaves

of bryophytes would be phyllid, but it is very rarely used in the botanical community. Hence, for the present book, we decided to use leaf or leaves for the sake of clarity, but as more researchers use moss for functional genomic and phenotypic analysis, we should at least debate whether a more specific term is needed. We have also included a glossary which includes terms used within the book which are bryophyte specific. Given the broad nature of disciplines that have contributed to this book, the glossary also includes some general terms which we hope will be useful to a wide audience.

To tackle global issues such as world food security and the impact of climate change, plant science research will increasingly focus on understanding how plants deal with environmental stress. As a bryophyte, *P. patens* represents a group of organisms that survive the stress of living on land with few structural adaptations to drought. However, bryophytes share physiological similarities with angiosperms. Because of its high frequency of gene targeting and the haploidy of its somatic tissues, analysis of gene function in *P. patens* can be more direct and informative than that in angiosperms. These attributes of *P. patens* complement other plant models and we hope this book will inform and inspire more to consider whether *P. patens* can help answer their questions.

We thank all of the authors for their excellent contributions and the staff at Wiley-Blackwell for realising this publication.

Celia Knight
Pierre-François Perroud
David Cove

GLOSSARY

The terms defined here are those used within this book. See also Glossarium Polyglottum Bryoligiae a multilingual glossary for bryology. R.E. Magill, Editor 1990 Missouri Botanical Garden.

Acrocarpous Having archegonia (q.v.) borne at the tip of gametophores. Gametophores are generally upright (cf. pleurocarpus). Species having this arrangement include *Physcomitrella patens* and *Ceratodon purpureus.*

Amphithecium The outer layer of cells of the sporangium (q.v.); cf. endothecium.

Antheridium (pl. antheridia) A male gametangium.

Archegonium (pl. archegonia) A female gametangium.

Arthrodontous A type of peristome (q.v.) that consists of one or two rings of teeth (cf. nematodontous). Many of the major moss model species have this type of peristome.

Brachycyte Drought-tolerant cell with thick cell wall developed in protonemata under stress (also called brood cells). A type of gemma.

Brood cells See Brachycyte.

Calyptra A cap on the sporophyte, formed from the archegonium (q.v.) by mitotic divisions.

Caulonema (pl. caulonemata) The adventitous filaments of the protonemal stage of gametophyte development. Compared to chloronemal filaments (q.v.), the cells of *P. patens* caulonemal filaments contain only fewer, less well developed chloroplasts. The cross walls of adjacent cells in caulonemal filaments are oblique to the filament axis.

Chloronema (pl. chloronemata) The assimilatory filaments of the protonemal stage of gametophyte development. Compared to caulonemal filaments (q.v.), the cells of *P. patens* chloronemal filaments contain many well developed chloroplasts. The cross walls of adjacent cells in chloronemal filaments are perpendicular to the filament axis.

Columella The central portion of the sporangium (q.v.).

Crown eukaryotes The six major clades of eukaryotes (green plants, animals, fungi, red algae, stramenopiles and alveolates.) See Knoll, A.H. (1992). The

early evolution of eukaryotes: a geological perspective. *Science*, **256**, 622–627 and http://www.tolweb.org/Eukaryotes/3.

Dioecious Having sporophytes that produce either megaspores or microspores.

Dioicous Having gametophytes that produce either female or male gametangia. The male and female gametophytes of dioicous species are often morphologically differentiated.

Diplolepideous A peristome characterized by two rows of teeth.

Diplolepideous-alternate A diplolepideous (q.v.) peristome with an interior ring of teeth (endostome) that alternate with the exterior (exostome) row of teeth. Species having this peristome structure include *Bryum argenteum.*

Diplolepideous-opposite A diplolepideous (q.v.) peristome with an interior ring of teeth (endostome) that are placed directly behind the exterior (exostome) row of teeth. Species having this peristome structure include *Funaria hygrometrica* and *Physcomitrium pyriforme.*

Embryophytes Plants having gametangia containing somatic cells. Most embryophytes are terrestrial.

Endohydric See Homeohydric.

Endothecium The inner layers of a sporangium (q.v.); cf. Amphithecium.

Ephemeral Having a short life cycle, characteristic of many 'weeds'.

Gametophore The leafy shoots that bear gametangia.

Gametophyte The haploid generation of plants having a life cycle showing alternation of generations. Gametophytes produce gametes mitotically.

GO Gene ontology; a compendium of hierarchical, inter-related functional descriptions for genes.

Green lineage The lineage of plants having chlorophyll a/b. Contains Viridiplantae, i.e. Chlorophyta (green algae) and Streptophyta (plants in the narrow sense and charophyceaen algae).

Haplolepideous A peristome (q.v.) characterized by a single row of teeth. Species having this peristome structure include *Ceratodon purpureus* and *Tortula ruralis.*

Heteroblastic An organ originating from multiple tissue layers.

Homeohydric A plant able to regulate its water content by means of structures such as a cuticle, stomata, roots etc.

Hydric A habitat having an abundant supply of water, but which is not flooded.

KEGG Kyoto Encyclopedia of Genes and Genomes; an annotation relating genes to biochemical pathways based on sequence homology.

Mesic A habitat having an adequate supply of water.

Monoecious Having a sporophyte that produces both microspores (which develop to form pollen grains containing male gametes) and megaspores (which develop to form embryo sacs containing the female gamete).

Monoicous Having a gametophyte that bears both female and male gametangia.

Nematodontous A type of peristome (q.v.) having teeth fused to a plate (epiphragum) forming a salt shaker-like ring of pores (cf. Arthrodontous).

Operculum The 'lid' of the sporophyte spore capsule through which spores are released. The *P. patens* sporophyte does not have an operculum.

Peristome Structure on spore capsule regulating the release of spores. The peristomes of some species are elaborate, but the *P. patens* sporophyte has no peristome. Peristome architecture has been used to distinguish major lineages of mosses, but loss of the peristome has occurred multiple times in moss evolution. (cf. Arthrodontous, Haplolepideous, Diplolepideous, Nematodontous).

Phenology The scientific study of cyclical biological events, such as flowering, breeding, and migration, in relation to climatic conditions. Phenological records of the dates on which seasonal phenomena occur provide important information on how climate change affects ecosystems over time.

Pleurocarpous Having gametangia borne on side branches of the gametophore. Gametophores are usually prostrate (cf. Acrocarpous).

Poikilohydric A plant, the water content of which, is directly adjusted to the water content of the environment that surrounds it.

Prasinophytes Flagellate green algal lineage, sister to the land plants and associated green algae.

Protonema (pl. protonemata) The filamentous stage of gametophyte development. Protonemal tissue is produced following spore germination or the regeneration of most tissues (whether gametophytic or sporophytic). Protonemal filaments comprise two cell types, caulonema (q.v.) and chloronema (q.v.). Both types of filament extend by the serial division of their apical cells. Sub-apical cells may branch.

Red lineage The Rhodophyta (red algae). Together with the green lineage they comprise the Viridiplantae (plants in the wide sense).

Rhizoid Filament that develops from a gametophore. In *P. patens*, the principal function of rhizoids is thought to be mechanical, anchoring and orienting gametophores.

Serpentine soils Soils that, because of their origin, are poor in some essential nutrients and may contain high concentrations of heavy metals.

Seta the stalk portion of a sporophyte. The seta of *P. patens* sporophytes is shorter than most other moss species.

Sporangium The spherical spore-containing part of a sporophyte.

Sporophyte The diploid generation of plants having a life cycle showing alternation of generations. Sporophytes produce spores meiotically.

SSR Simple Sequence Repeat; a short stretch of low complexity repeat, often used to amplify polymorphic marker regions.

Tmema (pl. tmemata) Specialized protonemal cells that develop in old protonema on either side of a brachycyte (q.v.). They undergo programmed cell death, allowing the release of the brachycyte.

Tracheophytes Plants having specialized lignified tissue for the long-distance conduction of solutes.

Vagility (Of plant species) Having a tendency to disperse and establish in a given environment.

Xeric Adapted to very dry habitats.

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Chapter 1

PUTTING *PHYSCOMITRELLA PATENS* ON THE TREE OF LIFE: THE EVOLUTION AND ECOLOGY OF MOSSES

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Abstract: *Physcomitrella patens* is an important model system for studies of genetics and physiology, and with its newly sequenced genome, it is perfectly placed phylogenetically to serve as a point of comparison for angiosperms. This chapter addresses three main questions: (1) How typical of a moss is *P. patens*? It is rather atypical, given the reasons that it was selected as a model system, such as its rapid life cycle and reduced morphology, yet it is representative in many ways. (2) Where does it belong in the phylogenetic history of land plants? The mosses are monophyletic, and share a common ancestor with hornworts and tracheophytes, with *P. patens* nesting within the 'true mosses'. (3) What are the special attributes of moss ecology and evolution that can lend special interest to the study of *P. patens*? When comparing *P. patens* with tracheophytes, it is important to understand both its similarities and differences with its larger cousins – we discuss how many processes influencing their physiology, ecology and evolutionary diversification seem to be quite different from the tracheophytes.

Keywords: desiccation tolerance; ecology; evolution; mosses; phylogeny; *Physcomitrella*

1.1 Introduction to *Physcomitrella*

The moss *Physcomitrella patens* (Hedw.) Bruch & Schimp. (sometimes called *Aphanorrhegma patens*) is becoming widely recognized as an experimental model of choice not only for basic molecular, cytological and developmental questions in plant biology, but also as a key link in understanding questions

in plant phylogeny, most recently those related to genome evolution. *P. patens* is well placed phylogenetically to provide important comparisons with the flowering plants. In terms of evolutionary distance, *P. patens* is to the flowering plants as *Drosophila melanogaster* is to humans! The completion of the sequencing of the *P. patens* genome (Rensing et al., 2008) allows bioinformatic and functional genomic comparisons to be carried out in plants, just as the *Mus musculus*, *Fugu rubripes*, *D. melanogaster* and *Caenorhabditis elegans* genomes have informed animal biology.

An increasing number of laboratories worldwide are studying this moss. *P. patens* has been developed into an important model for investigating gene function (Reski, 1998, 1999; Cove, 2000). A large reason for its growing popularity lies in the fact that it can be cultured rapidly on a simple medium containing only inorganic salts, its life cycle can be completed in culture in about 3 months, and since the dominant phase of the life cycle, the gametophyte, is haploid, mutant phenotypes can be observed directly in progeny. The simple morphology allows direct observation of intracellular processes involved in morphogenesis. Sexual crossing, somatic hybridization and the generation of transgenic plants are now routine.

The utility of *P. patens* is reinforced, since in contrast to vascular plants, transforming DNA containing a genomic sequence targets the corresponding sequence in the genome at very high frequency (see Chapter 4). The high frequency of gene targeting allows disruption and/or replacement of genes as efficiently as *Saccharomyces cerevisiae* and orders of magnitude more efficiently than any other plant system. *P. patens* is thus the first land plant that allows easy targeting of genes for replacement and elimination.

The increasing popularity of *P. patens* as a study system has focused new attention on the mosses, a diverse yet relatively unknown group of plants. To use this model properly to study general questions in plant biology, it is important to address several questions: How typical of a moss is *P. patens*? Where does it belong in the phylogenetic history of land plants? What are the special attributes of moss ecology and evolution that can lend special interest to the study of *P. patens*? Such questions will be addressed in the following sections of this chapter.

1.2 The position of *P. patens* on the Tree of Life

P. patens is a green plant, thus belonging to one of the six major clades of crown eukaryotes (the other five are: animals, fungi, red plants, stramenopiles and alveolates; Mishler, 2000). More specifically, *P. patens* is a land plant, and even more specifically, it is a moss. Our understanding of its precise position in this deeply nested phylogenetic hierarchy has been greatly enhanced over the last 15 years. Results to date suggest that the green plants appear to be composed of two major lineages and a residuum of unicellular prasinophytes (see Figure 1.1. For an elaborate tree shown in hyperbolic space, see

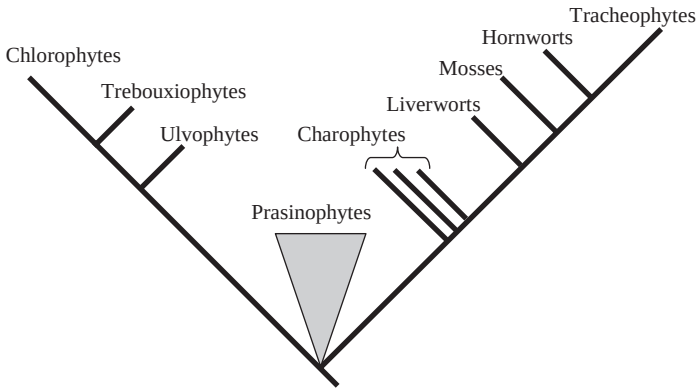

Figure 1.1 A summation of the currently hypothesized cladistic relationships of green plants. *Chlamydomonas* and *Volvox* are members of the farthest left clade, while flowering plants are nested well up in the farthest right clade. The marine green algae are in the Ulvophytes. The prasinophytes are a residual group of unicells whose relationships are not yet clear. The charophytes are a paraphyletic assemblage of land plant relatives that include *Chara*, *Coleochaete* and *Spirogyra*. *P. patens* is a moss.

`http://ucjeps.berkeley.edu/TreeofLife/hyperbolic.php`). One of these major lineages contains the bulk of the classical green algae (Chlorophyceae, Trebouxiophyceae and Ulvophyceae), including all the marine forms. The other contains the embryophytes (land plants) plus some of the former green algae, such as *Spirogyra* and *Chara*. Within the embryophytes, there are four major lineages, that is, liverworts, hornworts, mosses and the tracheophytes (vascular plants); the first three are collectively called bryophytes.

The relationships of these four major groups to one another are not fully agreed upon. Virtually all of the recently published studies agree that the traditional group 'bryophytes' is paraphyletic (i.e. this group does not contain all the descendants of its common ancestor since the tracheophytes are left out), and that the basal split in the embryophytes is between the liverworts and the rest. The relative positions of hornworts and mosses are more controversial, but most recent studies suggest that the mosses diverged first, and thus the hornworts and tracheophytes are sister groups (Kelch et al., 2004; Qiu et al., 2006). Considerable effort is now being expended to better resolve these relationships and other uncertain nodes in the land plant phylogeny (i.e. the Green Tree of Life Project funded by the National Science Foundation Assembling the Tree of Life (AToL) program; see `http://ucjeps.berkeley.edu/TreeofLife/`).

Within the tracheophytes, the lycophytes (club mosses) are sister to all other living tracheophytes, and within the tracheophytes, ferns and their allies are sister to the seed plants (Pryer et al., 2001). Within the seed plants, morphological and molecular data currently provide conflicting topologies for the five extant lineages (cycads, *Ginkgo*, conifers, Gnetales and angiosperms).

Further work is underway to resolve relationships among lineages of seed plants, also funded by the NSF AToL program (see `http://www.huh.harvard.edu/research/mathews-lab/atolHtmlSite/` and `http://www.flmnh.ufl.edu/angiospermATOL/`).

The rapidly developing green plant phylogeny described above can serve as a framework for evolutionary interpretations (Mishler, 2000). For example, it appears to support reasonably well that multicellularity arose at least twice in the green plants. The diversification of life-history strategies is becoming clearer; from a primitively haploid-dominant life cycle, both alternation of generations and diploid-dominant life cycles arose at least twice. The habitat transition in the movement of plants to land was from fresh water, not from salt water. Within the land plants, several morphological transformations can be reasonably postulated at present, such as the multiple elaborations of the gametophyte and sporophyte in all four main lineages, the radiation of types of conducting cells, and the single origin of a branched, multisporangiate sporophyte from unbranched, unisporangiate ones in the tracheophytes. We can also begin to understand genome evolution in the same manner, once major gaps in the phylogenetic coverage among green plants are filled. *P. patens* is placed in a strategic position in the phylogeny of green plants to provide insight into such topics.

1.3 Relationships within the mosses

Phylogenetic relationships are becoming well supported within the moss clade (see Figure 1.2; Newton et al., 2000; Cox et al., 2004). The first major phylogenetic split within the mosses is *Sphagnum* versus the remainder of mosses. The enigmatic *Takakia*, only recently discovered to be a moss rather than a liverwort (Smith and Davison, 1993), is still of uncertain placement, either as sister to *Sphagnum*, or diverging earliest among extant mosses. The next major split involves the divergence of the Andreales from the main line, followed by the Polytrichales. The 'true mosses' (see arrow 1 in Figure 1.2), that is, those mosses with articulated and hygroscopic peristomes are the most diverse group in terms of number of species. Within this group, the largest clade (see arrow 2 in Figure 1.2) is the group with alternate peristomial formulae (i.e. *Timmia* and the Haplolepideae, plus the diplolepideous-alternate mosses including the pleurocarps).

The sister to this group is the Funariales, where *P. patens* is located (shown with an asterisk in Figure 1.2). The Funariales have a diplolepideous-opposite peristomial formula (Goffinet et al., 1999), and a generalized leaf form with broad leaves and rectangular leaf cells. Interestingly, this leaf form is characteristic of juvenile leaves in the heteroblastic series of all the mosses (Mishler, 1988), perhaps indicating that it represents the primitive form from which the many diverse types of moss leaf morphology have evolved. Unlike many mosses, species within the Funariales often have an annual life history, with

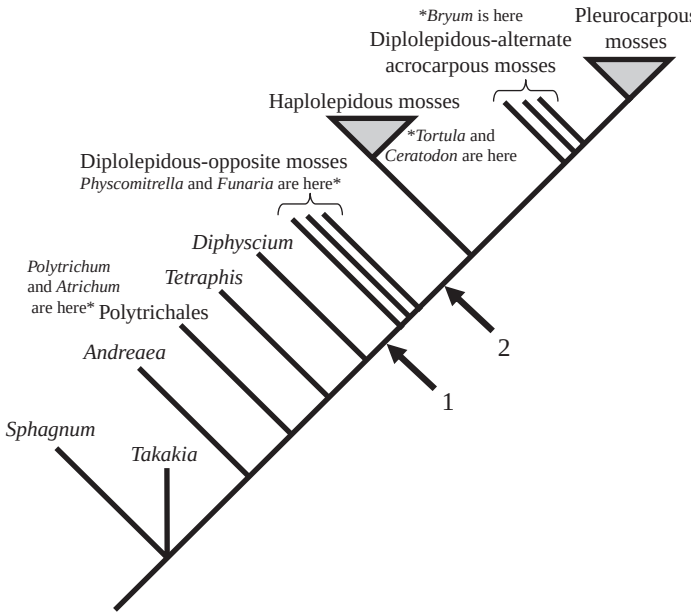

Figure 1.2 A simplified phylogeny of the mosses. Arrow 1 shows the mosses with a true hygroscopic peristome. The diplolepideous-opposite peristomial arrangement is primitive; arrow 2 shows where the alternate peristomial formula evolves, uniting the majority of moss species. See the text for further explanation.

P. patens showing an extreme version. Many species within the Funariales have a truncated sporophyte development, often lacking a peristome and operculum entirely as in *P. patens.*

This topology can be used to study evolutionary transitions within the mosses, although a considerable variety of ecologies characterize the basal clades making inferences difficult. *Sphagnum* is an extreme aquatic, likely with a highly derived morphology. The Andreales are highly desiccation-tolerant rock dwellers, and the Polytrichales show a variety of life histories ranging from small ephemerals to large perennials. There remains a need for detailed reconstructions of ecology and life history as the moss phylogeny becomes further resolved (e.g. Shaw et al., 2005). In the meantime, based on out-group comparisons, we suggest that the most likely reconstruction of the primitive moss ecology would be something like the growth habit that the Funariales generally show today: modest-sized plants occupying mesic, often ephemeral sites on soil near water.

From this starting point, some lineages evolved to occupy more hydric sites, others much more xeric sites. Accompanying these transitions, a number of specialized morphological and physiological traits evolved in various lineages. Mosses are not a collection of evolutionary dead ends; on the contrary, they are as highly evolved in their ways as the tracheophytes are in

theirs. The ways of mosses are different, not inferior, hence the aversion of moss biologists to hearing their organisms called 'lower plants'!

1.4 Evolution and ecology of the mosses

Mishler (2001) postulated that moss biology is different from that of tracheophytes in most ways, representing alternative strategies to being a land plant that are almost as different from each other as one might expect to see developing on different planets (suggesting that 'mosses are from Mars, vascular plants are from Venus'). We would like to elaborate here on some of the most important factors of this unique biology of mosses. When comparing the model moss *P. patens* with tracheophytes, it is important to understand both its similarities and differences with its larger cousins.

1.4.1 Desiccation tolerance and poikilohydry

As plants transitioned from an aqueous environment to the land, an event that is thought to have occurred from fresh water habitats rather than marine (Mishler and Churchill, 1985), the ability to survive periods of water depletion both from the plant and its surroundings became a critical aspect of their existence. All plants, and for that matter all organisms, require water in order to sustain metabolic activity and survive. Because of the strong selective pressure of dealing with desiccation, Oliver et al. (2000, 2005) postulated that early land plants had to have acquired desiccation tolerance as a heritable trait in order to survive in terrestrial environments. The need for desiccation tolerance was particularly critical for survival on land given that, in all likelihood, the water relations of early land plants were poikilohydric in nature because of their simple, generally one cell thick, architecture, similar to what we see in many modern-day bryophytes.

Poikilohydry is defined as the direct equilibration of a plant's water content to the water content of the environment (generally the air) that surrounds it, and is probably the most important single difference between bryophyte and tracheophyte biology – the latter being termed homeohydric or endohydric because of their ability to internalize water relationships by means of thick cuticles, stomates, efficient conducting tissues and roots. When free water is available, a poikilohydric plant is hydrated and can be physiologically active; when free water is lost, the plant, because it does not have the mechanisms in place to retain water, will equilibrate to the water content of the surrounding air, which is generally low, and thus desiccate. Bryophytes, including *P. patens*, have retained the original life cycle for land plants, with a dominant haploid gametophytic stage where the plants are a few layers thick. This architecture dictated that bryophytes are by nature poikilohydric. Other important biological correlates of this life cycle and poikilohydry are discussed below.

Poikilohydry is not necessarily combined with desiccation tolerance. Some aquatic bryophytes are poikilohydric yet not desiccation tolerant, while a few phylogenetically scattered tracheophytes are desiccation tolerant yet endohydric. However, poikilohydric plants must be desiccation tolerant in all but hydric situations in order to survive.

In order for growth to occur, plants must fix carbon for both structural development and energy storage. Carbon fixation requires that plant cells remain fully hydrated, which in dry environments (i.e. most terrestrial habitats) presents a challenge. In order to access CO_2 from the air, plants must expose themselves to a drying atmosphere with the associated water loss. The larger and more complex tracheophytes have evolved mechanisms to extract water from the environment, principally from the soil via roots, and transport it through the vegetative tissue via tracheids to the organs (leaves) that serve as the site of CO_2 uptake and fixation. Leaves of tracheophytes control the balance between CO_2 uptake and water loss by employing a waxy cuticle that severely limits evaporation from their surfaces and by utilizing a system of controlled openings into the leaf structure (stomates), for passage of gases and water vapor to and from the photosynthetically active cells. The loss of water vapor, that is, transpiration, from the leaves is also necessary, regardless of the risk of dehydration, for survival, as it is the only mechanism the plant has to regulate the temperature of the leaves (by cooling) that would otherwise overheat from the incoming radiant energy from the sun that they have evolved to capture and use. Bryophytes do not have the structural capacity to efficiently transport water internally from the soil to the site of photosynthesis in the leaves, and although some do have thin waxy cuticles to slow evaporation, most rely on the maintenance of a surface film of free water to remain hydrated. Several features of mosses, the most important of which are the establishment of a colonial lifestyle in clumps and the sacrificing of certain cells that act to form external capillaries for movement of water to the active leaf cells, serve to maintain a surface film of water. Consequently, gaseous exchange and carbon fixation in these plants occur primarily within an aqueous environment and thus these plants only grow when free surface water is available. To accommodate this, bryophytes either live in habitats that are essentially always wet or they have to suffer and survive desiccation in the same way as the primitive plants that first invaded the dry land.

While most mosses can tolerate some dehydration, not all are desiccation tolerant. Desiccation tolerance is defined as the ability to survive equilibration to the water content of dry air, and means that the plant can survive, repair and recover from the damage incurred during desiccation or during the inrush of water that occurs when the plant is rehydrated. Note that desiccation tolerance is not at all the same (either at the phenotypic or genotypic level) as 'water stress tolerance' or more correctly 'dehydration tolerance', defined as the ability to survive low water contents.

The difference between desiccation tolerance and dehydration tolerance is illustrated by the desiccation-tolerant moss *Tortula ruralis*. When placed

in an atmosphere that is at a constant 50% relative humidity and at 20°C, *T. ruralis* will equilibrate to the water content of the air approximately 4–5 h after the removal of free water from the surface of the leaves (M.J. Oliver, unpublished). *T. ruralis*, under these conditions, will dehydrate to a water content of close to 100 mg H_2O g^{-1} dry mass and a water potential of −100 MPa (Gaff, 1997), and yet still be able to recover when rewetted. By contrast, although *P. patens* does not tolerate desiccation under normal circumstances and is thus considered a desiccation-sensitive plant. *P. patens* is capable of surviving severe dehydration, reported as the loss of 95% of its fresh weight, for short periods of time (Frank et al., 2005).

The ability to tolerate severe dehydration, while not tolerating desiccation, appears to be common in mesic bryophytes. Interestingly, for the few mesic species that have been investigated, equilibration to water potentials of −100 MPa and lower (which would constitute true desiccation tolerance) can be achieved by the application of exogenous abscisic acid (ABA) (Alpert and Oliver, 2002; Oliver, 2007). For example, protonemal cultures of the mesic moss *Funaria hygrometrica* can survive dehydration to approximately −70 MPa if water loss is slow but cannot survive if water loss is rapid (Werner et al., 1991). Pre-treatment of the protonemata of this moss with ABA enables them to survive rapid desiccation rates and this alteration in phenotype is associated with the accumulation of specific proteins (Bopp and Werner, 1993), some of which resemble the dehydrins known from desiccation-tolerant species such as *T. ruralis* (Velten and Oliver, 2001). ABA treatment of the moss *Atrichum androgynum* results in a desiccation-tolerant phenotype and a significant reduction in dehydration-induced cellular leakage, indicative of an increase in membrane stability (Beckett, 1999). The effect of ABA on the membrane integrity of *A. androgynum* has been investigated further, and although there was no specific effect, ABA treatment does reduce the overall level of dehydration-induced alteration in lipid membrane components (Guschina et al., 2002). The implication of these studies is that ABA induces transcriptional changes that direct the synthesis of protective cellular components that increase the ability of these plants to survive severe dehydration; dehydrins appear to be a major aspect of this response (see Saavedra et al., 2006; for more details, see Chapter 11). Exploration of the evolutionary, ecological and genomic links between dehydration tolerance (water-deficit stress) and desiccation tolerance presents an exciting area for future studies.

The link between water and carbon fixation, as it relates to growth, can be best summarized by the Integrated Water-driven Carbon Budget (IWCB) model, as described in Figure 1.3. This model seeks to describe the relationship between water and carbon fixation for all plants, regardless of their mechanism for dealing with life on land and maintaining growth under conditions of limiting water resources. It also offers a way to quantify this relationship and establish a means by which species can be directly compared with regards to their ability to tolerate dehydration. The IWCB model describes the

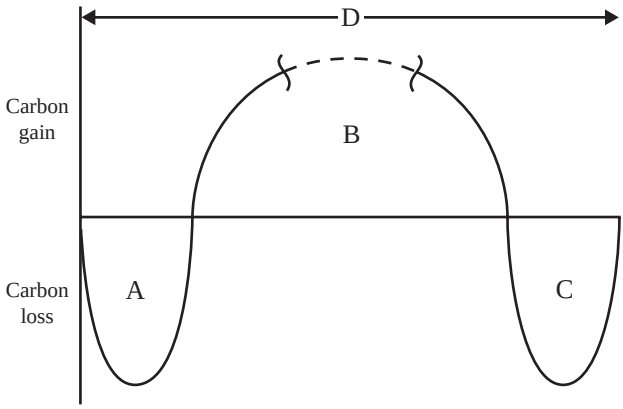

Figure 1.3 Integrated water-driven carbon budget (IWCB) model. This diagram represents the events following dryness until the next period of dryness, that is, one dry–wet–dry cycle (of time D) in the life of a plant. The dried plant is assumed to have a zero carbon balance as metabolism has ceased. When water is first added, there is an initial phase of net carbon loss (A). Then, as the plant repairs itself and begins photosynthesis, the compensation point is eventually reached, and the plant enters a phase of net carbon gain (B). Finally, as the plant begins to dry the curve starts to turn down since photosynthesis is limited, and the plant enters a second phase of net carbon loss (C), eventually drying completely. The net carbon gain for a given amount of water is the area under the curve in B minus the area under the curve in A+C. Plants can vary in many ways under this model; shapes of the curves for different plants can differ in each of these areas.

amount of carbon fixed for a given amount of water during one dry–wet–dry cycle within the lifetime of a plant. The traditional term 'water use efficiency' (WUE) only applies to the ability of a plant to fix a certain amount of carbon per unit water in phase B. Thus, the IWCB model is a broader measure of the overall water relationships of a plant than WUE. Although this model applies to the IWCB for all plants, it more completely describes the relationship between net carbon fixation and water use for poikilohydric plants, and in particular those plants that are desiccation tolerant. Endohydric plants (e.g. the majority of the angiosperms), those that are desiccation sensitive and have the capability to maintain the hydrated condition, spend their whole photosynthetic lifetime in phase B of the IWCB model. Desiccation-tolerant plants, whether poikilohydric or not, will experience many of the dry–wet–dry cycles within their normal life spans, many years in the case of most bryophytes. *P. patens* is, however, capable of surviving severe dehydration, reported as the loss of 95% of its fresh weight, for short periods of time (Frank et al., 2005). If this occurs under natural conditions, perhaps allowing the plant to survive brief periods of drying, the IWCB would reflect brief periods in both A and C stages of the dry–wet–dry cycle.

1.4.2 Reproductive biology

All bryophytes need free water for sexual reproduction as well as for metabolism. This is another residual feature remaining from the early land plants, a constraint imposed by swimming sperm inherited from their aquatic ancestors among the green algae. Swimming gametes have relatively short dispersal distances as compared to pollen grains, which presumably lead to frequent inbreeding in monoicous species (i.e. those bearing male and female gametangia on the same gametophyte) and potentially decreased sporophyte production in dioicous species (i.e. those bearing male and female gametangia on different gametophytes).

Due to the difficulty of achieving fertilization in the terrestrial environment, many bryophytes seldom reproduce sexually and some may even have evolutionarily lost functional sexuality. For example, a significant proportion (about 9%; Lane, 1985) of moss species in eastern North America have never been observed to produce a sporophyte. There is presumably a heavy reliance on asexual reproduction in many species; many types of specialized propagules are found in different species (Newton and Mishler, 1994), and all organs of a moss can serve as propagules by regenerating protonemata. Since mosses that are dioicous have a special problem with effecting fertilization, they appear to have more frequently evolved specialized asexual propagules (Mishler, 1990). Even in monoicous species such as *P. patens*, which frequently produce sporophytes in nature, it is important to keep in mind that the great majority of these sporophytes likely result from self-fertilization. In a haploid, selfing has very different implications from those in diploids where the result is complete homozygosity. Thus, spores from selfed sporophytes are genetically asexual propagules, perhaps explaining why monoicous taxa seem to have evolved specialized asexual propagules less often than dioicous taxa (Mishler, 1990).

1.4.3 Population biology

Many mosses are predominantly clonal because of several fundamental aspects of their biology. First, uniquely among land plants, they develop many stems from a single radiating protonemal system (derived from a single spore). Second, their various modes of branching, and persistence of older branches, mean that they expand clonally in a growth form characteristic of a species. Third, rampant asexual reproduction means that clones spread near and far. Newton and Mishler (1994) pointed out that the available evidence suggests that most new establishment events, especially close to the parent plant in crowded situations, are due to asexual propagules. They suggested that the ecological role of spores might be in longer distance dispersal and rare colonization of bare habitats.

Unlike tracheophytes, the green, vegetative part of the life cycle in bryophytes is haploid. Haploid dominance in the life cycle has major

implications. As described above, self-fertilization results in spores that are genetically similar to asexual propagules. Furthermore, without the genetic benefits of dominance, genes acting in the gametophyte are presumably subject to relatively severe selection. Haploidy plus clonal growth might suggest that levels of genetic variation within populations should be low. Some studies suggest this is the case (e.g. Shaw et al., 2002); however, other studies show significant variation within populations (McDaniel and Shaw, 2005). Since bryophytes grow from an apical cell, somatic mutation allows genetic variation even within clones (Klekowski, 1988), and indeed appreciable levels of genetic variation have been shown within and among populations of asexual species (i.e. those in which sporophytes have not been seen in nature).

Bryophytes tend to have very high amounts of morphological and physiological plasticity. Interestingly, while there appears to be considerable genetic variation within many bryophyte species, few examples of ecotypic differentiation (i.e. genetically based, locally adaptive variation) have been found in mosses to date, although more studies are needed (see Chapter 2). For example, the cosmopolitan moss *Bryum argenteum* has very similar physiological tolerances in the tropics as in the Antarctic (Longton, 1981), indicating that phenotypic plasticity can allow occupation of a broad range of environments. Studies of physiological variation within and among species of mosses present an exciting area for future research. Many genotypes of *P. patens* have been gathered from around the world and are now in culture in Freiburg (see `http://www.cosmoss.org/ecomap.content#`); these provide a rich source of material for such studies.

1.4.4 Ecology

Poikilohydry has a number of ecological correlates in addition to those discussed above. One is acute sensitivity to the chemical environment surrounding the plant, unlike anything seen in a rooted plant which brings its water to vegetative cells from below in the soil. Since water is crossing directly into the vegetative cells of mosses across the cell membrane, chemical entities in that water tend to enter the cell as well, and vice versa as the cell dries out and loses water into the immediate environment. Thus, mosses are quite sensitive to ions in the environment and are good ecological indicators of their substrate (e.g. pH, amount of calcium in a rock, or bark chemistry of a tree). Mosses are also sensitive to a range of pollutants, showing the impact of air pollution before endohydric plants. For example, the sulfur content of mosses is an exact indicator of SO_2 pollution levels in the air and thus can be used to monitor pollution from factories (de Caritat et al., 1997). Conversely, as the moss dries out, it loses cellular components to the environment, and this is thought to facilitate growth of symbiotic microbes, of which mosses appear to have many.

The combination of clonal growth with poikilohydry and external water holding and conduction means that we can best view most mosses as

essentially social organisms. The moss clump is the unit that interacts with the environment; the plants in a clump are subject to natural selection as a group. The moss clump is thus a 'super-organism' somewhat like a beehive. Intimate contact of each vegetative cell with the environment, due to poikilohydry, lends itself to interplant chemical communication via pheromones. Such communication has been suggested to explain the dwarfing of males in a number of dioicous mosses, giving them a special breeding system, and to explain the complete lack of germination of spores frequently seen within mature moss clumps (Mishler and Newton, 1988).

Mosses occupy microhabitats, which has many implications. Their small size, lack of roots and poikilohydry mean that mosses are in a close relationship with only their immediate microenvironment. Over a short time scale, this means that a suitable habitat for a particular moss species is likely to exist in most macro-environments. Over geological time, mosses may thus be less influenced by climatic change than the larger tracheophytes, and linger in refugial habitats along with other small organisms.

Establishment abilities of mosses seem relatively poor. They appear to be very slow to establish in available nearby habitats, thus available substrates are not filled in most mesic and xeric environments (although they may be in some hydric environments such as bogs). The few studies that have been aimed at a demonstration of competition among dry-land mosses suggest that instead of the expected negative effects of crowding, the presence of other moss species may enhance growth because of an increased water-holding capacity (e.g. McAlister, 1995). The main selective factors seem to be the ability of propagules to survive the physical environmental stresses and establish a new clump. Herbivory appears relatively low on bryophytes. Together, these observations suggest that for many mosses, there is less selection pressure from the biotic component of the environment than from the physical component, which again is at odds with the prevailing picture of tracheophyte ecology.

1.5 General implications for evolutionary processes in mosses

There is no evidence that mosses have slow rates of evolution at the molecular level, or at the physiological level. However, there does appear to be relatively slow evolutionary rates in morphology in mosses; their fossil record indicates that ancient forms are very similar to modern ones, and cryptic species are present, some of them apparently quite old (McDaniel and Shaw, 2003). This may indicate that developmental constraints play an unusually important role, and suggest that studies of evolution and development in mosses would be very productive to pursue in the future.

Biogeographically, bryophytes tend to follow the same historical patterns of disjunction as tracheophytes, but at a lower taxonomic level. In other words, a moss species often has a distribution historically comparable to an

angiosperm genus or family. There are also more molecular differences among geographically disjunct populations in moss species than one generally sees in tracheophyte species (Shaw, 2001). These recorded differences are probably the result of a difference in species concept held by those investigating the two groups. Bryologists tend to be conservative in naming species, only naming those with distinct morphological differences. Because of the relatively slow rate of morphological change, currently recognized moss species are thus undoubtedly older than many angiosperm species. It is likely that if the species level was applied in mosses at the same age level as it is in angiosperms, there might be as many species of mosses as angiosperms! Future research on mosses needs to continue probing into fine-scale phylogeny while looking more at physiological and developmental variation in order to explain their diversification. Like many other areas of moss biology, the processes influencing their evolutionary diversification seem to be quite different from the tracheophytes. Moss biologists need to declare independence from concepts imported from angiosperm and animal biology; these little plants are in a world of their own!

Acknowledgments

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Chapter 2

THE GENETIC BASIS OF NATURAL VARIATION IN BRYOPHYTE MODEL SYSTEMS

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Abstract: Natural phenotypic variation is widely documented within and among populations of bryophyte species. The tools necessary to identify the molecular genetic basis of this variation are now well developed in a few bryophyte model systems. Here, I review evidence for experimentally verified genetic variation in heavy metal tolerance, mineral nutrition, gametophyte growth and sexual dimorphism in four bryophyte model systems: the mosses *Ceratodon purpureus*, *Funaria hygrometrica*, *Physcomitrella patens* and the liverwort *Marchantia polymorpha*. Although classical genetic experiments needed to identify the basis of this variation have not been widely utilized among bryophyte researchers, genetic maps have recently been published for *C. purpureus* and *P. patens*. Bryophytes are well suited for such studies because a large number of recombinants can be generated with a single cross. Additionally, targeted allele replacement is a powerful technique for testing candidate genes identified by map-based cloning. However, additional techniques, such as association mapping or combining whole-genome expression studies with genetic mapping, may also facilitate testing candidate genes.

Keywords: association mapping; bryophyte breeding system; natural variation; population genetics; quantitative trait locus mapping

Abbreviations:

AFLP	amplified fragment length polymorphism
BAC	bacterial artificial chromosome
PCR	polymerase chain reaction
QTL	quantitative trait locus
SNP	single nucleotide polymorphism
SSR	simple sequence repeat

2.1 Introduction

Natural phenotypic variation among wild isolates represents a rich and largely understudied source of novel molecular variants (Salvi and Tuberosa, 2005; Weigel and Nordborg, 2005). The purpose of this chapter is to review recent literature pertaining to natural variation within and among moss and liverwort populations and attempts to identify the genetic basis of that variation and promising traits for future investigation. I focus on four species where both experimental evidence confirms that trait variation has a genetic basis, and sufficient genomic tools exist to facilitate progress toward identifying the underlying genetic variants: the moss *Ceratodon purpureus*; the moss *Funaria hygrometrica* and the closely related species *Physcomitrella patens*; and the liverwort *Marchantia polymorpha*. I will briefly mention other moss model systems where the laboratory cultivation protocols are underdeveloped (e.g. *Tortula ruralis*) or the genomic resources are scarce (e.g. *Bryum* spp.). Although the traits that have been surveyed to date are limited, the variety of traits for which demonstrable genetic variation exists suggests that polymorphism is likely to be evident in other traits of greater interest.

Several recent reviews attest to the accumulated knowledge of ecotypic differentiation (Longton, 1974; Shaw, 1991a), hybridization (Natcheva and Cronberg, 2004) and population structure (Shaw, 2001; Shaw et al., 2002) in bryophytes. Nevertheless, classical genetic analysis of natural variation has been underutilized in the moss research community of late, in spite of a rich history of such studies, particularly in the 1920s and 1930s (von Wettstein, 1932; Allen, 1935; 1945; Lewis, 1961). Charles Allen in his seminal work on inheritance in *Sphaerocarpus texanus* was the first to show the existence of sex chromosomes in plants (Allen, 1917; 1919) and conducted a thorough analysis of sex-linked traits in the species (Allen, 1924, 1926a, 1926b, 1930). Fritz von Wettstein carried out a series of crosses among genera of the moss family Funariaceae, demonstrating evidence for maternal and ploidy effects (von Wettstein, 1923, 1924a, 1926, 1930) and what we now describe as partial reproductive isolation in divergent crosses (von Wettstein, 1924b, 1928; Schmidt, 1931, reviewed in von Wettstein, 1932; Bryan, 1957). Many of these authors have noted that mosses are well suited for genetic analysis because they have a dominant haploid phase. A large segregating F2 population can be generated in a single cross, and the allelic effects of mutations expressed in the gametophytic stage cannot be masked by dominance. These features make it likely that forward genetic approaches will proceed rapidly in a bryophyte system, once appropriate material and molecular markers are identified.

2.2 Genetic variation among bryophyte populations

Taxonomic revisions and descriptive floras are a rich source of information concerning geographical patterns of morphological, ecological and

phenological variation within species. For example, in a study of herbarium samples of *P. patens* from the US, Tan (1978) reported variation in the speed of tissue re-hydration, spore size and ornamentation, and numerous aspects of leaf size and shape. In a global survey of nearly 1000 herbarium specimens, Burley and Pritchard (1990) documented extensive variation in gametophyte, sporophyte and spore morphology in *C. purpureus*. Specimens from different geographical regions were clearly distinguished by leaf and capsule geometry and size, spore diameter, peristome ornamentations, and much of this variation exhibited strong correlations with latitude of origin. *M. polymorpha*, as the name would suggest, is similarly variable in general appearance (Boisselier-Dubayle et al., 1995), and specimens conforming to different subspecific taxa exhibited different flavenoid profiles (Campbell et al., 1979).

However, the dependence of phenotype upon environmental conditions is well documented for several species of mosses and liverworts, and therefore variation in field-collected herbarium samples may not necessarily have a genetic basis. For example, Hoffman (1966a) showed in studies of *F. hygrometrica* that increasing the light intensity caused plants to mature faster and produce more biomass. Increased light also resulted in more compact stems (smaller stem length to width ratio), and increased photoperiod resulted in an increase in leaf size. Mineral nutrition also had major effects on plant growth, with nitrogen and phosphorus availability having the greatest effect on *F. hygrometrica* (Hoffman, 1966a, 1966b). The addition of heavy metals to growth media also had a strong, nonlinear effect on protonemal and gametophore traits in *F. hygrometrica* and the closely related *F. flavicans* (Shaw and Bartow, 1992). Furness and Grime (1982) found that the maximum growth rate of both *F. hygrometrica* and *C. purpureus* exhibited a strong sensitivity to temperature (although both have broad temperature tolerances, both produced maximum growth at approximately 25°C). Nakosteen and Hughes (1978) reported that *P. patens* and *F. hygrometrica* became sexually mature fastest at 7°C and 15°C, respectively, with higher temperatures delaying or completely suppressing sexual reproduction. In a series of papers, Voth and colleagues (Voth and Hamner, 1940; Voth, 1941, 1943) showed that biomass accumulation, allocation to reproductive tissues and features of the cell wall in *M. polymorpha* varied in response to altering the mineral nutrition. Benson-Evans (1961, 1964) similarly reported variation in growth in response to temperature.

It is clear, therefore, that in order to identify genetic differences among populations, rather than differences induced by the environmental conditions where the plants were found, natural isolates must be grown under common, controlled growth conditions. Several studies document such among-population or among-species differences in bryophytes, a representative sample of which I review here (Table 2.1). It is important to add that the evolutionary forces responsible for causing the among-population differences (i.e. natural selection, or genetic drift), with few exceptions, are largely unknown. The traditional gold standard for documenting ecotypic differentiation is a reciprocal transplant experiment to test whether alternate phenotypes performed significantly better in their native environment (Turesson,

Table 2.1 Studies of population variation in mosses and liverworts

Species	Trait(s)	Reference
Marchantia polymorpha	Metal tolerance	Briggs, 1972
	Reproductive isolation	Burgeff, 1943
Marchantia inflexa	Gametophyte growth, sexual dimorphism	McLetchie and Puterbaugh, 2000
	Reproductive output	Fuselier and McLetchie, 2002
Riella americana	Reproductive isolation	Proctor, 1972
Funariaceae	Reproductive isolation	von Wettstein, 1924b; von Wettstein, 1941; von Wettstein, 1932
Funaria hygrometrica	Metal tolerance	Shaw et al., 1987; Shaw, 1987, 1988
	Gametophyte growth	Dietert, 1980; Weitz and Heyn, 1981; Shaw, 1990c, 1991c
	Spore size, color	Weitz and Heyn, 1981
	Mineral nutrition	Dietert, 1979
Ceratodon purpureus	Gametophyte growth, speed of maturation, leaf size	Shaw and Gaughan, 1993; Shaw and Beer, 1999
	Genetic correlations among life history traits, sexual dimorphism	McDaniel, 2005
	Metal tolerance	Shaw et al., 1991; Jules and Shaw, 1994
	Population genetic analysis of *adk, phy2* and chloroplast DNA in worldwide sample	McDaniel and Shaw, 2005
	Reproductive isolation; Protonemal growth	McDaniel et al., 2008
Bryum argenteum	Metal tolerance	Shaw et al., 1989; Shaw and Albright, 1990
	Gametophyte growth	Longton, 1981

1922; Clausen et al., 1940, 1948). Such experiments have not been conducted in bryophytes, and nor have phylogenetically corrected associations between traits and habitats (Mishler, 1988; but see Vanderpoorten et al., 2002). Thus, the extent of ecotypic differentiation in bryophytes remains undocumented, much as it was two decades ago. Nevertheless, the material and tools now available make it possible to understand the evolutionary forces shaping variation in at least our model systems and their close relatives.

2.2.1 Heavy metal tolerance

Some of the best examples of ecotypic differentiation in bryophytes to date come from studies of heavy metal tolerance. These studies are appealing

because the selective force is clear and can be recreated in the laboratory with some fidelity. Several mosses and liverworts exhibit high tolerances of metals, some even thriving on the most contaminated soils (Shaw et al., 1987). In one of the earliest studies of ecotypic differentiation in a liverwort, Briggs (1972) found that urban populations of *M. polymorpha* were more tolerant of elevated lead levels than were individuals from rural and generally uncontaminated populations. The urban sites contained lead levels that were well above 1000 ppm. Plants from these populations grew equally well on media containing 400 ppm lead as on control media. In contrast, plants from rural populations, where lead levels were below 400 ppm, grew significantly less well on lead-containing media than on control media, strongly suggesting that these differences are a result of natural selection.

In an extensive series of experiments, Shaw and colleagues examined patterns of tolerance to heavy-metal contaminated soils in several species of mosses (Shaw, 1990a). Shaw et al. (1987) demonstrated that populations of *F. hygrometrica* exhibit local adaptation to zinc and copper-containing soils. Shaw (1987) found no strong or consistent effect of pre-treatment with heavy metals, suggesting that the different degrees of tolerance represent true genetic differences among individuals. The degree of tolerance of samples from a particular population was correlated with the concentrations of those metals in the soil from where the plants were sampled, also suggesting adaptive divergence. The reaction to metals was variable; for example, copper inhibited both spore germination and protonemal growth, but zinc had an inhibitory effect only at the protonemal stage. This suggests that copper and zinc may cause toxicity through different molecular mechanisms. Indeed, Shaw (1988) detected no correlation between zinc and copper tolerance in *F. hygrometrica*, suggesting tolerance to these metals does not share a common genetic basis.

In each of these experiments, individuals from contaminated sites and uncontaminated sites were grown on control media and media supplemented with zinc and copper. Surprisingly, however, plants from contaminated sites grew better than plants from uncontaminated sites on both metal-enriched and control media. Why then are these genotypes not found everywhere? This finding would suggest that success in the laboratory environment is probably an imperfect predictor of success in nature. Nevertheless, growth rate under permissive conditions is likely to be an important fitness component in species such as *F. hygrometrica* that colonizes open soils. Since tolerant genotypes generally appear to be restricted to metal-containing soils, this suggests that metal-tolerant genotypes may suffer a disadvantage on normal soil later in development or one that was not evident under these experimental conditions.

Populations of *C. purpureus* also exhibit local adaptation to heavy metal-containing soils. Preliminary experiments suggested that plants from moderately contaminated sites did not exhibit increased tolerance of metal-containing soils (Shaw et al., 1991). However, Jules and Shaw (1994) found clear evidence of tolerance in an experiment testing populations from sites

with higher levels of heavy metals. Plants from four natural populations – one a contaminated site – were inoculated on control soil and on metal-enriched soil collected from near a zinc smelter. Plants from metal-contaminated sites produced significantly more protonemata, gametophores and gametangia than plants from uncontaminated sites when grown on contaminated soil. In a second experiment, several isolates from four additional populations were brought into sterile culture and grown on media containing varying concentrations of copper, zinc, lead and cadmium (Jules and Shaw, 1994). Again, as measured by protonemal growth rate, plants sampled from contaminated soils exhibited significantly less sensitivity to metal concentration. The elevated metal levels in the soils, from which the tolerant populations were isolated, were a direct result of the smelter activity that began less than 90 years prior to when the experiment was conducted. This indicates that the rate of adaptation to contaminated soils was rapid in this species. Interestingly, unlike *F. hygrometrica*, *C. purpureus* plants from contaminated sites grew more vigorously on contaminated soil than on control soil, suggesting that such plants experience a cost to metal tolerance.

Local adaptation to elevated heavy metal levels, however, is not universal among bryophytes found on contaminated soils. *Bryum argenteum* exhibits genetic variation for both growth rate and uptake of lead within and among populations, but no genetic differentiation in lead tolerance among populations (Shaw et al., 1989; Shaw and Albright, 1990). Interestingly, this species also shows limited differentiation to latitude or origin in several traits (Longton, 1981). *Bryum bicolor* populations from mine sites exhibited no more genetic adaptation to metal contaminated soil than plants from uncontaminated sites (Shaw, 1990b). Populations of *Funaria flavicans* from metal-rich serpentine soils show no increased tolerance to nickel or cadmium (Shaw, 1991b). Nevertheless, mapping loci underlying variation in metal tolerance among populations of *F. hygrometrica*, *C. purpureus* or *M. polymorpha* is likely to be rewarding. The number of loci causing tolerance is likely to be small, as the selective pressure was strong and the rate of adaptation rapid, and the environmental factors associated with the fixation of such alleles within a population are well defined.

2.2.2 Mineral nutrition

Many mosses are endemic to substrata of particular mineral or organic compositions (Bates, 2000). In one of the few studies to examine population variation in mineral nutrition, Dietert (1979) examined the sensitivity of protonemal growth to nutrient allocation in plants from four populations of *F. hygrometrica* (Alaska, USA; Texas, USA; Massachusetts, USA; Alberta, Canada). Requirements for calcium, iron, sulfur and phosphorus were largely consistent among the sampled populations. However, the populations differed in tolerance or maximum growth on different sources of nitrogen. The Massachusetts and Texas populations tolerated 120 ppm ammonium, but the Alaska and Alberta populations failed to grow. Only the Massachusetts

population tolerated 120 ppm of nitrite, while the others grew best at 40 ppm. Similarly, the Massachusetts and Alberta populations grew best with one-third the concentration of nitrate (NO_3) than that for the Alaska and Texas populations. The Massachusetts population also had a different requirement of potassium for maximum growth; the Alberta population required three times the concentration of magnesium (22.2 ppm, as opposed to 7.1 ppm) than the other populations to achieve maximum growth. Dietert (1979) reported no correlations between growth rates on different media, although it is unclear whether these experiments had the statistical power to detect such correlations.

2.2.3 Gametophyte growth

Biogeographers have long noted that many single moss and liverwort species have broad geographical distributions, suggesting that the species tolerate a broad range of temperature, insolation, water availability or day length. For example, all four species considered in this review are widely distributed in temperate regions of both the Northern and Southern Hemispheres. Does the ability to grow under such diverse conditions result from phenotypic plasticity or local adaptation? A thorough investigation of trait variation across the distribution of any of these four species has not been conducted. However, patterns of trait variation within and among a more limited sampling of populations, in particular in *F. hygrometrica* and *C. purpureus*, are reasonably well documented.

In the first study to examine genetic variation in a sample of *F. hygrometrica* from a broad geographical sample, Weitz and Heyn (1981) cultivated populations of spores from several sporophytes from 18 Israeli populations and 33 additional populations from throughout Europe. There was no variation in germination rates, but plants from northern populations had a shortened summer dormancy period and later sporophyte maturation when grown together under common garden conditions. These results were consistent with day length response due to latitude of origin potentially suggesting adaptive differentiation. Weitz and Heyn (1981) also found differentiation in spore diameter and sporophyte pigmentation, which has no obvious association with climate or geography. In a similar experiment, Dietert (1980) reported that the temperature requirements for individuals of *F. hygrometrica* varied among four populations, depending upon the stage of growth. In some cases, the conditions promoting maximum growth for these individuals were consistent with the latitude of origin of the sample. For example, protonemal growth was greatest at 30°C, except for the sample derived from the northernmost population (Alberta, Canada), where it was 25°C. Sexual maturation proceeded most rapidly at 10°C in a 16-h photoperiod. At longer photoperiods, only individuals from the Alberta population – which was presumably exposed to the longest summer days – produced gametangia or sporophytes. Not all patterns of trait variation were predictable based on a simple

latitudinal model; gametophore growth at 25°C induced the most growth except in individuals from the Massachusetts population, where production was highest at 30°C.

It is important to recall that the Dietert (1980) and Weitz and Heyn (1981) studies were not designed to evaluate within-population variation. However, estimates of this component of variation are essential to perform a statistical evaluation of among-population variation. For example, although trends between individual trait values and latitude of origin are suggestive of among-population differences, these differences could in fact be present within populations. To eliminate this possibility, multiple individuals from each population must be measured to assess the structure of variation among populations compared to that within populations. Fuselier (2008) found that populations of *Marchantia inflexa* differed in growth rate, branching pattern and production of asexual propagules, although significant variation was present in most traits in all populations. Within populations, variation can be further partitioned into variation among siblings from the same sporophyte capsule, or family, and variation among gametophytes from different families. This arrangement gives increased power to evaluate the genetic architecture of variation using traditional quantitative genetic tools, relative to similar analyses in diploid dominant species (Shaw et al., 1997).

Shaw (1991c) studied patterns of natural variation in families of *F. hygrometrica* in two North Carolina (USA) populations, where one was from contaminated soils near a copper mine. Among capsules, there was variation in the number of spores, germination percentage, protonemal growth rate, as well as metal tolerance. Interestingly, much of the variation was among spores from the same capsule, rather than among spores of different capsules. Since a single isolate of *F. hygrometrica* produces both antheridia and archegonia, a single gametophyte can fertilize itself, in which case no allele should be segregating among the recombinant progeny. The significant within-family component of variation in *F. hygrometrica* suggests that the sampled sporophytes were either out-crossed, the spores experienced heterogeneous maternal effects, or that the variation was generated by mutation prior to gamete production or during sporophyte maturation. In an earlier experiment, Shaw (1990c) reported that spores produced by a single selfed sporophyte contained significant variation in growth rate and other traits. Whether this is the result of new mutations, meiotic abnormalities or some epigenetic phenomena is uncertain but may warrant further examination. Similar experiments with *P. patens* have not been published, but observation of single spore isolates from a small number of selfed capsules suggests that there is heterogeneity in protonemal growth rates within families in this species as well (P.-F. Perroud and S.F. McDaniel, unpublished observation).

The most complete description of quantitative genetic variation within and between bryophyte populations was conducted using a sample of families from two populations of *C. purpureus* from near Ithaca, New York (Shaw and Beer, 1999). This study involved intensive sampling of an average of more

than 15 haploid gametophytes from 29 and 39 sporophytes from populations in Ithaca and Danby, New York, respectively. There were significant among-family differences in speed of maturation, size at maturity and reproductive output in both populations, and the mean trait values differed significantly between the populations for all traits. Since the populations were not sampled across an obvious environmental gradient, it is unclear if the differences were correlated with any particular ecological feature. The Danby population, however, matured more slowly, but made bigger leaves, accumulated less overall biomass, had fewer fertile stems and each stem made fewer gametangia, which may be correlated with the overall stability of the site where this population was sampled (i.e. where water was more persistent throughout the year (Shaw and Beer, 1999)).

In addition to trait means, correlations among traits are of interest because they suggest that variation in multiple traits may share a common genetic basis. Genetic correlations among traits may be controlled by loci with pleiotropic effects on multiple traits, or by physical or statistical associations among alleles at multiple loci, a phenomenon called linkage disequilibrium. Negative correlations are of particular evolutionary interest because they represent a genetic trade-off that may constrain adaptation. For example, Fuselier and McLetchie (2002) found a negative correlation between plant size and number of asexual reproductive structures in *M. inflexa*, suggesting that plants maximize fitness by investment in either growth or reproduction, but not both (see also Fuselier, 2008). McDaniel (2005) used the Shaw and Beer (1999) dataset from *C. purpureus* to estimate correlations among traits within the families from the two populations. Many pairs of traits (e.g. estimates of gametophore growth rate, size and reproductive output) exhibited significant positive or negative correlations. Similar to the single trait values in the Danby and Ithaca populations of *C. purpureus*, the genetic correlations between the length of the juvenile phase and frequency of fertile stems and the correlation between leaf length and biomass accumulation differed significantly between the two populations. Distinguishing whether such correlations are caused by pleiotropy or linkage disequilibrium, and the nature of the underlying loci, may provide valuable information regarding the allocation of resources and the evolution of complex traits.

2.2.4 Sexual dimorphism

Mosses may be hermaphroditic or have separate sexes. In *F. hygrometrica* and *P. patens*, a single haploid individual is capable of producing both sperm and eggs, and therefore sporophytes and meiotic progeny. In contrast, *C. purpureus* and *M. polymorpha* are obligate out-crossers; hence, reproduction requires the presence of a male and a female plant. In both *C. purpureus* and *M. polymorpha*, sex is determined by the segregation of X and Y chromosomes (Ramsay and Berrie, 1982). The diploid sporophyte is always heterogametic, and the X and Y segregate to female and male spores, respectively. Because males and

females have different roles in reproduction – males produce sperm that swim to fertilize an archegonium, while females nurture a nutritionally dependent diploid sporophyte – the sexes may have different optimal phenotypes for a variety of traits. To test this hypothesis, Shaw and Gaughan (1993) examined four haploid gametophytes each from 40 sporophytes from a population of *C. purpureus* in Michigan, USA. Males produced more stems, but overall had less variability than females in growth rate and size (Shaw and Gaughan, 1993). Shaw et al. (1997) reported that sex had a detectable effect on the expression of variation in these populations, suggesting that trait variation in the sexes had a different genetic architecture. In a more detailed study, Shaw and Beer (1999) found that males had a longer juvenile phase, more fertile stems and more sex buds on each stem, significantly shorter leaves, but accumulated less biomass than females. McDaniel et al. (2008) reported significant sexual dimorphism in protonemal growth rate in the hybrid progeny of an inter-population cross between Ithaca and Otavalo, Ecuador. *M. polymorpha* is also sexually dimorphic, although no quantitative genetic studies of this species have been published. In the related species, *M. inflexa*, females are larger, with more meristematic tips, and generally produce fewer specialized asexual reproductive structures (McLetchie and Puterbaugh, 2000; Fuselier, 2008).

In *C. purpureus*, males and females also differed significantly in patterns of trait correlations, with males showing a strong negative correlation between leaf size and number of gametangial buds that were absent in females (McDaniel, 2005). This suggests that males can gain fitness by either dedicating resources to gamete production, which may result in the production of spores, or vegetative growth, which may improve survival to mate in subsequent years. The genetic basis of this negative correlation (i.e. pleiotropy or alleles at multiple loci in linkage disequilibrium), however, is unknown. Nevertheless, the maintenance of sexually dimorphic correlations suggests that selection acts differently on males and females. In addition to correlations among traits within a sex, McDaniel (2005) also reported correlations between male and female siblings for several single traits in the Danby and Ithaca populations. For all measured traits, the correlation between male and female trait values was significantly less than one, indicating that the trait values in males are imperfect predictors of patterns of trait variation in their sisters. This finding indicates that the degree of sexual dimorphism in gametophytes from a single sporophyte is variable within these populations, and therefore can evolve. This variation may be due to either the segregation of multiple X or Y-chromosome variants, or more likely the segregation of autosomal alleles that interact in a sexually antagonistic fashion with the sex chromosomes.

Alleles with sexually antagonistic effects in *C. purpureus* were evident not only in morphological and life history phenotypes, but also in the phenomenon of sex ratio distortion. Sex ratio in a wide variety of eukaryotes deviates from the expected 1:1 segregation of males and females (Lyttle, 1993). Sex ratio variation is common in bryophyte populations (Bisang and

Hedenas, 2005), although whether this is due to greater spore death of one sex, differential survival of males and females or differential sex expression is unknown except in *C. purpureus*. Some sporophytes of *C. purpureus* from the Danby and Ithaca experimental populations produced strongly biased progeny sex ratios, although the total population sex ratio did not deviate significantly from 1:1 (Shaw and Beer, 1999). The sex ratio bias in these sporophytes likely resulted from differential spore death. From the Michigan population, Shaw and Gaughan (1993) reported a nearly tenfold difference in number of spores within a sporophyte, considerably larger than that found within a single population of *F. hygrometrica* in the North Carolina experiment (although spore numbers from mine tailings were lower than those from the population on uncontaminated soils). The germination rates from this population of *C. purpureus* also varied greatly.

The prevalence of sex ratio distortion, and the concomitant negative effect on fitness, almost certainly results from ongoing genetic conflict between male and female sex chromosomes. Because the sex chromosomes are uniparentally inherited, alleles that favor the transmission of one sex at the expense of the other may be strongly favored. As one sex becomes rare, strong selection to increase the frequency of a repressor mutation arises; so alleles at other loci that suppress distortion become strongly favored as a distorter increases in frequency. The progeny from sporophytes that are heterozygous for both a distorter and suppressor should exhibit complete distortion in the non-suppressor background, and an equal sex ratio in the suppressor background. McDaniel et al. (2007) found segregation patterns consistent with this two-locus sex ratio distortion model in the Ithaca–Otavalo experimental cross, suggesting that loci linked to the sex chromosomes may be hot-spots for sexually antagonistic alleles, as predicted by theory (Rice, 1987).

2.3 Mapping genes underlying natural variants

In the previous sections, I have described experimentally verified trait variation in four model systems. In most cases, natural isolates with genetic variation for a trait of interest differ at many loci in the genome, in addition to allelic variation at loci that control the trait. The paradigm for identifying the molecular variants that underlie the trait of interest relies on (1) meiotic recombination to break up associations between causative loci and other unrelated allelic variation, and (2) polymorphic markers with a known pedigree that can be used to identify chromosomal regions containing the causative loci (Tanksley, 1993). Generally, two divergent individuals with extreme values at the trait of interest are crossed. This produces a large pool of progeny in which the alleles that are responsible for variation in the trait of interest are dissociated from allelic variants that do not affect the trait. Since few recombination events have occurred, the causative alleles will cosegregate with the surrounding chromosomal regions, which carry markers

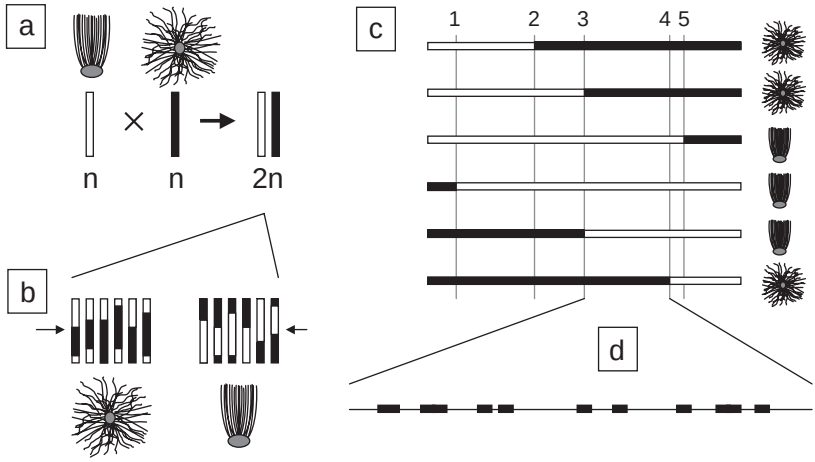

Figure 2.1 Map-based cloning in *P. patens*. (a) Generating a hybrid diploid between two phenotypically divergent haploid isolates. The oriented growth phenotype is associated with a white chromosomal segment, while the unoriented phenotype is associated with a black chromosomal segment. (b) A segregating mapping population used for coarse mapping the chromosomal region that causes the orientation phenotype. (c) Fine mapping the causative chromosomal region between molecular markers 1–5. (d) Nine candidate genes in the marker 3 to marker 4 interval.

from the same parent as the mutation of interest. By correlating trait and marker segregation, it is straightforward to associate variation at the trait of interest with particular chromosomal regions (Figure 2.1). In diploid dominant species, this process requires the initial cross to produce the F1 progeny, a second cross to make a segregating F2 population (or the F1 is backcrossed to one of the parents), followed by several generations of inbreeding to produce homozygous lines. The great advantage of a moss or liverwort model system is that this procedure is accomplished in a single cross. When a sufficient number of progeny have been screened such that the region containing the locus of interest is reduced to an experimentally tractable number of loci, candidate loci may be tested using transgenic methods (or association studies), which is another strength of the *P. patens* system (Quatrano et al., 2007).

While map-based cloning is routinely used for identifying induced mutations, relatively few natural variants have been identified by the procedure (Maloof, 2003; Salvi and Tuberosa, 2005; Ashikari and Matsuoka, 2006). Unlike induced mutations, which generally produce discrete phenotypes, natural variation among isolates is often quantitative rather than qualitative. Usually, allelic variants at multiple loci contribute to trait variation. As a consequence, substituting one allele for another at even the locus with the largest effect on the trait may explain only a modest portion of the phenotypic difference between the parents. Additionally, the genetic background may affect

the phenotypic expression of an allele; that is, a particular allelic substitution may have variable effects depending upon the allelic states at other loci, a phenomenon called statistical epistasis. Given this genetic complexity, it is essential to generate large mapping populations and employ statistical tools to identify genomic regions containing alleles with significant effects on the trait of interest. In conjunction with sophisticated statistical approaches, researchers often use repeated backcrosses to one parent to generate near isogenic lines that segregate for a single locus of interest. Needless to say, these are laborious experiments.

In spite of the challenges, natural variation has several attractive attributes for both applied and basic science. First, as we have seen, variation in wild accessions is generally abundant. Because natural variants cause trait changes that are not generally deleterious, and indeed may be adaptive in some environmental contexts, the alleles underlying this sort of variation may represent a qualitatively different spectrum of mutations compared with those generated from mutagenesis experiments. Indeed, natural variation in root morphology in *Arabidopsis thaliana* (Mouchel et al., 2004), heading time in *Oryza sativa* (Takahashi et al., 2001; Doi et al., 2004) and fruit shape (Liu et al., 2002) and weight (Cong et al., 2002; Frary et al., 2000) in *Lycopersicon esculentum* were controlled by loci that had not been predicted to be associated with these traits based on prior screens of induced mutants or knowledge of biochemical pathways from other species. Natural variation may be controlled by mutations that are less likely to be produced by experimental mutagenesis, such as regulatory changes, splice variants, copy-number variants or chimeric proteins. Comprehensive genomic resources, relatively inexpensive high-throughput genotyping and computationally sophisticated methods of associating markers and traits are now making this variation increasingly accessible for experimental manipulation (these developments fall outside the scope of this review).

2.3.1 Molecular genetic variation

Early molecular population genetic surveys based on allozyme variation suggested that bryophytes maintained little genetic variation. Shaw (1990c) examined 14 allozyme loci in populations of *F. hygrometrica*, and found no polymorphism. Shaw and Beer (1997) reported preliminary surveys of populations of *C. purpureus* in the eastern US that similarly showed no variation. Allozymes exhibited a similar lack of variation in *M. polymorpha*, although the various subspecies had distinct allozyme patterns (Boisselier-Dubayle et al., 1995). However, genomic data that facilitate PCR-based polymorphism screens are now available for several bryophyte species, most notably *P. patens* (Table 2.2). Although little DNA sequence variation was evident in a small sample at six loci in *P. patens* (S. F. McDaniel, M. von Stackelberg, G. Schween, S. Richardt, R. S. Quatrano, R. Reski and S. A. Rensing, unpublished data), von Stackelberg et al.(2006) reported variation in simple

Table 2.2 Genetic resources for moss and liverwort model systems

Species	Resource	Reference
Physcomitrella patens	SSRs from a global sample	von Stackelberg et al., 2006
	Genome sequence release v1	Rensing et al., 2008
	300 000 ESTs from Gransden and Villersexel	Nishiyama et al., 2003; Lang et al., 2005
Ceratodon purpureus	1700 ESTs	(unpublished)
	Linkage map (Ithaca x Otavalo)	McDaniel et al., 2007
Tortula ruralis	10 368 ESTs	Oliver et al., 2004
Marchantia polymorpha	Allozymes and random markers from a global sample	Boisselier-Dubayle et al., 1995
	Y-chromosome BAC sequencing	Okada et al., 2001; Yamato et al., 2007

sequence repeats (SSRs) among a similar panel of *P. patens* and closely related species. While it is unknown whether these loci will similarly be variable in *F. hygrometrica*, these data suggest that within- and among-population differences may have been missed in the earlier allozyme studies. Similarly, McDaniel and Shaw (2005) and Clarke et al. (2008) report finding considerable DNA sequence and microsatellite diversity, respectively, in several populations of *C. purpureus*. Interestingly, none of the variation resulted in amino acid substitutions, consistent with the previous allozyme study (Shaw and Beer, 1997).

2.3.2 Quantitative trait locus (QTL) mapping

Segregating mapping populations and genetic maps exist now for *C. purpureus*, between wild isolates from New York, USA, and Otavalo, Ecuador (McDaniel et al., 2007), and *P. patens*, between the widely studied Gransden laboratory strain and an isolate from Villersexel, France (Kamisugi et al., 2008). This represents the first step toward identifying genes (or genomic regions) that underlie induced or natural variants in these species. Using the *C. purpureus* mapping population, McDaniel et al. (2008) mapped quantitative trait loci (QTLs) that caused gametophytic growth rate differences between two populations. Consistent with the sexual dimorphism in the species, the sex chromosome had a large effect on growth rate; the protonemal area of a male spot innoculum was on average 85% the size of a female sibling after 3 weeks of growth. However, two other QTLs had significant effects on protonemal size variation. The allelic effects were consistent with the population-of-origin; that is, the allele from the slower-growing Otavalo population reduced the growth rate of progeny carrying that allele. The identification of the causative alleles underlying this phenotypic difference awaits

fine scale mapping. As an example of this procedure, kanamycin-resistance was mapped in the Gransden–Villersexel cross, a phenotype derived from a resistance cassette inserted in the *PpLEA-1* gene of the Gransden strain of *P. patens*. The resistance phenotype was mapped to linkage group 11, connecting this linkage group to the genomic scaffold containing the *PpLEA-1* gene. Were this an unknown variant, the physical region between the adjacent flanking markers would be scanned for candidate proteins (verified or predicted), and these would be tested for associations with the trait of interest using standard molecular methods (reviewed in Quatrano et al., 2007).

Both the *C. purpureus* and *P. patens* maps were constructed using a mix of amplified fragment length polymorphisms (AFLPs) and gene-based markers. One shortcoming of randomly generated presence or absence markers, such as AFLPs, is that their provenance in the genome is uncertain. Ultimately, we would like to identify the physical location of markers linked to loci of interest. Finding the physical localization of sequence-based markers is relatively straightforward on a genomic sequence, like that of *P. patens*, or a bacterial artificial chromosome (BAC) library; for an AFLP band, this process is more laborious. However, AFLP markers can be found on the sequenced *P. patens* genome using an *in silico* tool (Kamisugi et al., 2008). It is currently only possible to find those markers that are present in the Gransden parent (positive hits on the Villersexel genome cannot be found using this method). These authors have additionally mapped numerous SSR loci identified by von Stackelberg et al. (2006), many of which have already been matched directly to the *P. patens* genome. The identification of additional single nucleotide polymorphisms (SNPs) as well as other types of markers (SSRs, larger insertion or deletion polymorphisms) in *P. patens* and *C. purpureus* will be critical to advance map-based cloning in these species. The sequencing project underway in *M. polymorpha* will undoubtedly provide similar data for this species (Okada et al., 2001; Yamato et al., 2007).

2.3.3 Dissecting complex genetic architectures

A common observation in the wide crosses used to generate mapping populations is that the progeny trait values occasionally fall outside the range of the two parents. Several genetic mechanisms may cause this phenomenon, known as transgressive segregation. Suppose a trait is controlled by several loci, and two individuals contain alleles that increase a trait value at some loci and alleles that decrease the trait value at others. In a cross between these two individuals, the progeny by chance may carry more 'up' or 'down' alleles than either of the parents, and therefore exhibit a transgressive phenotype. Alternatively, transgressive phenotypes may result from interactions among alleles from the two parents, a model first proposed by Dobzhansky (1937) and Muller (1942). The simplest model involves deleterious interactions between alleles at two loci. Consider an ancestral population of haploids with a two-locus genotype *AB* at two interacting loci. Although such loci may fall into any

gene ontological category, a simple example is a transcription factor (*A*) and its binding site (*B*). In one daughter population, a new mutation arises in the transcription factor (genotype *aB*), while in a second daughter population a mutation arises in the binding site (genotype *Ab*). These mutations may increase in frequency due to natural selection, because they beneficially alter gene expression patterns, but this is not strictly necessary for the model. A cross between these populations produces four genotypes: the ancestral *AB* genotype, the parental *Ab* and *aB* genotypes, and a novel genotype *ab*. The model proposes that the recombinant *ab* genotype, which has not been tested by natural selection, has a transgressive phenotype. Both the new transcription factor and new binding site function appropriately with the native allele at the interacting locus, but the new transcription factor and new binding site fail to properly regulate gene expression when together in a recombinant individual. While many other arrangements are possible, the key feature of this model is that the transgressive phenotype is caused by deleterious gene interactions.

In the *C. purpureus* mapping population (McDaniel et al., 2007), approximately half of the progeny showed a growth rate lower than either of the parental isolates (McDaniel et al., 2008). McDaniel et al. (2008) used QTL mapping to identify four chromosomal regions that contributed to the difference between normal and small protonemata. The patterns of marker segregation fit the predictions for the interaction model; that is, particular QTLs were associated with the slow growth phenotype only in certain allelic combinations. Only one of these loci had pleiotropic effects on parental-type growth variation, suggesting that the slow growth phenotype and normal parental growth variation are independent traits.

The gene-interaction model, however, did not completely explain the segregation of the slow growth phenotype in the progeny of the Ithaca–Otavalo cross (McDaniel et al., 2008). In many interspecies crosses, F1 sterility (i.e. the failure of a hybrid to make functional meiotic products) is sometimes caused by meiotic recombination within chromosomal regions that are structurally different between the parents. Under this model, recombination in rearranged portions of a chromosome leads to the production of aneuploid meiotic products. Generally, such events are thought to be lethal. However, McDaniel et al. (2008) reported that a significant increase in the number of recombination events in three mapped intervals in the genetic map was associated with the small protonemal phenotype. Interestingly, one of these intervals contained a QTL that affected parental-type protonemal growth, and another interval contained a locus associated with the slow growth phenotype. While the meaning of these findings is uncertain, it suggests that some structural difference between the two parents, perhaps copy-number variation, was disrupted by recombination, leading to the small phenotype.

These low-fitness hybrid phenotypes are of interest for evolutionary reasons because this phenomenon is thought to be critical for the maintenance

of new species, which would otherwise fuse into a single species upon hybridization. However, the production of aberrant phenotypes from interactions between functional genes or gene products, or illegitimate recombination, is an interesting mechanistic problem. These unusual hybrid phenotypes have also been found in experimental crosses among genera of the Funariaceae (von Wettstein, 1924b, 1928, 1932; reviewed in Bryan, 1957), among isolates of both *M. polymorpha* (Burgeff, 1943), cited in Boisselier-Dubayle et al., 1995) and the liverwort *Riella americana* (Proctor, 1972). In addition to slow growth, hybrids from wide crosses may be more susceptible to stress, or exhibit other phenotypes that were not predictable based on parental phenotypes (Bomblies et al., 2007; Bomblies and Weigel, 2007). Map-based cloning of loci underlying traits that may arise serendipitously from wide crosses may complement other types of mutagenesis screens.

2.4 Assigning genes to phenotypes

Throughout this review, I have described genetic variation among populations of several experimental model systems, and a strategy for discovering the allelic variants that cause this variation. The use of map-based cloning to identify chromosomal regions containing causative natural variants is a laborious but ultimately a very effective process. The life cycle of a bryophyte means that it is possible to generate a large segregating mapping population with a single cross. This, combined with the genetic simplicity of studying haploid tissue, should facilitate rapid progress toward identifying chromosomal regions underlying traits of interest. However, meiotic recombinants become increasingly rare as the region containing the causative variant becomes smaller, and therefore the number of progeny that must be screened to narrow the chromosomal region further quickly becomes infeasible. For this reason, other approaches are generally favored for testing the association between particular genes within the chromosomal region identified by recombination mapping and the trait of interest.

Ideally, a narrow chromosomal region will contain an experimentally tractable number of genes. In both *P. patens* and *C. purpureus*, it is clear that targeted gene replacement is the method of choice for demonstrating an association between a trait and a gene (Trouiller et al., 2007). However, in many cases, transgenic approaches may be impractical. For example, the QTL region may contain too many genes to test, the causal mutation may be a deletion, duplication or translocation of a gene or genomic region that is difficult to engineer or the phenotype of interest may require the simultaneous manipulation of multiple loci. Although the list of candidate genes within a QTL region may be further narrowed based on previous functional information, a global analysis of gene expression or broad surveys of natural isolates offer two additional means of evaluating candidate genes. Although these

have not been undertaken in bryophytes, some of the groundwork necessary for these analyses is already in place.

2.4.1 Combining genetics and transcriptomics

A powerful way to survey candidates is to identify genes within a QTL region that have expression differences that correlate with alternate states in the trait of interest. Several recent studies in other model systems have used segregating mapping populations to map loci that control variation in gene expression (Brem et al., 2002; Schadt et al., 2003; Brem and Kruglyak, 2005). Indeed, some of the phenotypic variants that I have described in this review are likely to be caused by gene expression variation. Variation in transcription profile is common among individuals within a population and also segregates within hybrid mapping populations, suggesting that it has a genetic basis. The genetic architecture of expression variation can be mapped in much the same way as the phenotypes described previously. For example, Keurentjes et al. (2007) screened whole genome transcript accumulation in the progeny of a mapping population in *A. thaliana* to determine the genetic basis of gene expression variation between two wild accessions. In this cross, there were significant expression differences between the parents at 922 loci. Expression QTLs were found for 4066 loci; these eQTLs mapped to both changes at the gene itself (i.e. *cis*-regulatory variation) and unlinked *trans* regulators. Thus, surveys of gene expression can be used as a tool for exploratory analysis of control of gene expression. Additionally, targeted studies aimed at studying the control of variation at candidate loci of interest can be used as an experimental tool to verify network connections derived from other sorts of analyses (Keurentjes et al., 2007; Wentzell et al., 2007). This approach may be profitably exploited in *P. patens* to understand regulatory networks underlying a wide variety of physiological or developmental phenomena using currently available microarray technology.

2.4.2 Association mapping

An alternate means of testing whether variation in candidate loci underlies variation in a trait of interest relies upon natural associations among causative loci and phenotypic variants among natural accessions. This method generally involves PCR-based screening of a large number of natural variants at candidate loci in order to correlate a particular genotype with the phenotype of interest. These screens can be fast and inexpensive relative to more sophisticated transgenic manipulations. However, to employ such screens, it is critical to understand the population genetic context from which the natural isolates originate. In particular, linkage disequilibrium among polymorphic sites, due to inbreeding, undetected population structure, or recent natural selection, can cause spurious associations between genotypes and phenotypes.

Theory predicts that inbreeding species should be less heterozygous than outbreeding species and maintain less genetic variation within populations (reviewed in Charlesworth, 2006; Wright et al., 2008). Historically, bryophytes in general were thought to be highly inbred because sperm and spore dispersal distances were assumed to be very low and asexual reproduction prevalent. In self-fertile mosses, in particular, the proximity of antheridia and archegonia on the same plant presumably gives a statistical advantage to selfing. Thus, in contrast to hermaphroditic animals or angiosperms, where selfing generally unites two genetically distinct gametes, selfing in mosses results in a completely homozygous sporophyte that produces spores that have the same genotype as the parent. Recent studies of bryophytes, however, have revealed considerable heterogeneity in inbreeding levels and population structure. Allozyme variation in sporophytes from ten moss species showed that the five with separate sexes (including *C. purpureus*) were significantly less inbred than the five with combined sexes (including *F. hygrometrica*; Eppley et al., 2006). Consistent with this finding, McDaniel and Shaw (2005) found that in a global sample of populations of the outbreeding *C. purpureus*, the *adk* and *phy2* loci both showed evidence of intragenic recombination events. Moreover, the patterns of nucleotide variation suggested that the *adk* locus had experienced recent directional selection, while the *phy2* locus had a significantly different evolutionary history. These observations indicate that evolution at the two loci is decoupled by recombination, both within and between the loci, which can only occur in heterozygotes. In contrast, multilocus sequence data from the self-fertile *P. patens* showed low nucleotide diversity and no recombination, although close relatives that are also self-fertile showed greater nucleotide diversity and clear evidence of past recombination (S.F. McDaniel, M. von Stackelberg, G. Schween, S. Richardt, R.S. Quatrano, R. Reski, and S.A. Rensing, unpublished data).

These findings have significant consequences for the prospects of association mapping in these species. In *P. patens*, the self-mating system makes it likely that few loci will be polymorphic, and the loci that are polymorphic will exhibit strong linkage disequilibrium, perhaps over large genomic regions. Thus, associations between polymorphisms and traits may be correlative but not causative. In *C. purpureus*, in contrast, the outbreeding mating system and the high levels of polymorphism and recombination suggest that even physically proximate polymorphisms may be genetically unlinked from one another. Thus, few polymorphisms are likely to be in linkage disequilibrium with a phenotype-associated SNP, and these may be in the causative gene itself. Although the chance of finding a causative gene using association mapping as the sole approach in *C. purpureus* is remote, association studies using natural variants may be a valuable means for identifying the causative mutation within a region that was identified by QTL mapping or some other approach. Association mapping in carefully chosen populations that span a strong ecological gradient, for example, may prove a powerful use of this technique.

2.4.3 Are among-population differences adaptive?

Among the traits that I have reviewed, a reasonable case can be made for selection having shaped among-population differences in metal tolerance, sexually dimorphic genetic correlations and sex ratio distortion. For the remaining traits, it is impossible based on these phenotypic data alone to distinguish whether the among-population variation reflects ecotypic differentiation (i.e. resulted from natural selection) or random genetic drift. In fact, some of this variation may be deleterious; Taylor et al. (2007) report that crosses between siblings (gametophytes from the same parent sporophyte) in *C. purpureus* resulted in smaller sporophytes than crosses between unrelated gametophytes, consistent with recessive deleterious alleles segregating within population of the species. This phenomenon, called inbreeding depression, was not found in experimental crosses in *F. hygrometrica* (Taylor et al., 2007). This is presumably because recessive deleterious alleles are purged from predominantly inbreeding populations faster than from obligate outbreeding populations.

A critical factor for determining the efficacy of natural selection at both purging deleterious mutations and fixing adaptive mutations is the effective population size. If the historical population sizes for these species were small, or migration among populations was limited, genetic drift is likely to drive phenotypic differentiation. In contrast, if population sizes were high, or migration among populations was frequent, even slight phenotypic differences could reflect natural selection. The fixation of an adaptive mutation itself can locally reduce the effective population size; this was evident at the *adk* locus in *C. purpureus* (McDaniel and Shaw, 2005). Changes in population size, stochastic demographic events or selection at unlinked sites can profoundly influence the kind of variation that segregates within a species or is fixed over the course of evolution. Based on the limited evidence currently available, *P. patens* likely has a low effective population size, due to inbreeding, while the population size for *C. purpureus* is higher, but heterogeneous throughout the genome due to the effects of selection on linked variation and variable recombination rates. Clearly, evaluating the evolutionary processes shaping genetic variation in nature is not directly relevant for understanding the molecular basis of phenotypic variation discussed in this chapter. However, any causal explanation for this variation must be grounded in population genetic analysis, which requires studying the ecological and genomic milieu in which the mutation evolved.

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Chapter 3

COMPARATIVE GENOMICS

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Abstract: The moss *Physcomitrella patens* has been developed as a model organism during the last two decades. Analyses of its transcriptome and genome have revealed differences and commonalities with the genetic systems of seed plants. Comparisons with land plants and algae allowed the inference of evolutionary incidents that occurred with the transition of plant life from water to land. The established molecular tool kit and the available genome sequence together enable future research of plant evolution and development. In this chapter, we elaborate on *P. patens* biology and evolution, give an overview of the analyses that have been carried out in the last decade, and envision future prospects.

Keywords: functional genomic; gene history; *Physcomitrella patens* genome; plant comparative genomic

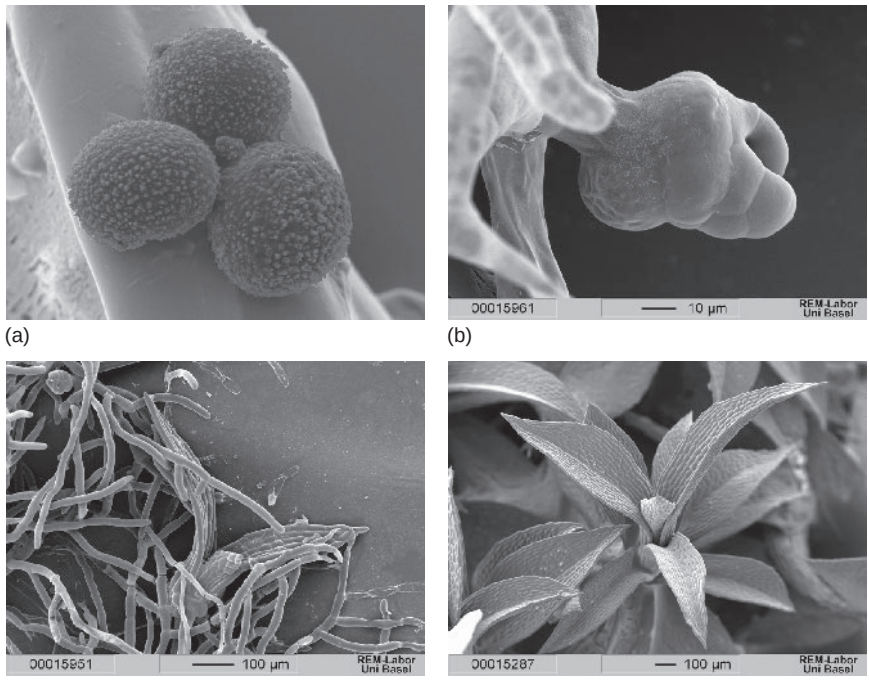
Abbreviations:

GO	gene ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
LCA	last common ancestor
SSR	simple sequence repeat CDS coding sequence

3.1 Introduction

3.1.1 Ecology and life cycle

The moss *Physcomitrella patens* is haploid, monoicous and self-fertile. *P. patens* is an annual opportunist occupying open and disturbed habitats, often close to the waterline. The distribution pattern is disjunct and approximately circum-temperate, with the noteworthy exception of South America. In contrast to the diploid or polyploid seeds of flowering plants, the sexual propagation bodies of mosses are haploid spores (Figure 3.1a),



(a) (b) (c) (d)

Figure 3.1 Cryo-SEM (scanning electron microscopy) images of *P. patens* tissue. (a) *P. patens* spores (~20 µm diameter); (b) bud; (c) protonemata (with bud and developing gametophore); (d) gametophore.

from which the dominant haploid gametophytic generation develops. The filamentous protonemata (Figure 3.1c) form buds (Figure 3.1b) from which the leafy gametophores (Figure 3.1d) develop. On the latter, sexual organs (antheridia and archegonia) develop. The motile male gametes swim to the archegonia and fertilize the egg, from which the diploid sporophyte develops, growing epiphytically on the gametophore. Several thousand spores are produced by meiosis within each sporophyte, the germination of which yields protonemata.

3.1.2 The early evolution of land plants

Based upon spores found in the fossil record, the first plants had occupied the land in the middle Ordovician, approximately 460 million years ago (MYA (Kenrick and Crane, 1997); Figure 3.2). However, the fossil record can only provide us with a minimum estimation, that is, with the date that a certain specimen appeared at the latest. Because of the close resemblance of Late Paleozoic bryophytes to extant taxa, it has been argued that the origin of mosses should date back even further than 460 MYA (Frahm, 1994) and some molecular clock analyses date the separation of vascular plants and mosses

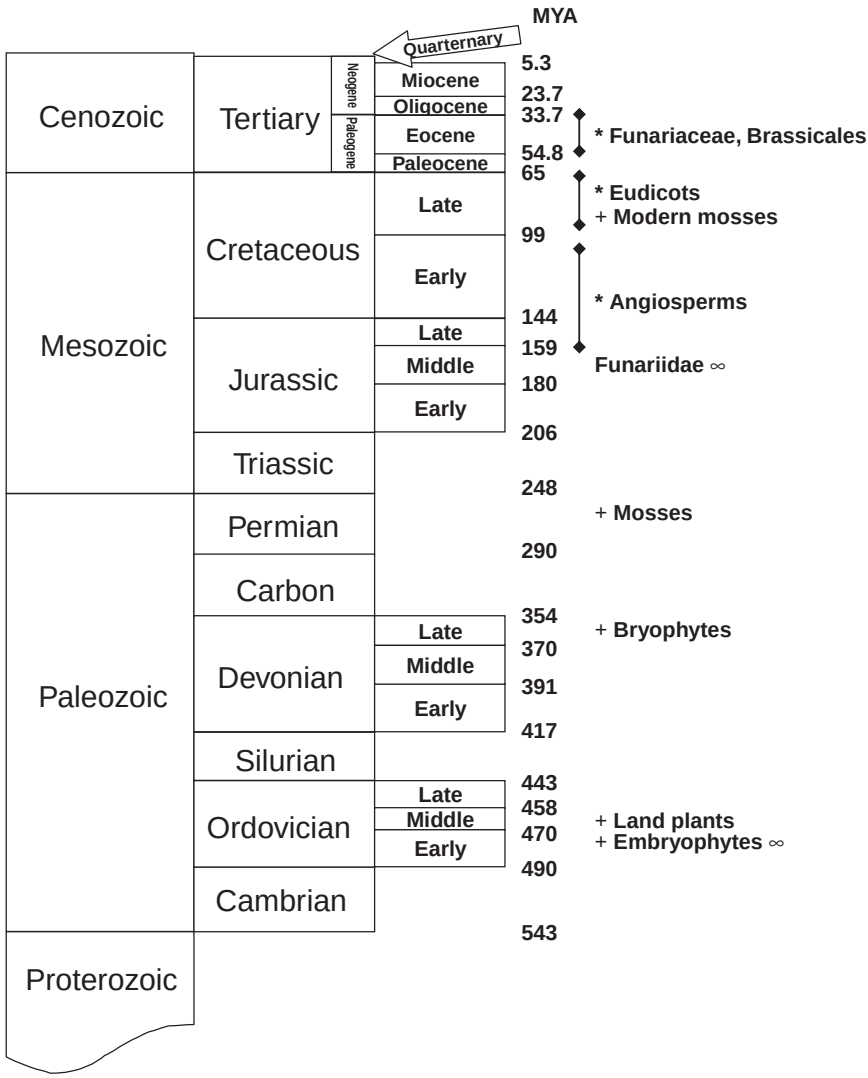


Figure 3.2 Geological timescale summarizing some evolutionary events (+, fossil record; *, genome duplication; ∞, divergence).

to even ~700 MYA (Hedges et al., 2004). Therefore, it seems safe to say that the last common ancestor (LCA) of mosses and seed plants was present at least 460 MYA.

The land plants (Embryophyta) exhibit alternating multicellular generations, that is, the sexual, haploid gametophyte, and the asexual, diploid sporophyte. In early land plant fossils, the gametophytic and sporophytic generation share about equal morphological complexity. Starting from these LCA of all land plants, according to the homologous theory, the gametophyte was reduced while the sporophyte became the dominant generation in

vascular plants (Kenrick and Crane, 1997; Graham et al., 2000; Taylor et al., 2005), while in bryophytes (mosses, hornworts and liverworts), the sporophytic generation was reduced and the gametophyte became dominant. The alternative antithetic theory suggests that the diploid sporophyte generation was interpolated into the life cycle of charophytes through a delay of meiosis after fertilization. This sporophytic generation subsequently evolved to different complexity and dominance (reviewed by Qiu et al., 2006). In any case, the first splits among the Embryophyta separated the Marchantiophyta (liverworts), Bryopsida (mosses) and Anthocerotophyta (hornworts) from the remainder of the land plants, the vascular plants (Tracheophyta). Based on molecular phylogenetic studies, the current understanding is that liverworts are sister to all other land plants, while hornworts are sister to the vascular plants and mosses branch off between the two other bryophyte groups (Qiu et al., 2006).

3.1.3 The age of mosses and their resilience to change

The first deposits containing remnants of modern mosses are from the Jurassic and Cretaceous, demonstrating that some extant species exhibited limited morphological change in the past 80 MY (Schuster, 1983; Crum, 2001) (Figure 3.2). Most of the mosses deposited in European Miocene (24 MYA) are morphologically identical to extant European genera and even species (Dickson, 1973; Crum, 2001). Mosses embedded in Caribbean amber (20–45 MYA) could also be traced to a large extent to extant genera and species (Frahm, 1994). Therefore, some moss species might be 40–80 MY old, whereas some genera might even be 80–100 MY old (Schuster, 1983), which is also seconded by recent phylogenetic analyses (Newton et al., 2006; Heinrichs et al., 2007). Based on the observation that not as many morphological changes during their evolution can be observed in mosses than in seed plants, it is traditionally believed that this is also reflected at the molecular level. Howard Crum even wrote: 'They are, in a sense, evolutionary failures that have changed rather little since the end of the Paleozoic when the major groups were already differentiated' (Crum, 2001). But, are mosses really resilient to changes in their genomes? We try to give an answer to that question in the remainder of this introduction.

3.1.4 Genome evolution

Organisms evolve by the continuous modification of their genomes' functional regions by the fixation of mutational events. Duplication events, which strongly contribute to this fluidity, arise on different chromosomal and temporal scales, ranging from small-scale events like duplicative transposition and tandem duplication via mid-scale events like segmental duplications and chromosome duplication (i.e. aneuploidy) to large-scale events like whole-genome duplication (i.e. polyploidization) (Li et al., 2001; Taylor and Raes, 2004; De Bodt et al., 2005). Depending on the time that has passed since these

events occurred, the three types become harder to discriminate and might overlap due to multiple events at the same locus. Since this is particularly true for mid- and large-scale events, they are often referred to under the common term genome duplication.

Genome duplication, due to its effects on gene regulation and developmental processes, is considered a major mechanism for speciation and adaptation through genetic divergence in plants (Van de Peer, 2004). In the case of haploid mosses, however, other effects appear even more relevant; for example, the allopolyploidization of dioicous gametophytes might yield a monoicous plant. Indeed, moss genera or species seem to have become hermaphroditic several times and independently during evolution, given the dissipated pattern of monoicous and dioicous species across taxonomic groups (Schuster, 1983). A second advantage might be that the duplication of the genome would free the formerly haploid plant from the necessity to preserve the function of crucial single-copy genes under all circumstances, thus enhancing the potential for development of new functions.

Following regeneration of *P. patens* protoplasts, a small percentage of regenerants are diploid. However, these cannot always be distinguished from haploid plants using morphological traits alone (Schween et al., 2005). Recently, evidence was presented that polyploidization and subsequent haploidization also occurred in the history of *P. patens* (Rensing et al., 2007, 2008); the plant potentially becoming hermaphroditic through this process. Therefore, mosses seem to share with seed plants (Blanc and Wolfe, 2004; Cui et al., 2006) the ability to take advantage of whole-genome duplication. Intriguingly, the properties of tandemly arrayed genes in *P. patens* were recently found to be different from those in flowering plants (Rensing et al., 2008) and hint at the involvement of homologous recombination and gene conversion, mechanisms that could additionally serve to explain how haploid genomes survive the millennia.

Chromosome counts among bryophytes are especially divergent among mosses. Among the Funariales, to which *P. patens* belongs, the basal chromosome count of the LCA has been hypothesized to be seven, based on the fact that the majority of observed chromosome numbers is seven or a multitude of this number (for details, see Rensing et al., 2007). The haploid chromosome count of *P. patens* is 27 (Bryan, 1957; Reski et al., 1994), which would make it a putative paleopolyploid and paleoaneuploid (Rensing et al., 2007). A scheme for the hypothesized genome evolution of *P. patens* is presented in Figure 3.3, Plate 1. Given the peculiar paralogue situation in many *P. patens* gene families, namely that two pairs of two closely related paralogues exist, at least one additional polyploidization event seems to have occurred among the Funariaceae. However, this event might be hidden in the first peak of the duplication, as is the case for recent polyploidization events among domesticated crops such as cotton, wheat or sunflower (Blanc and Wolfe, 2004), or within the trailing edge of the secondary peak, as is the case for *Arabidopsis thaliana* (Maere et al., 2005). In a recent phylogenetic analysis, the age of the

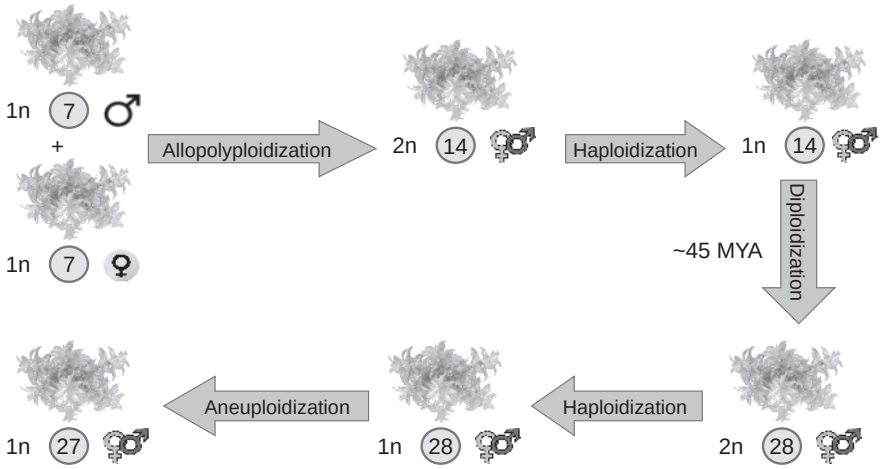


Figure 3.3 Hypothesized genome evolution of *P. patens* 1n/2n depicts a functional haploid/diploid; chromosome numbers are shown in circles; the male/female symbol shows dioicous plants, the hermaphrodite symbol shows monoicous plants. The age of the most recent genome duplication (~45 MYA) is derived by Rensing et al. (2007). (For a color version of this figure, see Plate 1)

Funariidae was determined at ~172 MYA (Newton et al., 2006). Therefore, the last whole-genome duplication, at ~45 MYA (Figures 3.2 and 3.3, Plate 1), most probably represents a duplication event that occurred after speciation among the Funariaceae. Consequently, the different chromosome counts found in extant Funariaceae species such as *P. patens*, *Funaria hygrometrica* and *Physcomitrium pyriforme* (Fritsch, 1991) have most probably occurred and been fixed several times independently during evolution.

3.1.5 Opportunities

Mosses, although their phenotype apparently is not subject to a lot of visible change across the millennia, use the same mechanisms of genome evolution as flowering plants. Therefore, they are certainly not the evolutionary failures that Crum referred to (Crum, 2001) but have instead used other strategies than their tracheophyte kin to occupy a vast number of niches and survive hundreds of millions of years. Moreover, due to its phylogenetic position, the analysis of the *P. patens* genome offers the chance to infer ancestral states of land plant evolution and to unravel the evolution of dominance among the alternating generations of land plants.

3.2 A short history of *P. patens* genomics

The first attempts to study *P. patens* genes date back to the late nineties. Genes with significant sequence similarity to annotated genes of flowering plants, as well as putative species-specific genes that were not homologous to known

genes, were found by sequencing cDNAs isolated by subtractive hybridization (Reski et al., 1998). Sequencing of cDNAs from tissue treated with abscisic acid (ABA) revealed the same and also demonstrated that the same classes of stress-induced genes were activated in moss as those known from flowering plants (Machuka et al., 1999). After that, several large-scale EST sequencing projects were undertaken around the world (e.g. UK, The *Physcomitrella* EST programme; USA; Germany (Rensing et al., 2002a), `http://www.cosmoss.org` and Japan (Nishiyama et al., 2003), `http://moss.nibb.ac.jp/`). Moreover, some of the libraries were normalized and/or subtracted, yielding a transcriptome representation of low redundancy (Lang et al., 2005).

Based on large-scale EST data, the amount of protein-encoding genes in the *P. patens* genome was estimated to be ~25 000 (Rensing et al., 2002a), that is in the same range as that of, for example, *A. thaliana*. Interestingly, the fraction of genes without detectable homologues was found to be larger for gametophore and sporophyte tissue than for protonemata (Rensing et al., 2002a), which might reflect that the protonemata express most of the house-keeping genes, whereas the gametophore and the sporophyte also express a lot of other genes that are not found in rice and *A. thaliana*. The number of genes represented in the transcriptome at this time was already very high (Rensing et al., 2002a), but most of the longer virtual transcripts were fragmentary. As more ESTs became available, the number of full-length transcripts was increased to approximately 8% (Rensing et al., 2005) and is improving.

More recently, an annotation pipeline has been developed that has allowed high-quality functional annotation of approximately 63% of the virtual transcripts (Lang et al., 2005). In addition, a Web interface has been developed that allows easy access to the virtual transcriptome (`www.cosmoss.org`). To date, nearly 400 000 ESTs have been generated from libraries spanning the complete life cycle of *P. patens* and are available as the virtual transcriptome representation 12/06 (`www.cosmoss.org`). Table 3.1 gives a detailed overview of nuclear sequence resources for *P. patens* in the Genbank database.

Table 3.1 Transcriptome resources of selected photosynthetic eukaryotes in Genbank (as of September 2007)

	ESTs	mRNA	Proteins
Populus trichocarpa	89 943	200	487
Arabidopsis thaliana	1 279 945	107 089	130 768
Oryza sativa	1 211 418	78 548	223 025
Physcomitrella patens	194 851	362	1160
Chlamydomonas reinhardtii	167 641	629	1743
Ostreococcus tauri	n.a.	n.a.	8184
Phaeodactylum tricornutum	89 139	25	341
Thalassiosira pseudonana	n.a.	26	n.a.
Cyanidioschyzon merolae	n.a.	4	629

Based on the interesting findings that were enabled through the analysis of the transcriptome and on the fact that a lot of questions can only be answered through the availability of a sequenced genome, it was suggested in 2002 that 'this is a good time to establish an international moss-genome-sequencing project to exploit *P. patens* to its fullest as a model organism for functional and comparative genomics' (Rensing et al., 2002b). At the annual moss meeting in 2004, the International Moss Genome Consortium was initiated and plans to sequence the genome formed and pursued since then.

3.2.1 The chloroplast genome

Analyses of complete chloroplast DNA have shown that algal chloroplast genomes vary across a broad range, from 89 kbp in the case of the green siphonous alga *Codium fragile* to over 1500 kbp for the genus *Acetabularia mediterranea* (Sugiura et al., 2003). In contrast to this situation, the chloroplast genomes of land plants, including bryophytes, range from 120 to 160 kbp. Within the bryophytes (liverworts, hornworts and mosses), the ~123 kbp chloroplast genome of *P. patens* (Table 3.2), sequenced in 2003 (Sugiura et al., 2003), is situated between the ~121 kbp of the liverwort *Marchantia polymorpha* plastome and the hornwort *Anthoceros formosae* (~160 kbp). The overall G/C content (28.5%) of the *P. patens* cpDNA is closer to the two other bryophytes (~29% and ~33%) and algae (~30–33%) than to the vascular plants (~38–39%) (Sugiura et al., 2003). The moss cpDNA comprises 83 protein-coding genes and 31 tRNA and 4 rRNA and a pseudo-tRNA gene (Table 3.2). Chapter 8 of this book details their specific nature and function. But the most striking difference of the *P. patens* chloroplast genome to those of other plants is the absence of the RNA polymerase alpha chain gene (*rpo*A) from the cpDNA, which has been transferred to the nuclear genome. It was revealed that this gene is present in plastid genomes of other mosses and was lost twice during the evolutionary history of bryophytes (Sugiura et al., 2003; Goffinet et al., 2005). A large inversion in the *P. patens* chloroplast genome was found that encompasses a 71-kbp region in the large single-copy (LSC) region (Sugiura et al., 2003). This inversion is the largest inversion documented in plants to date (Goffinet et al., 2007). Initially, this inversion was considered to be *P. patens* specific but afterward shown to exist in members of the Funariaceae, Disceliaceae and Encalyptales (Goffinet et al., 2007), but not in Gigaspermaceae (which, together with the three genera mentioned above, form the Funariales). Therefore, this large fragment inversion in the plastid genome is assumed to have occurred once in the ancestor of the Funariaceae, Disceliaceae and Encalyptales (Goffinet et al., 2007).

3.2.2 The mitochondrial genome

The sequence of the *P. patens* mitochondrial genome (~150 kbp) was determined in 2006 as the second bryophyte mitochondrial genome and the

Table 3.2 Overview of sequenced genomes of land plants and algae

	Nuclear genome								Plastid genome		Mitochondrial genome	
	Genome size (1C) (Mbp)	Chromo-somes (1C)	Ploidy (x)	GC (%)	Protein-coding genes	miRNAs		Repetitive/ transposable elements (% of the genome)	Genome size (kbp)	Encoded proteins	Genome size (kbp)	Encoded proteins
						#	Families					
Populus trichocarpa	485	19	2	36.7	45 555	215	33	37%	157	101	803	52
Arabidopsis thaliana	157	5	2	36.0	26 819	184	109	10%	155	87	367	117
Oryza sativa	490	12	2	43.6	~32 000	243	62	30%	135	64	492	54
Physcomitrella patens	511	27	1	38.7	~30 000	220	99	48%	132	83	105	42
Chlamydomonas reinhardtii	120	17	1	64.0	15 143	49	47	11%	204	69	15	8
Ostreococcus tauri	12	20	1	58.0	7618	n.a.	n.a.	n.a.	72	86	44	65
Phaeodactylum tricornutum	26	33	2	48.5	10 010	n.a.	n.a.		117	130	n.a.	n.a.
Thalassiosira pseudonana	32	24	2	47.0	11 390	n.a.	n.a.	2%	129	127	44	40
Cyanidioschyzon merolae	16	20	1	55.0	5014	n.a.	n.a.	6%	150	207	32	34

smallest one among the sequenced land plant chondriomes to date (Terasawa et al., 2007); 42 protein-coding as well as 24 tRNA and rRNA genes were found (Table 3.2). The presence or loss of genes could not be correlated to the phylogenetic position of *P. patens*. Nevertheless, the organellar genome was found to have retained prototype features of land plant chondriomes (Terasawa et al., 2007). Comparisons with Charophyta and liverworts resulted in the detection of a relatively close relationship between these. The multipartite structure of angiosperm chondriomes is absent in bryophytes and charophytes, indicating that this feature was established later during the evolution of seed plants. The phylogenetic analysis of the *P. patens* chondriome supports the bryophytes as a sister group of the flowering plants (Terasawa et al., 2007).

3.2.3 The amount of nuclear DNA in land plants

There is a broad range of variation in the amount of nuclear DNA across the land plants (Table 3.2). The lowest amount reported so far was for *Fragaria viridis* (strawberry; 14 chromosomes, diploid) with about 0.1 pg (1C) in contrast to ~127 pg (1C) in *Fritillaria assyriaca* (Fritillary; 48 chromosomes, tetraploid) (Leitch et al., 2005). Different land plant groups show a characteristic DNA profile. The highest and lowest C-values for land plants are observed among angiosperms, spanning three orders of magnitude (Leitch et al., 2005). In contrast, mosses occupy a comparatively narrow range from 0.2 to 2.1 pg (one order of magnitude) with an average of about 0.5 pg (1C). This small range and the comparatively low maximum C-value among the bryophytes were suggested to be the result of natural selection (Renzaglia et al., 1995). The low C-values of a narrow range were hypothesized to constitute a selective advantage and to be correlated to the biflagellate gametes of bryophytes. A significant increase in the quantity of nuclear DNA would enlarge the size of the sperm cells, limit their motility and therefore affect the efficiency of fertilization. This assumption could be supported by the fact that the lycophyte *Isoetes lacustris* (quillwort) has an up to 75 times greater C-value than those generally found in Selaginellaceae and Lycopodiaceae (Leitch et al., 2005). *I. lacustris* has multiflagellate sperm cells, suggesting that the amount of nuclear DNA is under lower selective pressure in species with multiflagellate sperm than in those with only two flagella (Leitch et al., 2005).

3.2.4 The nuclear genome

The haploid genome of *P. patens* has an estimated size of ~510 Mbp (C-value: 0.53 pg; Table 3.2), dispersed on 27 small chromosomes (Reski et al., 1994; Schween et al., 2003). The sequencing of the *P. patens* nuclear genome was funded as part of the community sequencing program of the US Department of Energy, one of the reasons being `'[t]he` moss *Physcomitrella patens* is becoming widely recognized as an experimental organism of choice

not only for basic molecular, cytological and developmental questions in plant biology, but also as a key link in understanding plant evolutionary questions, especially those related to genome evolution. *P. patens* is well placed phylogenetically to provide important comparisons with the flowering plants. [...] Having the full *P. patens* genome available will greatly inform bioinformatic comparisons and functional genomics in plants, just as the mouse, Fugu and Drosophila genomes have informed animal biology.' (`http://www.jgi.doe.gov/sequencing/why/CSP2005/physcomitrella.html`). The *P. patens* genome has been sequenced by whole-genome shotgun at the Joint Genome Institute in 2005. In addition, several fosmid clones have been sequenced as quality control and a total of nearly 300 000 ESTs have been used to improve the accuracy of gene prediction. The version 1.1 of the assembled and annotated genome was released to the public in April 2007 and is available via `http://genome.jgi-psf.org/Phypa1_1/Phypa1_1.home.html` and `http://www.cosmoss.org/cgi/gbrowse/physcome/`. The draft genome sequence of *P. patens* has been published recently (Rensing et al., 2008) and, as expected, revealed insights into the conquest of land by plants.

3.3 Features of the *P. patens* nuclear genome

3.3.1 Gene structure

In terms of gene structure, *P. patens* introns are larger than those of *A. thaliana*, yet the position and amount of introns are approximately the same (Table 3.3). In *P. patens*, the average G/C content of the coding regions is 50%, while it is ~44% in *A. thaliana*. The average codon bias is the same in the two plants, but the distribution pattern is different, with 15% of moss genes being unbiased (Rensing et al., 2005). In *P. patens*, highly expressed genes contain shorter introns, a situation known from animal, yet not from seed plant, genomes

Table 3.3 Features of *P. patens* protein-coding genes in comparison with other land plants

	***Physcomitrella* V1.1**	***Arabidopsis* Tair 7.0**	***Oryza sativa* Tigr 5.0**	***Populus trichocarpa* (Tuskan et al., 2006)**
Protein-coding genes	35 938	26 819	41 042	45 555
Average exons per gene	4.9	5.8	4.9	4.3
Average exon length (bp)	246	268	309	254
Average intron length (bp)	311	165	412	379
Average transcript length (bp)	1195.8	1466.3	1697.0	1079.0
Average CDS length (bp)	1088.5	1228.2	1348.5	987.0

(Stenoien, 2007). Interestingly, orthologues of *A. thaliana* genes show a significant drift of codon fraction usage toward the seed plant. Tools developed for splice site prediction of flowering plants were shown to exhibit low accuracy when applied to *P. patens* sequences (Rensing et al., 2005); the same is true for the prediction of sub-cellular localization of proteins. Therefore, species-specific splice site prediction has been developed and is available as an online tool (`www.cosmoss.org`).

3.3.2 Transposons

Repetitive sequences are classified into three groups (Bao and Eddy, 2002): (1) tandem repeats and simple sequence repeats (SSRs), (2) transposable elements (TEs) and (3) duplicated genomic fragments. Transposable elements alone contribute up to 80% of some plant genomes (Feschotte et al., 2002), and, together with repeats, are especially interesting in terms of evolution as they enhance the diversity of genomes. Transposons are commonly classified into two categories. Class 1 elements transpose via an RNA intermediate and either have long terminal repeats (LTR retrotransposons, prevalent families in plants are copia- and gypsy-like retrotransposons) or are terminated by a SSR that is usually polyA (the non-LTR retrotransposons, long and short interspersed nuclear elements LINEs and SINEs, respectively). Class 2 (DNA) elements transpose via a DNA intermediate, have terminal inverted repeats and are grouped according to whether they are autonomous or not.

On the basis of the *P. patens* draft genome, full-length LTR retrotransposons have been identified using LTR_seq (Kalyanaraman and Aluru, 2006). In total, 6963 full-length LTR retrotransposons with an average length of 7038 bp were identified on 676 scaffolds, with a total length of about 44 Mbp (∼10% of the assembled genome). Long-terminal repeats were found to be 778 bp long on average. Based on application of LTR_STRUC (McCarthy and McDonald, 2003), *P. patens* contains about three times more full-length LTR retrotransposons than *A. thaliana*, but about three times fewer than rice (Rensing et al., 2008). In terms of density, *P. patens* contains the lowest amount of full-length LTR transposons per Mbp. Together with nested and deteriorated TEs and repeats, nearly half of the *P. patens* genome is expected to consist of repetitive and transposable elements (Rensing et al., 2008) (Table 3.2).

3.3.3 Non-coding RNAs

Endogenous small RNAs, including microRNAs (miRNAs) and short interfering RNAs (siRNAs), are critical components of plant gene regulation. In-depth analysis of these two types of RNA in *P. patens* is described in Chapter 5 of this book. Briefly, some abundant miRNAs involved in developmental control are conserved among land plants, while many other less-abundant miRNAs appear to have emerged more recently. As indicated by the data presented in Table 3.2, 220 miRNA genes from 99 families have been described

for *P. patens* (Axtell et al., 2007; Fattash et al., 2007), which is comparable to the situation found in seed plants. In addition, the entire complement of moss small RNAs has been analyzed by high-throughput sequencing of samples from three developmental stages yielding a total of 561 102 small RNA reads that represent 214 996 unique sequences, 127 135 of which could be uniquely mapped to the genome (Axtell et al., 2006).

Using a combination of tRNAScan-SE (Schattner et al., 2005) and Aragorn (Laslett and Canback, 2004), 432 putative tRNA genes could be identified within the *P. patens* genome (`www.cosmoss.org`). A total of 417 of the tRNAs code for one of the 20 standard amino acids. In all cases, at least one of the anti-codons per amino acid was found, including a gene for a selenocysteine tRNA, but not all of the possible 57 anti-codon tRNAs seem to be used in *P. patens*. The remainder of the genes comprise two possible suppressor tRNAs (CTA, TTA), two tRNAs with undetermined/unknown isotypes and ten pseudogenes.

3.3.4 *P. patens* genes analyzed in detail

A total of 257 *P. patens* gene entries have been submitted to Genbank in the pre-genome era since the early 1990s (first entry: a Lhcb on 1 September 1989 (Long et al., 1989)), characterized in a total of 66 articles (Table 3.4). These entries correspond to about 60 different gene families, which have been described in detail for *P. patens* so far.

Besides genes for chlorophyll a/b-binding proteins, work has been done on some of the *P. patens* cytoskeletal components including FtsZ (Rensing et al., 2004), beta-tubulin (Jost et al., 2004) and ARPC4 (Perroud and Quatrano, 2006). Cell wall synthesis and flexibility has been subject to investigation in the work on expansins (Schipper et al., 2002) and cellulose synthase (Roberts and Bushoven, 2007) families. Because of *P. patens*' exceptionally high rate of gene targeting, the DNA-repair machinery has been analyzed extensively (e.g. RAD51 (Markmann-Mulisch et al., 2002), MSH2 (Trouiller et al., 2006) and RecA (Odahara et al., 2007)). A lot of work also has gone into the comparative and phylogenetic analysis of transcription factors and their role in development (e.g. HD-Zip (Sakakibara et al., 2001), MADS (Riese et al., 2005), FLO/LFY (Maizel et al., 2005), GOLDEN2-LIKE (Yasumura et al., 2005), ABI3-like (Marella et al., 2006) and Rhd6-like (Menand et al., 2007)), establishing *P. patens* as an 'evo-devo' model. Furthermore, several aspects of *P. patens*'s primary metabolism (e.g. Delta6-elongase (Zank et al., 2002), APR (Koprivova et al., 2002) and hexokinase (Olsson et al., 2003)), secondary metabolism (e.g. polyphenol oxidase (Richter et al., 2005), chalcone synthase (Jiang et al., 2006), ent-kaurene synthase (Hayashi et al., 2006) and tRNA-isopentenyltransferase (Yevdakova and von Schwartzenberg, 2007)), signaling (PLC (Mikami et al., 2004), phytochrome (Mittmann et al., 2004), Snf1-related protein kinase and SKI (Thelander et al., 2007) and stress response (LEA, (Kamisugi and Cuming, 2005)), Shaggy kinase (Richard

Table 3.4 Pre-genome publications describing *P. patens* gene families

PubMed ID	Title	Authors	Referenced genes
17689888	Comparative analysis of the SBP-box gene families in *P. patens* and seed plants.	Riese et al., 2007	7
17556514	Exclusion of Na+ via sodium ATPase (PpENA1) ensures normal growth of *Physcomitrella patens* under moderate salt stress.	Lunde et al., 2007	1
17627823	Step-by-step acquisition of the gibberellin-DELLA growth-regulatory mechanism during land plant evolution.	Yasumura et al., 2007	2
17450376	Characterization of a prokaryote-type tRNA-isopentenyltransferase gene from the moss *Physcomitrella patens*.	Yevdakova and von Schwartzenberg, 2007	1
17533513	The moss genes PpSKI1 and PpSKI2 encode nuclear SnRK1 interacting proteins with homologues in vascular plants.	Thelander et al. 2007	2
17556585	An ancient mechanism controls the development of cells with a rooting function in land plants.	Menand et al., 2007	8
17283080	A Pentatricopeptide repeat protein is required for RNA processing of clpP Pre-mRNA in moss chloroplasts.	Hattori et al., 2007	2
17396019	Involvement of mitochondrial-targeted RecA in the repair of mitochondrial DNA in the moss, *Physcomitrella patens*.	Odahara et al., 2007	2
17289796	Distinct roles of nitrate and nitrite in regulation of expression of the nitrate transport genes in the moss *Physcomitrella patens*.	Tsujimoto et al., 2007	18
17006591	The cellulose synthase (CESA) gene superfamily of the moss *Physcomitrella patens*.	Roberts and Bushoven, 2007	19
17083952	Cloning and characterization of chalcone synthase from the moss, *Physcomitrella patens*.	Jiang et al., 2006	1
17064690	Identification and functional analysis of bifunctional ent-kaurene synthase from the moss *Physcomitrella patens*.	Hayashi et al., 2006	1
16805735	Characterization and functional analysis of ABSCISIC ACID INSENSITIVE3-like genes from *Physcomitrella patens*.	Marella et al., 2006	3

(*Continued*)

Table 3.4 (*Continued*)

PubMed ID	Title	Authors	Referenced genes
16618924	Genes for the peptidoglycan synthesis pathway are essential for chloroplast division in moss.	Machida et al., 2006	12
16450411	The role of ARPC4 in tip growth and alignment of the polar axis in filaments of *Physcomitrella patens*.	Perroud and Quatrano 2006	1
16429263	Gene structure and expression pattern analysis of three monodehydroascorbate reductase (Mdhar) genes in *Physcomitrella patens*: implications for the evolution of the MDHAR family in plants.	Lunde et al., 2006	3
16397301	MSH2 is essential for the preservation of genome integrity and prevents homeologous recombination in the moss *Physcomitrella patens*.	Trouiller et al., 2006	2
16367967	A dehydrin gene in *Physcomitrella patens* is required for salt and osmotic stress tolerance.	Saavedra et al., 2006	1
16270226	The evolution of the abscisic acid-response in land plants: comparative analysis of group 1 LEA gene expression in moss and cereals.	Kamisugi and Cuming, 2005	2
16113222	A novel plant major intrinsic protein in *Physcomitrella patens* most similar to bacterial glycerol channels.	Gustavsson et al., 2005	2
16007489	Organization and expression of the GSK3/shaggy kinase gene family in the moss *Physcomitrella patens* suggest early gene multiplication in land plants and an ancestral response to osmotic stress.	Richard et al., 2005	8
15923345	A conserved transcription factor mediates nuclear control of organelle biogenesis in anciently diverged land plants.	Yasumura et al., 2005	2
15912446	The family of CONSTANS-like genes in *Physcomitrella patens*.	Zobell et al., 2005	3
15912448	Identification and characterization of a bryophyte polyphenol oxidase encoding gene from *Physcomitrella patens*.	Richter et al., 2005	1
15912451	Isolation and characterization of new MIKC*-Type MADS-box genes from the moss *Physcomitrella patens*.	Riese et al., 2005	4

Table 3.4 (*Continued*)

PubMed ID	Title	Authors	Referenced genes
15743879	Diversification of gene function: homologues of the floral regulator FLO/LFY control the first zygotic cell division in the moss *Physcomitrella patens*.	Tanahashi et al., 2005	2
15604751	Germin-like protein gene family of a moss, *Physcomitrella patens*, phylogenetically falls into two characteristic new clades.	Nakata et al., 2004	14
15563615	Differential expression on a daily basis of plastid sigma factor genes from the moss *Physcomitrella patens*. Regulatory interactions among PpSig5, the circadian clock, and blue light signaling mediated by cryptochromes.	Ichikawa et al., 2004	1
15588585	Identification and characterization of cDNAs encoding pentatricopeptide repeat proteins in the basal land plant, the moss *Physcomitrella patens*.	Hattori et al., 2004	11
15556303	A large plant beta-tubulin family with minimal C-terminal variation but differences in expression.	Jost et al., 2004	6
15365180	Targeted knockout in Physcomitrella reveals direct actions of phytochrome in the cytoplasm.	Mittmann et al., 2004	4
15310072	Loss of GH3 function does not affect phytochrome-mediated development in a moss, *Physcomitrella patens*.	Bierfreund et al., 2004	4
15247376	Phototropins mediate blue and red light-induced chloroplast movements in *Physcomitrella patens*.	Kasahara et al., 2004	4
15260436	A novel aspartic proteinase is targeted to the secretory pathway and to the vacuole in the moss *Physcomitrella patens*.	Schaaf et al., 2004	1
15073208	Isolation of cDNAs encoding typical and novel types of phosphoinositide-specific phospholipase C from the moss *Physcomitrella patens*.	Mikami et al., 2004	2
15057278	Snf1-related protein kinase 1 is needed for growth in a normal day-night light cycle.	Thelander et al., 2004	4
15063776	Calmodulin-binding proteins in bryophytes: identification of abscisic acid-, cold-, and osmotic stress-induced genes encoding novel membrane-bound transporter-like proteins.	Takezawa and Minami, 2004	2
15042335	Diversification of ftsZ during early land plant evolution.	Rensing et al., 2004	2

(*Continued*)

Table 3.4 (*Continued*)

PubMed ID	Title	Authors	Referenced genes
14749485	Cloning and characterization of glycine-rich RNA-binding protein cDNAs in the moss *Physcomitrella patens*.	Nomata et al., 2004	3
14749487	Circadian expression of the PpLhcb2 gene encoding a major light-harvesting chlorophyll a/b-binding protein in the moss *Physcomitrella patens*.	Aoki et al., 2004	2
14617094	Molecular cloning and characterization of a sodium-pump ATPase of the moss *Physcomitrella patens*.	Benito and Rodriguez-Navarro, 2003	8
12941966	A novel type of chloroplast stromal hexokinase is the major glucose-phosphorylating enzyme in the moss *Physcomitrella patens*.	Olsson et al., 2003	2
12860996	Characterization of a novel plant PP2C-like protein Ser/Thr phosphatase as a calmodulin-binding protein.	Takezawa, 2003	1
12917289	Involvement of auxin and a homeodomain-leucine zipper I gene in rhizoid development of the moss *Physcomitrella patens*.	Sakakibara et al., 2003	4
12559580	Sucrose-phosphatase gene families in plants.	Lunn, 2003	1
12399933	A novel gene family in moss (*Physcomitrella patens*) shows sequence homology and a phylogenetic relationship with the TIR-NBS class of plant disease resistance genes.	Akita and Valkonen, 2002	18
12374308	Expansins in the bryophyte *Physcomitrella patens*.	Schipper et al., 2002	1
12070175	Functional knockout of the adenosine 5′-phosphosulfate reductase gene in *Physcomitrella patens* revives an old route of sulfate assimilation.	Koprivova et al., 2002	2
12164806	Cloning and functional characterization of an enzyme involved in the elongation of Delta6-polyunsaturated fatty acids from the moss *Physcomitrella patens*.	Zank et al., 2002	2
12062804	Two RpoT genes of *Physcomitrella patens* encode phage-type RNA polymerases with dual targeting to mitochondria and plastids.	Richter et al., 2002	2
12032236	Two ancient classes of MIKC-type MADS-box genes are present in the moss *Physcomitrella patens*.	Henschel et al., 2002	8
11976967	Characterization of three PDI-like genes in *Physcomitrella patens* and construction of knock-out mutants.	Meiri et al., 2002	3

Table 3.4 (*Continued*)

PubMed ID	Title	Authors	Referenced genes
11884681	Cryptochrome light signals control development to suppress auxin sensitivity in the moss *Physcomitrella patens*.	Imaizumi et al., 2002	3
11880641	The organization of *Physcomitrella patens* RAD51 genes is unique among eukaryotic organisms.	Markmann-Mulisch et al., 2002	4
11516147	Photoaffinity labelling with the cytokinin agonist azido-CPPU of a 34 kDa peptide of the intracellular pathogenesis-related protein family in the moss *Physcomitrella patens*.	Gonneau et al., 2001	1
11418118	Characterization of two genes, Sig1 and Sig2, encoding distinct plastid sigma factors(1) in the moss *Physcomitrella patens*: phylogenetic relationships to plastid sigma factors in higher plants.	Hara et al., 2001b	3
11342113	Cloning and characterization of the cDNA for a plastid sigma factor from the moss *Physcomitrella patens*.	Hara et al., 2001a	1
11264400	Isolation of homeodomain-leucine zipper genes from the moss *Physcomitrella patens* and the evolution of homeodomain-leucine zipper genes in land plants.	Sakakibara et al., 2001	10
11076976	Visualization of a cytoskeleton-like FtsZ network in chloroplasts.	Kiessling et al., 2000	3
10743663	c-MYB oncogene-like genes encoding three MYB repeats occur in all major plant lineages.	Kranz et al., 2000	2
9744093	Identification of a novel delta 6-acyl-group desaturase by targeted gene disruption in *Physcomitrella patens*.	Girke et al., 1998	2
9680981	Cloning and characterization of an adenosine kinase from Physcomitrella involved in cytokinin metabolism.	von Schwartzenberg et al., 1998	1
9539743	Plant nuclear gene knockout reveals a role in plastid division for the homologue of the bacterial cell division protein FtsZ, an ancestral tubulin.	Strepp et al., 1998	1
8224238	Mosses do express conventional, distantly B-type-related phytochromes. Phytochrome of *Physcomitrella patens* (Hedw.).	Kolukisaoglu et al., 1993	1
8401607	Expression of myb-related genes in the moss, *Physcomitrella patens*.	Leech et al., 1993	2
2473942	Cloning and nucleotide sequence analysis of genes coding for the major chlorophyll-binding protein of the moss *Physcomitrella patens* and the halotolerant alga *Dunaliella salina*.	Long et al., 1989	1

et al., 2005) and dehydrin (Saavedra et al., 2006) have been analyzed in more detail. This list of genes studied nicely reflects the evolution of the moss as a model system for nearly all aspects of modern plant biology. Table 3.4 gives a more detailed overview of publications according to the PubMed articles that are referred to in the Genbank *P. patens* sequence entries.

3.4 Comparisons with seed plants and algae

While the number of protein-coding genes in *P. patens* and *A. thaliana* is similar, the *P. patens* genome is about four times larger, that is, the genome is less tightly packed (Table 3.2). Mapping of homologues in general and orthologues and paralogues in particular against the *A. thaliana* chromosomes revealed a random pattern, that is, no hot or cold spots (Rensing et al., 2002a, 2005). Based on the fact that homologues of a high proportion of *A. thaliana* genes are expressed in the *P. patens* gametophyte, it was hypothesized that gametophytic genes were recruited by the sporophyte during land plant evolution (Nishiyama et al., 2003).

3.4.1 Ancestry of genes and gene transfer

Based on virtual transcripts, only ~50% of the *P. patens* protein genes had detectable homologues in other genomes and the average gene family size in the moss was found to be smaller than that in *A. thaliana* (Rensing et al., 2002a). Based on mate-pair connected ESTs, that is, virtual transcripts that represent a larger fraction of the actual transcript, the rate of protein-coding genes with homologues in other sequenced genomes increased to ~60% (Nishiyama et al., 2003). In this study, evidence for retained genes (i.e. ancient genes present in mosses that have been lost during seed plant evolution) and genes that were acquired by horizontal gene transfer (HGT) was found. Also, a total of 8% of the *P. patens* transcripts was found to have their closest sequence homologue not among the genes of seed plants. In a later analysis, more than 130 genes were found that were either lost during seed plant evolution or acquired by HGT (Rensing et al., 2005). The distribution of functional classes among these genes was found to be uneven among taxa. Several genes with intriguing putative functions, such as transport, cytotoxicity (e.g. murein degradation), metabolism, signaling and DNA repair, were found. Also, a novel plant major intrinsic protein in *P. patens,* most similar to bacterial glycerol channels, has been reported to have been introduced most likely by HGT (Gustavsson et al., 2005). An analysis based on Gene Ontology (GO) annotation revealed that the fraction of gene products involved in metabolism is significantly higher in mosses (70–80%) than in seed plants (10–44%) (Lang et al., 2005).

3.4.2 Orthologues shared among photosynthetic eukaryotes

In terms of evolutionary distance, mosses are to flowering plants as fish are to mammals (Benton and Donoghue, 2007). Therefore, *P. patens* holds

a key position that bridges the phylogenetic gap between algae and flowering plants. This can be nicely exploited to inform comparative analyses and provides insight into the evolution of land plants. In a comparative genomics approach using extended reciprocal BLAST searches, a set of common orthologues shared among all photosynthetic eukaryotes has recently been analyzed (Zimmer et al., 2007). All major phyla were represented by using the protein-encoding genes (besides *P. patens*) of *A. thaliana* (eudicotelydons), *Oryza sativa* (Liliopsida), *Pinus taeda* (Gymnospermae), *Chlamydomonas reinhardtii* (Chlorophyta) and *Cyanidioschyzon merolae* (Rhodophyta). The evolutionary time span covered by this set of organisms is approximately 1.5 BY (Hedges et al., 2004).

As shown in Figure 3.4, 9497 shared orthologous genes were observed between *O. sativa* and *A. thaliana*. These two plants have 2105 such genes in common with the gymnosperm *P. taeda.* Adding *P. patens* reduces the amount of shared orthologues among the Embryophyta to a quarter (536 genes). Only about 204 genes could be detected if the green alga was included in the analysis to complete the green lineage. With the inclusion of the red alga

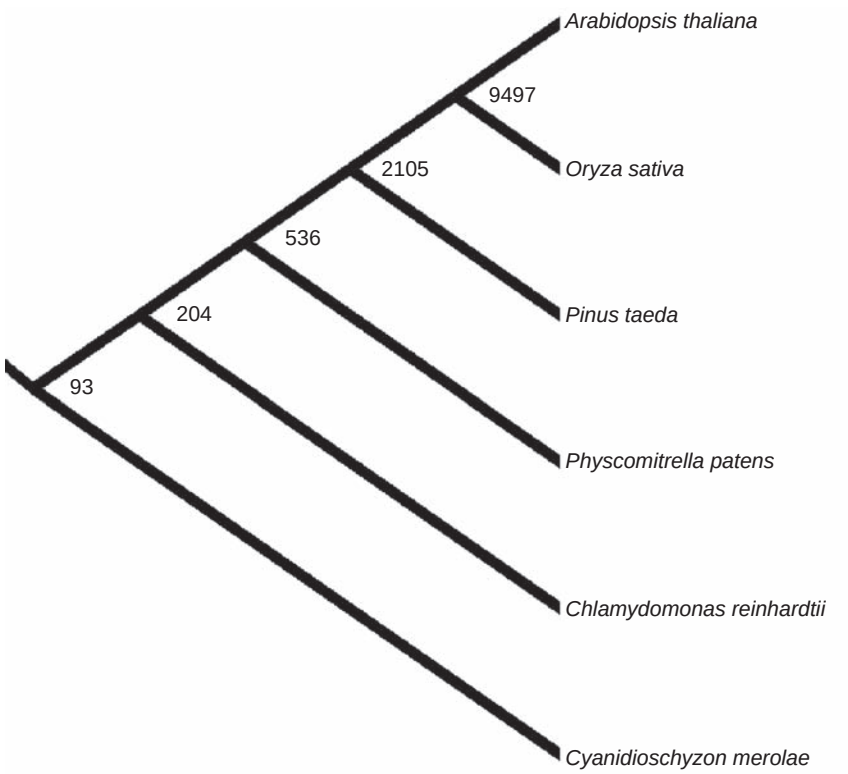


Figure 3.4 Phylogenetic relationship among the six eukaryotic organisms analyzed. Orthologues were determined by comparisons between all six species and counted only if present in every species that contributes to the respective node. Numbers at the nodes represent the number of orthologues shared by all members of that cluster.

C. merolae, the number of orthologues finally dropped to 93 clusters among the photosynthetic eukaryotes, of which 51% are single-copy genes. These genes had already been established before the split of the red and the green lineage at least 1 BYA (Hedges et al., 2004).

Plastids have arisen by engulfment of a free-living cyanobacterial-like prokaryote and subsequent establishment of endosymbiosis. During evolution, the majority of prokaryotic genes have been transferred to other compartments, mainly to the nucleus, while the gene products are targeted to the plastid as well as other destinations (Martin et al., 2002). The same holds true for mitochondria, which share their LCA with extant alpha-Proteobacteria and were established prior to plastids. The contribution of the ancestral genomes to the common orthologues of living photosynthetic eukaryotes was investigated using BLAST searches against all known proteins of Cyanobacteria, alpha-Proteobacteria and non-photosynthetic eukaryotes.

According to this analysis, 9.7% of the orthologues were inherited from the chondriome, 25.8% from the plastome and 64.5% from the ancestral eukaryotic nuclear genome (Figure 3.5a). Interestingly, the inheritance of a given gene is not correlated with the present-day localization of its gene product. It seems as if the assignment of specialized functions to the semiautonomous organelles (like energy metabolism to mitochondria and lipid metabolism to plastids) eventually determined into which compartment the gene products are targeted (Figure 3.5b). Those pathways that are significantly overrepresented among the different gene inheritance and protein localization categories shown in Figure 3.5b demonstrate that while the main function of mitochondria is energy metabolism, they contributed genes from other categories (metabolism of cofactors and vitamins, nucleotides and amino acids) as well. While plastids contributed genes for lipid metabolism and still host the related pathways, they are also the plant cell's production area of secondary metabolites, cofactors and vitamins. The ancestral nuclear genome provided the genetic complement of protein biosynthesis and degradation and these processes are still carried out in the cytoplasm.

3.4.3 The peptidoglycan pathway is still functional in non-seed plants

Treatment of *P. patens* with different β-lactam antibiotics causes the occurrence of macrochloroplasts (Kasten and Reski, 1997). It was observed that the antibiotics inhibit the division of chloroplasts and therefore lead to enlarged chloroplasts. Upon removal of the antibiotics, the enlarged chloroplasts start to divide again. Treatment of the liverwort *M. polymorpha* (Tounou et al., 2002) and the pteridophyte *Selaginella nipponica* (Izumi et al., 2003) with β-lactam antibiotics also leads to the appearance of macrochloroplasts. This is not the case, for example, in the angiosperm *Solanum esculentum* (Kasten and Reski, 1997); β-lactams are even used as antibiotics against contaminating bacteria

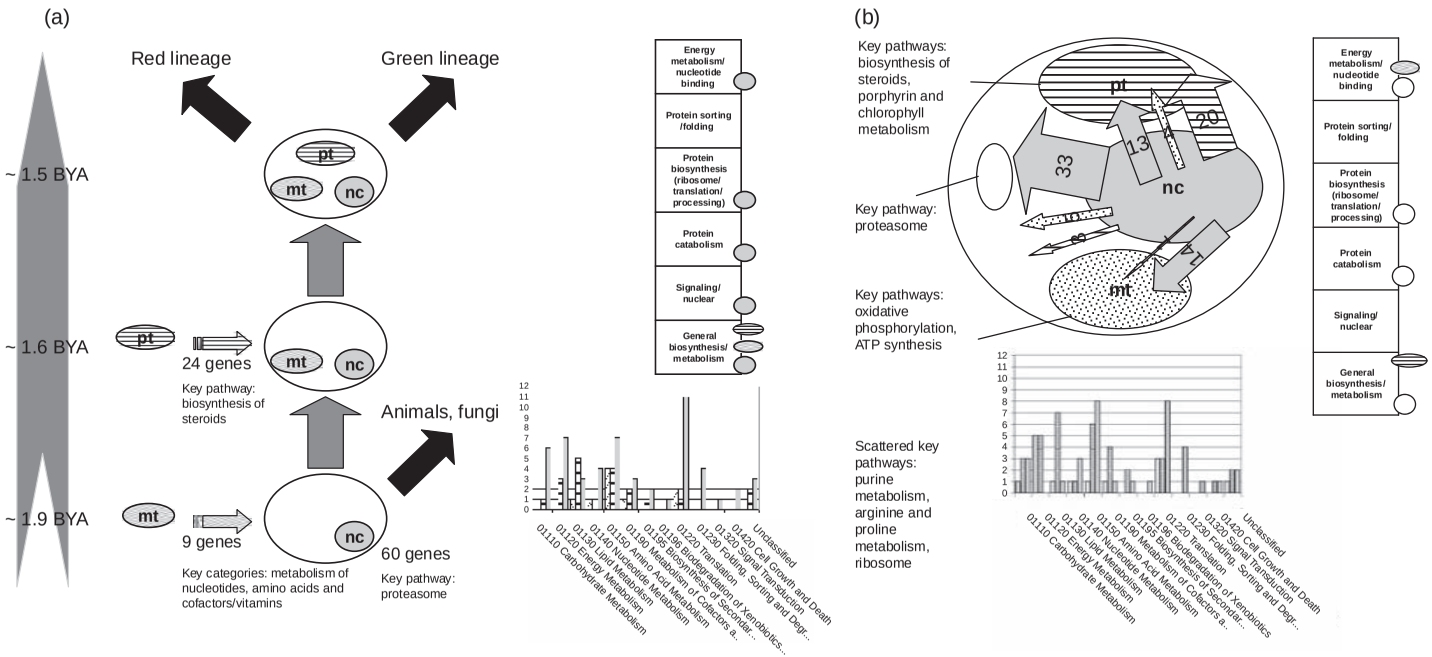


Figure 3.5 Early evolution and pathway annotation of (predominantly single-copy) orthologues shared by photosynthetic eukaryotes. (a) Early evolution of plant cells: endosymbiosis of an alpha-proteobacterial-like prokaryotic cell (dotted) by an ancestral eukaryotic host cell and subsequent engulfment of a cyanobacterial cell (dashed) led to the establishment of mitochondria and plastids (mt, mitochondrion; pt, plastid; nc, nucleus). The number of common orthologous genes that was inherited from the three genetic compartments is shown as well as the pathways that are enriched among these sets (percentage of KEGG terms significantly deviating in terms of absolute average deviation, AAD). The vertical box displays GO categories that contain more than 5% of the 93 orthologues within the respective gene set (depicted by icons for mitochondria, plastids and nucleus). The bar chart displays the KEGG terms assigned to each of the three gene sets. (b) Schematic compartmentation of a plant cell. The origin of the present-day nuclear-encoded orthologous genes is shown by the shading described in (a) together with the localization of the gene products (arrow head toward mitochondrion, plastid or cytoplasm) and the number of genes comprising that category (numbers on the arrows). The pathways that are enriched among the gene products are shown, divided by sub-cellular localization (significant deviation in terms of AAD). The vertical box displays GO categories that contain more than 5% of the 93 orthologues within the respective protein location (depicted by icons for mitochondria, plastids and cytoplasm). The bar chart displays the KEGG terms assigned to each of the three localization sets.

during the transformation of seed plants. Thus, the peptidoglycan synthetic pathway seems not to be involved in the plastid biogenesis of seed plants. While the genome of *A. thaliana* contains five genes that are essential for peptidoglycan synthesis, the function of those genes has not been determined. The *P. patens* genome contains nine homologues related to the peptidoglycan pathway (Katayama et al., 2003; Machida et al., 2006). These results suggest that not just the single gene responsible for penicillin binding is involved in chloroplast division in moss, but nearly the whole peptidoglycan biosynthesis pathway. The results even indicate that the *P. patens* chloroplasts have bacterial cell wall-like structures. However, these could not be visualized by electron microscopy. Apparently, bacteria, cyanelles (Kies, 1988) as well as fern and bryophyte chloroplasts share peptidoglycan genes involved in their division mechanism that is sensitive to β-lactam antibiotics and that may have been modified or lost in seed plant chloroplasts and is also absent in red algae (Matsuzaki et al., 2004; Machida et al., 2006).

3.4.4 Transcription-associated proteins

As already indicated by the mere number of examples in the above list (Table 3.4), *P. patens* is widely used to study transcription factors and transcriptional regulation. Its phylogenetic position predetermines its use for evolutionary developmental studies ('evo-devo') to unravel the conquest of the land by plants and enables inference of the ancestral state of land plant transcriptional regulation. Reprogramming the set of transcribed genes during development or physiological adaptation requires modulated activation and deactivation of regulatory factors. In eukaryotes, the transcription of protein-coding genes is controlled by complex networks of transcription-associated proteins (TAPs). Specific transcription factors (TFs) activate or repress transcription of their target genes by binding to *cis*-active elements. Further transcriptional regulators (TRs) include the following: (1) co-activators and co-repressors, which bind and influence TFs; (2) general transcription initiation factors, which recognize core promoter elements and recruit components of the basal transcription machinery; and (3) chromatin remodeling factors, which affect the accessibility of DNA through histone modifications and DNA methylation.

Recently, the PlanTAP database (Richardt et al., 2007) was constructed; it is a phylogeny-based resource (`http://www.cosmoss.org/bm/plantapdb`) of plant TAPs incorporating TAP sequences (besides *P. patens*) from the diatom *Thalassiosira pseudonana*, the red alga *C. merolae*, the green alga *C. reinhardtii*, the Liliopsida *O. sativa* and the eudicotyledonae *A. thaliana*. Starting with the complete set of *P. patens* candidate TAP genes, homologues were collected and annotated manually. From the resulting ample pool of TAP genes, taxonomic distribution, lineage-specific expansion and high-quality phylogenies were inferred. In total, PlanTAPDB contains 138 TAP protein families, 59 of which are TFs, 56 TRs and 23 are families with unknown

function and/or domains that are possibly associated with transcriptional regulation (putative TAPs, PT).

The analysis revealed that the *P. patens* genome contains most of the TAP proteins found in seed plants. Only six TF families found in seed plants (C2C2-YABBY, NOZZLE [NZZ], PBF-2-like/Whirly, S1Fa-like, STERILE APETALA [SAP] and ULTRAPETALA [ULT]) appear to be missing from the moss gene complement. The TF gene families that are absent from moss are all of small size and have specialized functions in flower development and lateral organ formation of the sporophyte in flowering plants and thus have probably emerged after the evolutionary split of mosses and seed plants.

There is a trend that total amounts of TAPs are associated with the number of cell types in the respective organism. A correlation of numbers of TFs with organism complexity has previously been described for animals (Levine and Tjian, 2003). The fraction of TAPs per genome depicts the same trend of association with the number of cell types. On average, TAP gene families are two to three times larger in *P. patens* than in the three algae, while in *A. thaliana* and rice, the TR and PT families show an approximately fourfold increase and TF families a ninefold increase as compared to the algae. Figure 3.6 shows a comparison of the 14 largest TF families between *A. thaliana*, *P. patens* and *C. reinhardtii*.

A relationship between the increasing number of plant TAP families and the gain in morphological complexity has been hypothesized before (Doebley

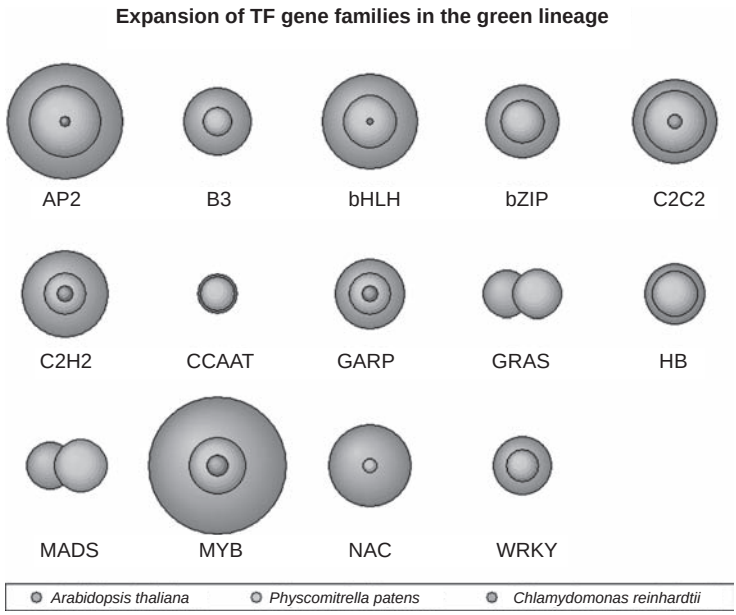


Figure 3.6 Comparison of transcription factor family sizes between *A. thaliana*, *P. patens* and *C. reinhardtii*. (*Source*: Courtesy of S. Richardt.) (For a color version of this figure, see Plate 2)

and Lukens, 1998; Hsia and McGinnis, 2003; Gutierrez et al., 2004). Basal multicellular metazoans already contain most of the TF families present in mammals (Riechmann et al., 2000; Messina et al., 2004; Reece-Hoyes et al., 2005; Larroux et al., 2006). This, however, does not seem to be the case for the comparison of single-cellular algae and land plants. Thus, the explosion of gene family number in land plants might also be related to the switch from unicellularity to multicellularity. The inclusion of the forthcoming genomes of multicellular algae such as *Volvox carteri* (closely related to *C. reinhardtii*) and *Ectocarpus siliculosus* (a brown alga) will further inform these analyses.

Gene families that were subject to individual expansion against the background of lineage-specific evolution, that is, families in which above-average expansion of distinct gene families per species occurred, were also determined. In total, 29 families exhibit species-specific expansion, 2 of which are present in *A. thaliana*, 1 in rice, 10 in *P. patens*, 4 in *C. reinhardtii*, 9 in *C. merolae*, and 3 in *T. pseudonana*. Moss and the algae contain more specifically expanded TAP families (e.g. HIT and CONSTITUTIVE PHOTOMORPHOGENIC1 [COP1] in *P. patens*, PcG and SBP in *C. reinhardtii*, FHA and TFb2 in *C. merolae*, and DUF833 in *T. pseudonana*) than the two seed plants, which might be due to the fact that the overall expansion rate is less pronounced in the non-seed plants organisms.

Visualization of the taxonomic profiles can be used to yield further insight into the distribution of certain families or clusters of families among taxonomic groups. It can, for example, be applied to narrow down the probable function of PTs, such as PT007 (DUF296 and HMG DNA-binding domain containing), which is located in a significantly correlated cluster that is mainly composed of plant-specific TFs. The hypothesis that PT007 might represent a novel TF family is strengthened by the domain structure of the members, most of which contain the two PFAM domains AT_hook (PF02178) and DUF296 (PF03479), which are known to be present in this particular order in a class of proteins that is thought to have DNA-binding activity. Over-expression of a protein containing DUF296 led to late flowering and modified leaf development in *A. thaliana* (Weigel et al., 2000).

3.5 Computational resources for *P. patens*

BLAST services that allow searching against the assembled transcriptome (Physcobase, `www.cosmoss.org`) and the genome (traces, assembly, gene models, JGI and `www.cosmoss.org`) are available as well as visualization of functional annotation (genome: JGI, `www.cosmoss.org`; transcriptome: `www.cosmoss.org`). The *P. patens* ecotype map, containing isolates from around the globe, is available at `www.cosmoss.org`; the International Moss Stock Center (IMSC) enables cryo-storage and retrieval of isolates and mutant lines. Information about SSR markers, the genetic linkage map (under development), UTR repeats, miRNAs, species-specific splice site prediction and

literature can be retrieved from www.cosmoss.org. PlanTAPDB, a phylogeny-based resource of plant transcription associated proteins offers precomputed phylogenies, domain structure visualization and more. A genome browser is available at the JGI and an integrated genome browser, including information on repeats, transposons, miRNAs, markers, linkage groups, tRNAs, plant homology evidence and many more features, is available at www.cosmoss.org.

3.5.1 Links

Cosmoss http://www.cosmoss.org
Cosmoss genome browser http://www.cosmoss.org/cgi/gbrowse/physcome
IMSC http://www.moss-stock-center.org
JGI genome browser http://genome.jgi-psf.org/Phypa1_1/Phypa1_1.home.html
Physcobase http://moss.nibb.ac.jp/
PlanTAPDB http://www.cosmoss.org/bm/plantapdb
The moss genome homepage http://www.mossgenome.org/

3.6 Conclusions and outlook

The notion that large-scale sequencing projects of the *P. patens* transcriptome and genome would greatly inform our understanding of early land plant evolution has certainly been justified (Rensing et al., 2008). The availability of the first genome of a non-seed plant has already led to interesting revelations, such as evolution of the floral regulator LEAFY (Maizel et al., 2005), the abundance and redundancy of metabolism in moss (Lang et al., 2005; Rensing et al., 2007) or the ancestry of regulators for rooting functions in plants (Menand et al., 2007). Future research is expected to unravel the early evolution of plants further. An intriguing question is how much of the increase in complexity of transcriptional regulation is due to the switch from unicellularity to multicellularity as opposed to the water to land transition. Another open question is the evolution of sporophyte dominance: Did the land plant sporophyte evolve multicellularity, independence and dominance gradually, as is suggested by some (H. Schneider, personal communication)? How did the approximate isomorphic phases of early land plants (e.g. Rhynophytes) evolve into extant flowering plants? Which divergently evolved ancestral gene families enabled mosses to occupy drastically different ecological niches than flowering plants? The availability of the *P. patens* genome and comparison with genomes that have recently been sequenced, such as the lycophyte *S. moellendorffii* and the multicellular brown alga *E. siliculosus*, are expected to help answer these questions.

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Chapter 4

GENE TARGETING

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Abstract: *Physcomitrella patens* is a model plant for comparative genomic analysis, and is the only plant model in which 'gene targeting' – the replacement of an endogenous gene by a genetically altered variant transgene – occurs with high efficiency. We describe the parameters that determine the efficiency of gene targeting and discuss the nature of the integration events that occur following transformation of *P. patens*. Integration of transgenes utilises endogenous mechanisms for the repair of DNA double-strand breaks, and the preferential integration of transforming DNA at homologous sites by *P. patens* indicates that the predominating mechanism for DNA repair is based on homologous recombination, rather than non-homologous end joining, as is the case in flowering plants. *P. patens* thus provides a useful experimental model for the study of the HR-mediated DNA repair pathway in plants, and understanding how this mechanism operates in *P. patens* may pave the way for the development of more efficient gene targeting strategies for the genetic modification of crop species.

Keywords: allele replacement; DNA double-strand break repair; gene targeting; homologous recombination; non-homologous end joining; *Physcomitrella patens*

4.1 Introduction

'Gene targeting' describes the delivery of exogenous DNA to an organism such that this DNA integrates, as a transgene, at a precisely defined site in the recipient genome. The key feature of such genetic transformation is that the site of DNA integration is predetermined. This distinguishes the procedure from experimental approaches in which populations of transgenic organisms are generated through the delivery of DNA vectors that integrate essentially at random, but whose site of integration in the genome may subsequently be determined through DNA sequence analysis of the transgenic locus. Gene targeting requires that the exogenously supplied DNA be able to seek out specific sequences in the target genome with which it shares sequence homology, and that it is then inserted into the homologous genomic sequence.

Gene targeting is thus an elegant and powerful tool for genetic manipulation. The technique is elegant, because precise changes to specific genes can be designed and undertaken, without the recourse to large-scale, high-throughput transformation strategies used, for example, in the creation of collections of insertionally mutagenised organisms by random DNA integration. The technique is powerful because of the range of modifications that can be made to individual genes. Whilst these might include insertional mutagenesis through the delivery of a gene disruption cassette (gene 'knockout'), the technique also enables other modifications. These include the deletion of all, or part of a gene, the insertion (or 'knock-in') of reporter sequences such as enzymes (β-glucuronidase, luciferase, GFP and other fluorescence derivatives), and affinity- or epitope-tag sequences (His-tags, TAP-tags, myc-tags, etc.), and the defined alteration of coding sequences by as little as a single base pair, in order to engineer the structure or activity of a specific gene or gene product with surgical precision.

4.2 Gene targeting in eukaryotes

In one of the first studies of the genetic transformation of *Saccharomyces cerevisiae*, Hinnen et al. (1978) demonstrated that when a plasmid DNA containing a wild-type *LEU2* gene was introduced to a *leu2* mutant strain, that not only did the plasmid DNA integrate stably into the genome, thus restoring the wild-type phenotype, but also that in some instances, the transforming DNA replaced the mutant gene within the genome. This was the first demonstration that an endogenous eukaryotic locus could be targeted, and subsequently the efficiency of gene targeting was found to be substantially enhanced if a double-strand DNA break was introduced into the targeting sequence prior to delivery (e.g. by cutting with a restriction enzyme: Orr-Weaver et al., 1981), to create a vector of a type generically described as an 'ends-in' vector (Figure 4.1a). This implied that the incidence of gene targeting was intimately associated with the activity of an endogenous DNA-damage repair mechanism that utilised homologous sequences as a means of repairing double-strand breaks (DSBs) in DNA.

The wider utility of this strategy was further extended to the targeted transformation of mammalian cells (Thomas et al., 1986), in which cells carrying a transgenic recombination target showed a relatively high rate of targeted transformation relative to random insertion (ca. 1:100), following microinjection of an 'ends-in' vector. This approach was followed by the development of an alternative approach utilising so-called 'ends-out' vectors – in which a linear transforming molecule comprised a selectable marker cassette flanked by termini that were directly homologous with the targeted locus flanking (Thomas and Capecchi, 1987) (Figure 4.1b).

Such vectors cause the disruption of the target locus, and in the case of ends-in vectors, the insertion of plasmid vector backbone. This strategy thus

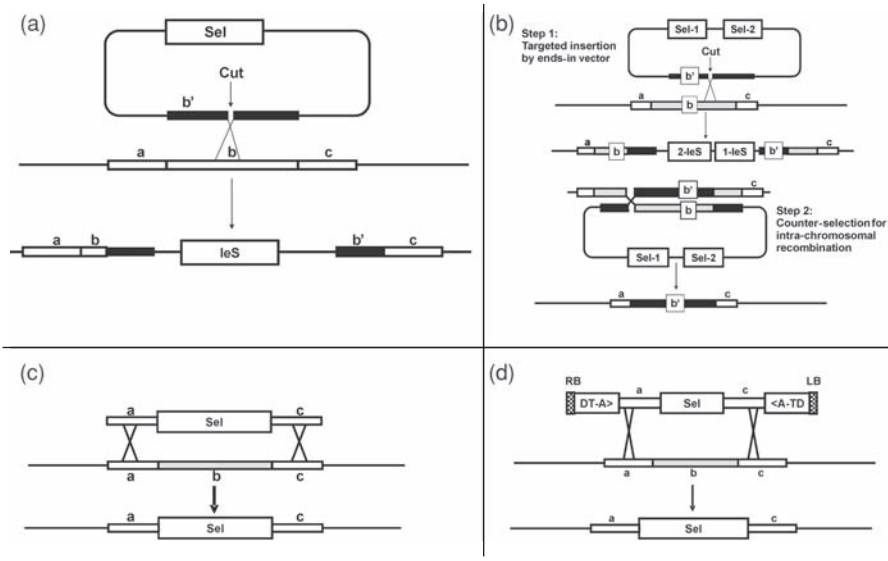

Figure 4.1 Different types of gene targeting vector. (a) 'Ends-in' targeting vectors comprise plasmids containing a length of genomic sequence homology (sequence 'b') that contains a unique site for a restriction enzyme, enabling the creation of a DNA DSB that can be used to initiate DSB repair with its genomic target (sequence 'b'). The entire plasmid is thus inserted at the targeted site. (b) The 'hit-and-run' strategy for targeted mutagenesis. An 'ends-in' vector integrates in the genome and its presence is selected by positive selection ('Sel1'). The vector also contains a counter-selective marker ('Sel-2'). Targeted transformants are then incubated under conditions that render the counter-selective marker cytotoxic. Intra-chromosomal recombination events that delete the vector and selection markers are thus selected for (c) 'Ends-out' targeting vectors are delivered as linear DNA fragments. A selectable marker gene ('Sel') is flanked by terminal sequences homologous with their genomic targets ('a' and 'c'). The central region of the targeted gene ('b') is replaced by the selection cassette. (d) Gene targeting in rice was achieved by using strong counter-selection. By incorporating an 'ends-out' targeting vector between two copies of a highly cytotoxic gene (encoding diphtheria toxin ('DT-A') within an *Agrobacterium*- delivered T-DNA flanked by right and left border sequences ('RB' and 'LB'), only transformants in which DNA fragments that incorporate into the targeted sequence by homologous recombination should survive. Ectopically incorporated T-DNAs containing the diptheria toxin gene will result in cell death. This counter-selection strategy follows that developed for mammalian cell gene targeting.

allows effective gene disruption, and the creation of null alleles. More subtle genetic manipulation is possible if an endogenous gene can be surgically replaced by a specifically engineered variant sequence, with the minimum additional disruption of the host genome. This became achievable by including a strong counter-selective marker in the vector, so that it was possible to select for a subsequent excision of vector sequences from the targeted locus by intra-chromosomal recombination. This process – termed 'hit and run' (Figure 4.1c) – enables the replacement of an endogenous sequence with a

modified allele and leaves the rest of the host genome otherwise unperturbed (Hasty et al., 1991a; Valancius and Smithies, 1991).

The discovery of gene targeting in mammalian cells was a breakthrough technique in biomedical research, especially when applied to pluripotent embryonic stem (ES) cells that enabled the regeneration of transgenic mice. This was recognised by the award of the 2007 Nobel Prize in Physiology or Medicine to the pioneers of the technology, Mario Capecchi, Martin Evans and Oliver Smithies. Transgenic strains of 'knockout mice' are now a staple for the 'reverse genetic' functional analysis of mammalian genes.

Whilst mammalian targeting vectors require relatively long homologous termini (kilobases: Hasty et al., 1991b) for efficient gene targeting, this is not the case for *S. cerevisiae*, in which the frequency of gene targeting, relative to random insertion, is very high. For *S. cerevisiae*, gene targeting the flanking sequences can be very short: Wach et al. (1994) obtained efficient targeting by using an *NptII* cassette flanked at either end by only 35 bp, enabling the rapid construction of targeting vectors by a single PCR amplification of the selectable marker cassette by using primers with a short 5′-gene-specific sequence. This technology has allowed every gene in the *S. cerevisiae* genome to be disrupted for functional genomic analysis (Scherens and Goffeau, 2004).

The application of gene targeting techniques to flowering plants has been less successful. Plant genetic transformation was first successfully achieved in 1983, following the delivery of transforming DNA by the 'natural genetic engineer', *Agrobacterium tumefaciens*, to tobacco (Bevan et al., 1983; Fraley et al., 1983; Herrera-Estrella et al., 1983). In 1984, the transformation and regeneration of transgenic plants from protoplasts was described, following the uptake of naked DNA (Paszkowski et al., 1984). However, integration of the transforming DNA using either method occurred essentially at random, and the only reports of successful gene targeting events in higher plants appeared to have occurred at low frequency, and probably as a random event (Miao and Lam, 1995; Kempin et al., 1997). The application of a stringent positive–negative selection approach in an *A. tumefaciens*-mediated transformation strategy for rice enabled the isolation of transgenic lines in which a very high proportion of the resulting stable transformants were targeted (Terada et al., 2002, 2007). However, this approach involved selecting specifically for targeted integration by a region of a larger vector carrying homologous targeting sequences, and selecting against the random integration of the rest of the vector sequences due to the presence of a cytotoxic gene (encoding the diphtheria toxin) outside the homologous region (Figure 4.1d). This method of counter-selection follows protocols established for the identification and selective regeneration of the rare homologous recombination (HR) events that occur following transformation of mouse ES cells. Thus, although this allows efficient recovery of targeted transgenic plants, the overall efficiency of transgenic plant regeneration remains very low, by contrast with the efficiency with which gene targeting occurs in more proficient organisms such as *S. cerevisiae* and the moss *Physcomitrella patens*.

Currently, true high-efficiency gene targeting in plants has been possible only in mosses, in particular *P. patens* (although gene targeting in *Ceratodon purpureus* has also been undertaken successfully: Brücker et al., 2005; Trouiller et al., 2007). The discovery of *P. patens*' remarkable ability to undertake gene targeting is an example both of serendipity and of fortune favouring the prepared mind. A protocol for the genetic transformation of *P. patens* was developed in 1991 (Schaefer et al., 1991). In this procedure, plasmid DNA was delivered directly to protoplasts in the presence of polyethylene glycol and calcium ions, and the resulting stable transformants were found typically to contain multiple copies of the transforming DNA at a single locus. In this respect, the outcome of transformation of *P. patens* did not differ in detail from the outcomes obtained when protoplasts of flowering plants were similarly transformed. However, it was observed that if a transgenic strain carrying a selection cassette conferring resistance to the antibiotic hygromycin was retransformed with a second vector carrying a selection cassette conferring resistance either to G418 or to sulfadiazine, then the frequency with which stable retransformants were obtained was substantially greater than that which occurred if the same plasmids were delivered to an untransformed strain (Schaefer, 1994). Moreover, the site of integration of the second vector was genetically linked with that of the first, suggesting that the two vectors had been associated by HR (Kammerer and Cove, 1996) between their shared bacterial replicon sequences. Molecular evidence supporting this conclusion was obtained by Schaefer and Zrÿd (1997), who demonstrated that vectors containing cloned moss genomic DNA became targeted to the cognate genetic loci with high efficiency.

4.2.1 Applications of gene targeting in *P. patens*

The ability to direct transforming DNA to a precise locus, with high efficiency, provides a tool for the targeted disruption and functional analysis of that gene. The first use of this method to create a mutant phenotype was the disruption of a *P. patens FtsZ* gene by Strepp et al. (1998). The *FtsZ* genes (for 'filamentous temperature-sensitive Z') encode bacterial-tubulin-like proteins that are associated with chloroplast division. There are four related *FtsZ* genes in *P. patens*, which make up two subfamilies. However, although the genes within each family encode near-identical proteins (Rensing et al., 2004), they are functionally distinct, as was demonstrated by the striking phenotype obtained when the *PpFtsZ2–1* family member was disrupted: plants exhibited a failure of normal chloroplast division, resulting in the accumulation of giant chloroplasts in each cell. This demonstration, as well as providing insights into the mechanism of organelle division and the evolution of organelle function following their symbiotic capture, stimulated a wider interest in the use of *P. patens* as an experimental model for the study of plant development, through the application of reverse genetics. The efficacy with which gene targeting could be achieved was limited only by knowledge of the available

gene sequences, and consequently the recognition that *P. patens* could add a powerful tool to gene functional analysis was instrumental in stimulating the establishment, first of EST collections, and ultimately the initiation of the *P. patens* genome programme.

The construction of gene knockouts is relatively simple, and consequently the technique has been widely applied to a range of genes in *P. patens*, in order to determine their biological function. However, this relies crucially on the ability to identify and interpret the resulting phenotype, and this has sometimes been less successful. Because gene disruption by the insertion of a selection cassette will frequently result in a null mutant, there is a danger that the disruption of an essential gene will result in lethality. This is a particularly acute problem when working with haploid tissues, where the consequences of mutation will be directly revealed in the absence of an additional wild-type gene copy. Indeed, this explanation is frequently invoked to account for the inability to obtain any targeted transformants, by workers whose experience of working with *P. patens* is relatively limited. More often, the inability to target a specific gene results from a generally low frequency of stable transformation: in a number of cases in our laboratory, we have been able to isolate targeted replacements of genes which have proved recalcitrant to less experienced groups.

Probably, a more frequent (indeed, possibly the most frequent) outcome of a gene targeting experiment is the isolation of targeted transgenic lines that exhibit no apparent phenotype. Such a result can lead to the conclusion that the disrupted gene is redundant in function. This interpretation may often be at least partly true, but it may also depend on the nature of the screen applied to the mutant strains. It is clearly important, before undertaking any directed mutagenesis, to have a good idea of the normal pattern of expression of the candidate gene: prior knowledge of the spatial, temporal and developmental specificity of gene expression (gained, for example by analysing expression patterns using promoter–reporter fusions) can be very helpful in informing a functional screen. It is easier to find something if one has a good idea of when and where to look!

4.3 Gene targeting in *P. patens*: practical aspects

In setting out to undertake a gene targeting experiment in *P. patens*, several questions must be addressed. These concern the design of the gene targeting construct, the physical form of the DNA to be delivered, the means of delivery, the state of the tissue to be transformed, the nature of the locus to be targeted, the likely consequences of disruption, and the methods for analysis of the transformants. We shall start by considering the simplest form of gene targeting experiment: the construction of a 'gene knockout' by the targeted insertion of a selectable marker cassette, with the aim of creating a null mutant of the targeted gene.

4.3.1 Selection of transformants

The first consideration is to ensure that transformants can be recognised and selected. Usually, this requires that the transforming DNA contain a dominant selectable marker gene, although in a few instances, gene disruption may itself generate a selectable phenotype (the best example of this is the targeted disruption of the *PpAPT* gene encoding adenosyl phosphoribosyl transferase: such disruptants become resistant to the nucleotide analogue, diaminopurine (Schaefer, 2001; Trouiller et al., 2006), whilst Brücker et al. (2005) were able to use a wild-type sequence to target a mutant heme oxygenase gene in *C. purpureus* and to identify targeted transformants by using the restoration of a phototropic phenotype as a screen. The most commonly used selectable transgene is the bacterial neomycin phosphotransferase (*NPTII*) gene that confers resistance to the aminoglycoside antibiotic kanamycin, and its analogue G418 (also known as geneticin). In practice, G418 is preferable to kanamycin, as it is both more stable and less costly. This gene is widely used for the selection of transformed cells of flowering plants, and consequently a number of expression cassettes containing this gene are available. In our laboratory, we have principally used a cassette in which the *NPTII* gene is under the control of the Cauliflower Mosaic Virus '35S' promoter. Whilst this promoter is a very powerful and constitutively active promoter in dicotyledonous angiosperms, its activity is weaker in *P. patens* (which in this – as in other aspects of its molecular biology – seems to share a greater similarity with cereals). Nevertheless, the CaMV35S promoter is still sufficiently active to enable the selection of transformed moss tissue on medium containing G418. For correct expression in eukaryotic cells, the coding sequence should also be terminated by a sequence specifying transcription termination and the addition of a polyadenylate tail to the transcript. The 'pMBL' series of plasmids constructed at Leeds therefore contain a selection cassette comprising the *NPTII* gene under the control of the CaMV35S promoter, and flanked at the 3′-end by the termination sequence derived from the CaMV 'gene 6' ('g6ter') (Figure 4.2). Other promoters and terminators may also be used. The nopaline synthase terminator ('noster') from *A. tumefaciens* T-DNA is an equally effective terminator sequence, whilst the promoter from the rice actin-1 gene has been shown to be both highly active and effective in driving high levels of transgene expression in *P. patens*. The most active form of the rice actin promoter – in both transgenic cereals and transgenic moss – incorporates not only the rice DNA sequence that lies immediately upstream of the rice actin mRNA coding sequence, but also that sequence encoding the gene's 5′-untranslated region (5′-UTR). Although non-coding, this sequence includes an intron, whose processing is required for maximal levels of transgene expression (McElroy et al., 1991). Processing appears to be effective even in *P. patens*, and this promoter is among the most powerful available for driving transgene expression in moss (Horstmann et al., 2004). Other selectable markers used include the *APHIV* gene, encoding hygromycin phosphotransferase and conferring hygromycin resistance, the zeocin resistance gene,

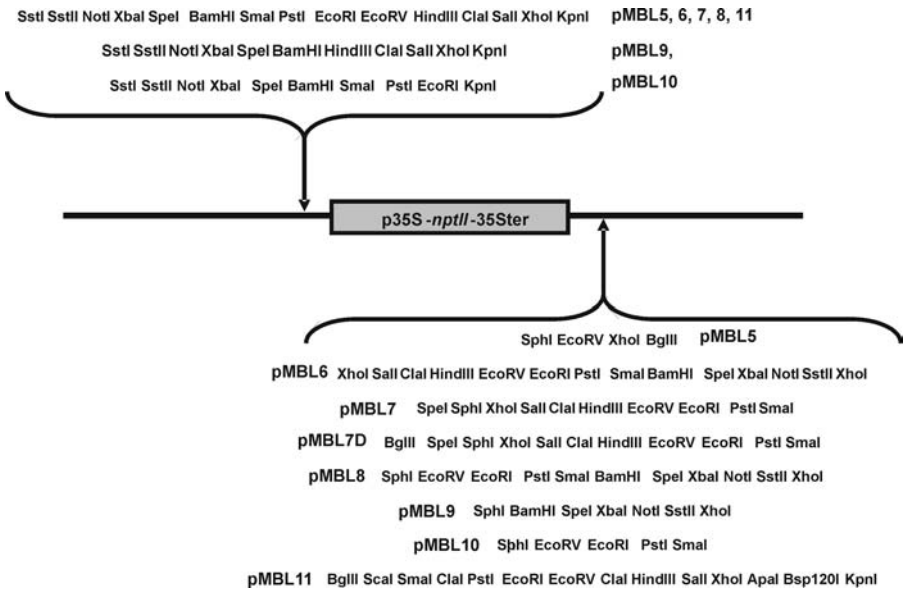

Figure 4.2 The pMBL series of vectors. This series of vectors comprise a CaMV35S promoter – *NPTII* – CaMV gene 6 terminator cassette cloned in pUC replicons, and flanked by different combinations of restriction enzyme sites, derived from the pBluescript replicon. The vectors are available with the selection cassette in both orientations ('a' and 'b') relative to the restriction enzyme sites: this figure shows the 'a' orientation. These cassettes can be inserted into cloned genomic sequences in order to construct targeting vectors. The pMBL7D and pMBL11 cassettes contain substantially truncated terminator sequences, based on deletion to the first *BglII* site present in his sequence. We have observed that use of the longer terminator sequences can (very occasionally) result in DNA rearrangement in some constructs.

encoding resistance to the glycopeptide antibiotics of the bleomycin family (*P. patens* is normally highly sensitive to low concentrations of bleomycin: Markmann-Mulisch et al., 2007) and the sulfonamide resistance gene conferring resistance to agents such as sulfadiazine (Markmann-Mulisch et al., 2007). By contrast with flowering plants, *P. patens* is remarkably tolerant of herbicides that have been used as selective agents for angiosperm transformation. Neither glyphosate ('Roundup') nor phosphinothricin-based herbicides (including the naturally occurring compound 'bialaphos' and the commercial derivatives 'Basta' and 'Liberty') have been effective in our laboratory.

4.3.2 Positioning of the selection cassette

In the simplest form of gene targeting experiment, a selection cassette is inserted into the cloned sequence of the gene to be disrupted. However, to be sure of obtaining a knockout of the gene's function, certain precautions should be taken. The most effective approach is to replace a substantial segment of the coding region with the selectable marker cassette. Simple insertion of

the cassette into an otherwise unmodified targeting sequence may not be sufficient. Insertion of the cassette into an intron may result in its removal during the production of a spliced transcript. Additionally, many gene targeting events are asymmetric, resulting in the insertion of the transforming DNA at one end of the targeted gene, but with the accompanying retention of a wild-type copy of the gene. This 'targeted insertion' will be addressed in more detail subsequently. An effective targeting construct will comprise a selectable marker cassette flanked by sequences corresponding to the 5′- and 3′-genomic sequences on either side of the replaced sequence.

4.3.3 DNA delivery

Stable transformation of *P. patens* was first achieved by the delivery of purified DNA to isolated protoplasts, and this remains the method of choice in most laboratories. Protoplasts are easily derived by digestion of chloronemal tissue with 'Driselase': a commercially available cocktail of fungal enzymes that digest the cell wall. Protoplasts generated in this way retain their totipotency, regenerating into individual plants with very high efficiency. Regeneration frequencies exceeding 90% are possible, for an experienced tissue culturist. However, as with all transformation techniques based on the uptake of naked DNA by protoplasts, the efficiency of transformation is much lower. Additionally, transformed *P. patens* protoplasts typically fall into one of three classes. The first, and most numerous class comprises protoplasts that take up the transforming DNA, express genes encoded within it, but fail to retain it for longer than a few days. This transient transformation enables the rapid analysis of expression constructs, e.g. promoter–reporter constructs used to determine the activity of different promoter constructs and to analyse *cis*-acting regulatory sequences required for gene expression (Horstmann et al., 2004; Kamisugi and Cuming, 2005), but following the transfer of transfected protoplasts to a selective medium, such cells will not survive. The second class of transformant is generically described as 'unstable' transformants. Such protoplasts will regenerate and divide, producing antibiotic-resistant plants, but only so long as they are maintained on medium containing the selective antibiotic. Relaxation of selection for approximately 2 weeks results in loss of the transforming DNA, so that the return of such plants to selective conditions results in their death. Such unstable transformants may retain the transforming DNA for very long periods, so long as selection is maintained. Unstably transformed plants have been maintained on continuous selection for up to 10 years, during which time they retain the transforming sequences within their cells, but without integrating this DNA into their genome. Instead, the transforming DNA remains – and replicates – in the form of extra-chromosomal concatenated repeat sequences (Ashton et al., 2000). Notably, the proportion of transformants falling into this 'unstable' class is greatest when the transforming DNA is a covalently closed circular molecule. The final class of transgenic plants recovered from transformation experiments is made up of stable transformants: in these plants, the DNA

integrates into the genome and is replicated along with it. Selection for stable transformants is achieved by subjecting transfected protoplasts to at least two successive rounds of growth on selective medium. Following an initial regeneration period, to allow the synthesis of new cell walls, we embed the transformed protoplasts in an agarose regeneration medium which we overlay onto medium containing the selective antibiotic. Two weeks of culture is sufficient to permit the identification of resistant colonies that are then sub-cultured onto drug-free medium for a further 2 weeks. Finally, explants from each colony are returned to selective medium for a further 2 weeks, during which any colonies that were unstable transformants will die. Stably transformed plants can subsequently be maintained on drug-free medium indefinitely. Rates of stable transformation are relatively low, in terms of the numbers of protoplasts initially transfected, stable transformants being obtained at a rate of approximately 10^{-5}–10^{-3}. However, the frequency can be variable between experiments, and additionally can depend on the nature of the transforming DNA. Typically, delivery of linear molecules is more effective than the delivery of circular molecules, and the presence in the transforming DNA of a sequence homologous with a native gene (and thus able to undergo homology-dependent integration) can result in a tenfold higher rate of stable transformation than a completely novel sequence (Hohe et al., 2004).

Other methods of DNA delivery are also possible. Biolistic transformation can be carried out, by bombarding protonemal tissue directly with microprojectiles bearing DNA constructs. As with direct uptake of DNA by protoplasts, transformed cells may be either transiently, unstably or stably transformed, and stably transformed plants may be recovered by successive rounds of selection. Biolistic transformation and protoplast transfection can both result in the targeting of endogenous loci, when the transforming DNA includes a homologous sequence (Y. Kamisugi and A. Cuming, unpublished data).

The same does not appear to apply to transformation by *A. tumefaciens*. Until recently, attempts to achieve *Agrobacterium*-mediated transformation had been unsuccessful. However, the development of 'supervirulent' strains of *Agrobacterium*, and the discovery that the phenolic compound acetosyringone was a natural inducer of the *A. tumefaciens* virulence genes, facilitated the development of *A. tumefaciens*-mediated moss transformation. If *A. tumefaciens* containing supervirulent Ti plasmid vectors is co-cultivated with *P. patens*, in the presence of acetosyringone, and under conditions essentially similar to those used for high-efficiency transformation of tobacco protoplasts, then stable transformants can be recovered with relatively high frequency (Y. Kamisugi and A. Cuming, unpublished data). The limited amount of currently unpublished data available from such experiments indicates that DNA insertion into the *P. patens* genome appears (i) to occur essentially randomly, (ii) to occur as single-copy insertions, by contrast with the integration of long concatenates that occur frequently when protoplasts take up naked DNA and (iii) to result in non-targeted integration, even when the transforming DNA comprises a construct designed to target specific genes (P.-F. Perroud,

D. Cove, Y. Kamisugi and A. Cuming, unpublished data). There is not yet sufficient evidence to indicate why *A. tumefaciens*-mediated transformation might not result in gene targeting, but three possibilities suggest themselves. The first is that the (highly specialised) mechanism of T-DNA integration, relying as it does on the coordinated interaction of a number of *A. tumefaciens* Ti-encoded virulence determinants, including the establishment of a bacterial Type IV secretion complex for the transmembrane delivery of the T-DNA (Cascales and Christie, 2004) and the covalent interaction between the T-strand and the $VirD_2$ protein required to 'pilot' the T-strand to the nucleus, militate against homology-dependent integration and in favour of integration at pre-existing DSBs in the genome, using a non-homologous end-joining DNA (NHEJ) repair process (Chilton and Que, 2003). The second possibility is that the presence of additional non-homologous sequences at the termini of the T-strand (the right (RB) and left (LB) borders) interfere with the homology-search mechanism required for integration by homology-dependent recombination. The third also implicates the RB and LB sequences, but in a more direct manner, with the possibility that microhomologies between the borders and genomic sequences might initiate the formation of the required DSBs for T-DNA integration, preferentially directing the T-DNA away from sequences with sub-terminal homology (Thomas and Jones, 2007). No explanation is wholly satisfactory, however. Whilst it is evident that the random integration of T-DNA in higher plants is certainly linked to the activity of components of the NHEJ DNA-repair pathway (Chilton and Que, 2003; Friesner and Britt, 2003; van Attikum et al., 2003; Li et al., 2005), it is also clear that even in plants largely incompetent to undertake gene targeting, a low incidence of *A. tumefaciens*-mediated targeting events can be achieved. This is either through the application of stringent counter-selective screens (Terada et al., 2002, 2007) or through the over-expression of exogenous HR-associated genes (Shaked et al., 2005). Additionally, in moss, gene targeting following naked DNA delivery remains efficient even in the presence of ca. 40 bp of terminal non-homology (Kamisugi et al., 2005).

4.3.4 The nature of the transforming DNA

The most critical feature in ensuring the success of gene targeting in *P. patens* is the nature of the targeting construct. As outlined above, a typical targeting construct will comprise a selection cassette, flanked by cloned sequences corresponding to genomic regions that lie 5′- and 3′- to a segment replaced by the selection marker. This construct should be delivered in the form of linear DNA. Circular constructs do exhibit gene targeting, but only at low frequency. Indeed, the overall recovery of stable transformants is greatly reduced when circular DNA is delivered, in favour of a predominance of unstable transformants.

The most important feature determining the efficiency of gene targeting is the length of homology between the transforming DNA and the targeted

sequence. In an analysis of the relationship between targeting efficiency and overall homology length, we determined that an homology length of approximately 600 bp was sufficient to ensure that 50% of the stable transformants were targeted to the desired locus (the 'T_{50}' length). For constructs that contained homology with the target sequence that extended to their termini, there was a direct linear relationship between the length of homology and the frequency of gene targeting. When the constructs (derived by digestion from a plasmid) contained 40 bp of terminal non-homology, the relationship between targeting frequency and homology length was better described by a logarithmic relationship (Kamisugi et al., 2005).

However, the term 'gene targeting' refers simply to the integration of the DNA at the targeted locus. It does not indicate the precise nature of the targeting event. We can distinguish two types of targeted transgene integration, that we describe as 'targeted insertion' ('TI') and 'targeted gene replacement' ('TGR'). A simplified overview of these two types of targeting events is illustrated in Figure 4.3. Targeted insertion occurs apparently (although not

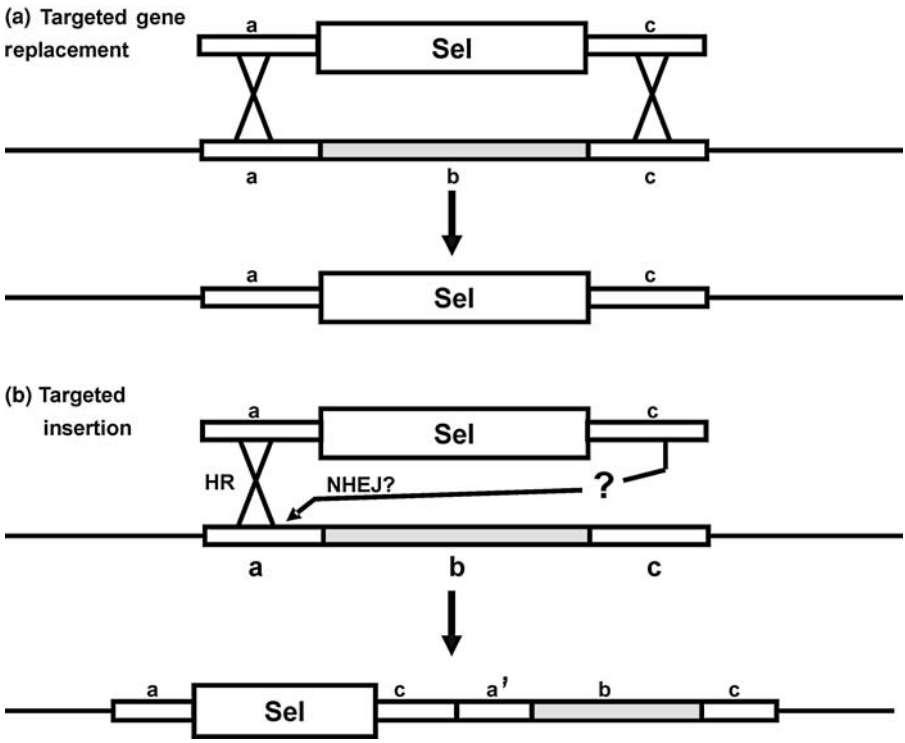

Figure 4.3 Targeting events in *P. patens*. (a) TGR occurs by homologous recombination between the target locus and both ends of an 'ends-out' vector. (b) Targeted insertion results in the insertion of the transforming cassette in one or other of the targeted sequences. This will not necessarily generate a gene knockout.

actually) as the result of an HR event by one 'arm' of the targeting construct and the cognate sequence in the targeted locus. This results in the precise integration of this end of the construct into the genomic locus. However, the other end of the targeting construct appears to undergo a NHEJ event at the same site (in the 5′-end, in this diagram), resulting in the insertion of the targeting construct at one end of the targeted gene. Depending on the position of integration, such a targeting event may not result in an alteration of the expression or function of the targeted gene.

By contrast, TGR occurs as a result of an HR event occurring between the homologous sequences at either end of the targeting construct and its cognate locus. We envisage this to occur in a manner similar to the analogous procedure in *S. cerevisiae*, in which each end of the targeting construct forms a heteroduplex strand-invasion complex with its homologous sequence in the genome (Langston and Symington, 2004). In this case, the native gene is replaced by the targeting construct. If the selection cassette disrupts the coding sequence sufficiently, a true gene knockout will result.

An important factor in determining the relative frequencies of targeted insertion and TGR is the symmetry of the targeting construct. Highly asymmetric constructs favour targeted insertion events, biased strongly towards targeting in the longer of the two arms. By contrast, TGR is favoured when the two homologous arms of the targeting construct are of approximately equal length. Thus, for any targeting construct, the frequency with which TGR occurs is directly proportional to the length of the shorter arm. This is clearly illustrated in Figure 4.4, which shows the relationship between the incidence of TGR and the length of the shorter homologous sequence, with a T_{50} for the shorter arm of 400 bp in the absence of terminal non-homology, extending to 600 bp when non-homologous termini are present (Kamisugi et al., 2005).

It is interesting, although not wholly surprising, that terminally non-homologous constructs can still mediate gene targeting with high efficiency, albeit at a slightly reduced frequency by comparison with terminally homologous sequences. In *S. cerevisiae*, relatively short terminal non-homology is sufficient to significantly depress the efficiency of gene targeting (Wach, 1996), but the action of the RAD1/RAD10 excision nuclease and the MSH2/MSH3 mismatch repair enzymes can remove these non-homologous termini to allow targeted integration (Colaiacovo et al., 1999). *P. patens* contains homologues of these genes, and it will be interesting to determine whether their inactivation alters the ability of terminally non-homologous DNA to be targeted to specific loci. Certainly, mismatch repair genes are required for precise gene targeting. In *S. cerevisiae*, mutants in *MSH2* showed enhanced levels of gene targeting by mismatched sequences (Negritto et al., 1997), and the same is true in *P. patens*. In an elegant experiment, in which an increasing number of random point mutations were introduced into a *PpAPT* (adenosine phosphoribosyl transferase) targeting construct, to create a series of homeologous variants, Trouiller et al. (2006) showed that *Ppmsh2* mutants were able to

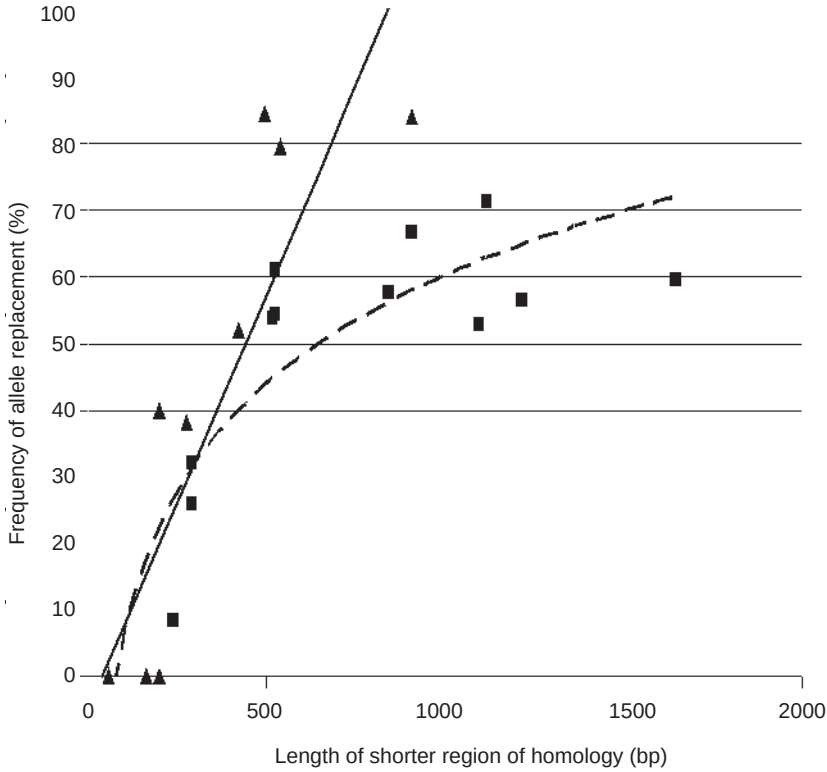

Figure 4.4 Relationship between frequency of TGR and length of homology. This summarises the results of a series of transformation experiments in which *P. patens* was transformed with a series of 'ends-out' constructs, each with different lengths of 5′- and 3′-homology. The regression lines are of frequency of TGR on the length of the shorter homologous arm (solid line, triangles) for targeting constructs whose arms were homologous throughout their length and of frequency of TGR on the logarithm of the length of the shorter homologous arm for constructs that contained 40 bp of terminal non-homology at each end (dashed line, squares). (*Source*: Data reproduced from Kamisugi et al., 2005.)

integrate the variant targeting sequences at the *PpAPT* locus as efficiently as wild-type targeting sequences.

4.3.5 Analysis of targeting events

The analysis of gene targeting occurs both at the level of selection of transformants, and at the molecular level. The first stage in the procedure is to distinguish stable from unstable transformants, by successive rounds of selection, relaxation and re-selection, as outlined above. By observation of the growth rate of transformants, it is sometimes possible to predict which transformants

may turn out to be stable, at the first selective stage. We have frequently observed that the growth rate of unstably transformed plants is slower than those that have undergone a stable integration of the transforming DNA into the genome, and that the stable transformants are therefore identifiable by the larger colony size of the regenerants. However, this 'rule of thumb' must be tempered with caution, when the possible effects of a targeted gene disruption are also considered. Thus, a gene disruption in a critical developmental pathway – such as cell division or enlargement – might well result in a slow-growth phenotype. It is therefore important, at the first round of selection, to replicate as many regenerants as are practically possible for the subsequent rounds of screening.

Molecular analysis is initially most conveniently undertaken by PCR. The first necessity is to distinguish between the three outcomes of stable transformation: TGR, targeted insertion and ectopic integration at a non-targeted site. This is achieved by using PCR primers that lie external to the targeted sequence in combination with 'outward-pointing' PCR primers derived from the selectable marker cassette. A further PCR reaction, using primers designed to amplify only the selection cassette, provides an additional assurance that the transgene is present (Figure 4.5a).

A TGR will result in an amplification product being obtained with both the 5′-located pair of primers, and the 3′-located pair. Targeted insertion results in only one of the end-specific pairs of primers generating an amplicon, whilst the inability to amplify a product with either end-specific primer pair is indicative that the transgene has integrated at another site. Inability to generate an amplicon might also be the result of a PCR failure, but in practice, it is necessary to consider this only if none of the three primer combinations yield a product (Figure 4.5b).

If PCR failure can be ruled out, a failure to identify a targeted disruption of a gene may indicate that mutation of that gene results in early lethality – perhaps during protoplast regeneration. However, in many cases, initial failure to obtain a targeted gene usually reflects a low overall rate of transformation, and should not be automatically taken as an indication that a specific gene is essential for viability. Repeated failures to obtain gene disruption may well indicate an important role for the gene in question. In such cases, alternative strategies should be considered. These might include co-transformation of plants with both a targeting construct designed to achieve disruption, simultaneously with a construct that will complement the null allele generated by disruption (e.g. a cDNA expression construct). Alternatively, instead of attempting to obtain a null allele of the targeted gene, differently mutated gene replacement constructs might be delivered. These could include sequences carrying specific point mutations, with the aim of generating a less severe or conditional allele, or with the selection cassette inserted at different sites within the coding sequence.

The strategy of generating multiple different mutant alleles of a gene should in any case be considered by any researcher wishing to undertake a

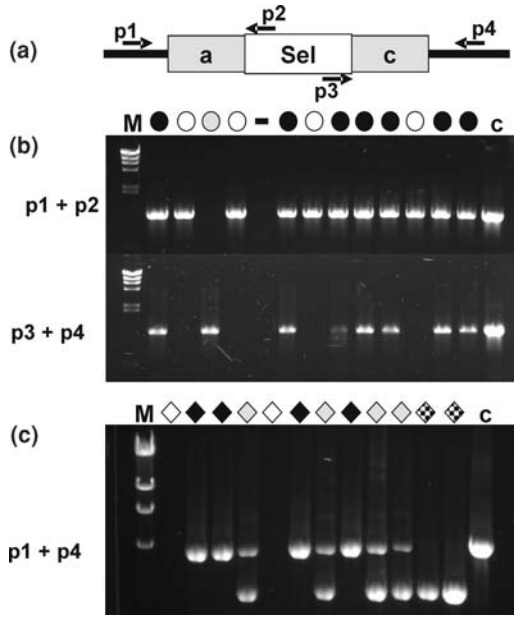

Figure 4.5 PCR analysis of targeting events. (a) PCR analysis is undertaken using combinations of external, 'inward-pointing' gene-specific primers 'p1' and 'p4', and selection cassette-specific 'outward-pointing' primers, 'p2' and 'p3'. (b) Analysis of targeting events. The upper set of PCR products was generated by amplification of genomic DNA from transformants using primers p1 and p2 in combination, and the lower set using primers p3 and p4 in combination. Tracks indicated by white circles show targeted insertion into sequence 'a'. Tracks indicated by grey circles show targeted insertion into sequence 'c'. Tracks indicated by black circles show that targeted replacement has occurred. In one track ('-'), no targeting has occurred. Track 'M' corresponds to the DNA size standards (a *HindIII* digest of λDNA) and track 'C' is a control amplification using the cloned, disrupted locus from which the targeting vector was derived. (c) Identification of single-copy TGRs. Genomic DNA from plants in which TGR had occurred was amplified with primers p1 and p4. Where no amplification is observed (white diamonds), multiple copies of the transformation vector have been integrated. Black diamonds indicate that a single-copy TGR has occurred, with the amplification of a predicted ca. 4 kb fragment. Some single-copy (grey diamonds) and multicopy (hatched diamonds) events are accompanied by the detection of an additional ca. 3 kb wild-type fragment. These plants contain polyploid DNA content, resulting from somatic fusion of protoplasts during the transformation procedure. Tracks M and C are the size standard and control, as in 4B.

comprehensive functional analysis of a particular gene. Genetic analysis is a very powerful technique, particularly where different mutant alleles of a particular gene are available, as different mutations may have varying effects on the severity of a mutant phenotype. This is the case even for genes which might be expected to be disrupted by the insertion of long sequences, since the severity of the phenotype may depend on the position and nature of the insertion. There are many examples of genes in higher plants that have been

disrupted by transposon or T-DNA insertions at different sites, resulting in mutants with phenotypes that differ markedly in severity and function. The availability of such allelic series of mutations additionally aids functional characterisation.

Identifying TGRs by terminus-specific PCR reactions is only the first stage in the characterisation of targeted gene disruptions. Both targeted insertion and TGR may involve the integration of multiple copies of the transforming DNA at the targeted locus. Indeed, it is likely that targeted insertion events always result in the insertion of multiple copies of tandemly repeated transgenes at the integration site (Kamisugi et al., 2006). Additionally, targeting events may also be accompanied by ectopic insertion of transgenes, elsewhere in the genome. Because it is important to rule out the possibility that any mutant phenotype that is observed may be derived from a disruption of an accidental insertion into another gene, it is therefore desirable to ensure that analyses are carried out using mutant strains in which precise TGR has occurred. The integration of a single copy of the transforming cassette at the targeted locus can be confirmed by PCR amplification using the 5′- and 3′-primers external to the targeting construct's sequence, in combination (Figure 4.5c). Provided that the targeting construct is not too long (and an overall length for such a construct need not be more than approximately 4 kb, including a 2 kb selection cassette), then it should be possible to identify plants in which only a single copy of the transforming DNA undergoes TGR. Plants in which two or more copies of the transgene are inserted at the target locus usually result in a failure to amplify the larger insertion in the genome, under standard PCR conditions. In our experience, between 15% and 50% of plants in which TGR has occurred exhibit the integration of single copies at the targeted locus.

There is another very important reason to undertake such an analysis. This PCR amplification will also identify whether the transgenic plant contains not only a locus that has been correctly targeted, but also a residual copy of a wild-type locus. At first sight, this possibility does not seem possible. If targeting occurs by an HR event between both termini of the transforming DNA and its target locus, the result should be that the wild-type locus is replaced by the mutant gene. How then could such a plant contain a residual wild-type sequence? The answer to this conundrum lies in the manner in which transformation is most usually undertaken. The method used by most researchers is to transform protoplasts with naked DNA, in the presence of calcium ions and polyethylene glycol (Schaefer et al., 1991). However, this procedure is similar to that used to obtain hybrid plants by protoplast fusion (Grimsley et al., 1977a, 1977b). This technique was used to construct diploid strains for complementation analysis of mutants derived by chemical mutagenesis (largely because many of the mutants isolated following severe mutagenic treatments were also compromised in their fertility, ruling out the use of sexual crosses for such tests). Consequently, it is not unusual to recover a number of transformants that have abnormal ploidy, following PEG-mediated protoplast transformation. Analysis of nuclear DNA content

by flow cytometry in transgenic plants in which TGRs are accompanied by an additional wild-type target sequence indicates that such wild-type loci most probably result from a protoplast fusion, resulting in the regeneration of diploid or variously aneuploid plants. Fortunately, such events are relatively infrequent, easily detectable by PCR analysis, and usually also predictable from a close inspection of the phenotype of the transformants (Schween et al., 2005): we have observed such plants to have a characteristic 'fluffy' appearance compared with wild-type plants, probably as a result of a greater proportion of caulonemal filaments. It is clearly important to be able to distinguish between an abnormal colony phenotype resulting from altered ploidy and an abnormal colony phenotype that results from the disruption of the targeted gene, so PCR assays using external gene-specific primers should always be undertaken (Figure 4.5c).

Finally, it is necessary to ensure that the only copy of the transforming DNA that is present in the genome is that (or those) which have replaced the target locus. This can most reliably be ascertained by Southern blot hybridisation, although PCR-based methods used to amplify and analyse the sites of random transgene insertion (iPCR, TAIL-PCR, anchored AFLP analysis, etc.) could also be used, providing the appropriate controls are incorporated. In our experience, only a minority of transgenic plants in which a single-copy TGR has occurred contain additional, untargeted copies of the transgene. An analysis of 73 transgenic plants containing TGRs at 10 different loci revealed that only 15 (20%) contained ectopically integrated transgenes (Kamisugi et al., 2005; Kamisugi and Cuming, unpublished data).

It is important to emphasise that possible consequences of random transgene insertion should be ruled out, especially if the DNA used for transformation uses a targeting construct released by digestion of a cloned sequence, without further purification of the targeting construct from its vector background. Plasmid DNA can frequently be found integrated into the genome of transgenic plants, when such precautions are not undertaken, despite the absence of a selection cassette in the plasmid sequence (Kamisugi et al., 2006).

If it is not possible to exclude the presence of non-specifically integrated DNA in the genome of plants that have also undergone targeting events, then it will be necessary to take further steps to demonstrate that any mutant phenotype observed is the result of the targeting event, and not to an adventitious incorporation of DNA elsewhere in the genome. For this reason, it is always desirable if multiple independent transgenic plants containing accurately targeted loci can be identified. It would be very unlikely for such plants to have integrated non-targeted DNA at other, identical genomic loci, and so the observation that a mutant phenotype was consistently associated with the mutant phenotype would reinforce the likelihood that such a phenotype was a direct consequence of gene targeting events. If only a single correctly targeted plant is obtained, evidence that any phenotype results as a direct consequence of gene targeting would be provided by crossing the transgenic plant with a wild-type strain. The co-segregation of the mutant phenotype with the selectable marker would support the hypothesis that targeting was

responsible for the mutant phenotype. Finally, one would expect that a mutation resulting from a gene targeting event would be complemented if the mutants were retransformed with a wild-type copy of the targeted gene. Such a retransformation would not require a further targeting event to occur – the expression of additional copies of the wild-type gene in an unstable transformant might be sufficient to bring about complementation of the mutant phenotype.

4.3.6 Targeting multi-gene families

Genetic redundancy is a common phenomenon, frequently resulting from the presence in the genome of several closely related genes. Like most (possibly all) plants, *P. patens* has undergone genome-wide duplication events in its evolutionary history, and individual gene families have also undergone evolutionary expansions and contractions (Rensing et al., 2007). Consequently, some duplicated genes may be insufficiently differentiated in their coding and regulatory sequences to be greatly differentiated in function, and therefore a knockout of one gene may be compensated for by the residual expression of unmutated family members. To overcome such problems, it may be necessary to undertake multiple gene targeting experiments, to obtain knockouts of all possible gene family members. One way in which this can be achieved is by successive retransformation of a line carrying a knockout with a further targeting constructs. Whilst this is theoretically feasible, there are limitations to its application. First, each successive transforming construct would need to carry a different selectable marker cassette. The relatively small number of selectable marker genes available limits the number of times retransformation can be undertaken. This can be partly overcome by using an initial targeting vector in which the selectable marker gene is flanked by sequences that act as targets for bacteriophage recombinase enzymes, allowing the deletion of the selectable marker and its subsequent reuse. One such system that has potential for marker recycling is the bacteriophage P1 Cre-lox recombination system (Figure 4.6) (Chakparonian, 2001; Perroud and Quatrano, 2006). Following the isolation of a correctly targeted transgenic strain, delivery of a transiently expressed Cre-recombinase gene will result in the deletion of the selectable marker gene. So long as the original targeting vector generated a replacement of part of the target gene, a deletion mutant will result which can subsequently be retransformed with another vector containing the same selectable marker. However, this is not an approach that can be continually repeated. The presence of multiple *loxP* sites in the moss genome would act as targets for Cre-recombinase, leading to unwelcome chromosome deletions and rearrangements.

An alternative strategy is to simultaneously deliver multiple targeting constructs, containing the same selectable marker (Hohe et al., 2004). The frequency with which multiply targeted strains can be recovered should correspond to the products of the frequencies with which singly targeted strains

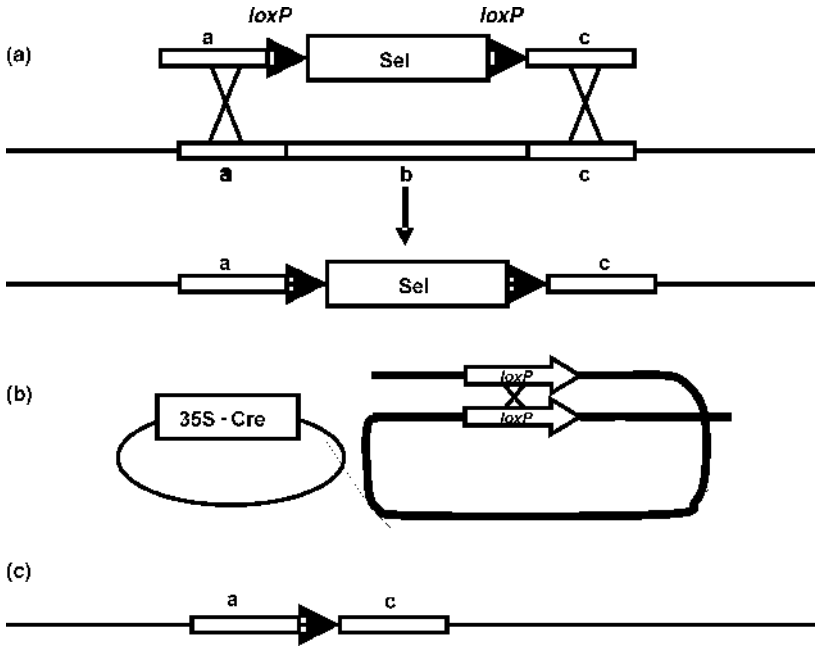

Figure 4.6 Marker excision by the Cre-lox recombination system. (a) A TGR is constructed using a vector in which the selection cassette is flanked by *loxP* sequences. (b) Following isolation of plants in which TGR has occurred, protoplasts from these plants are transformed with a plasmid directing the expression of the Cre-recombinase. This results in recombination between *loxP* sites causing the excision of the selection cassette. (c) Protoplasts are regenerated to produce plants from which the selection cassette has been deleted. (Note: This outcome can be selected, if the Cre-recombinase containing plasmid contains a second selection marker. So long as the Cre-transformed plants remain as unstable transformants – an outcome favoured if the Cre-construct is delivered as a supercoiled plasmid – then the intermediate 'Cre^{+}/Sel^{-}' plants can be regenerated and subsequently 'cured' of the Cre-containing replicon by relaxation of selection.)

can be recovered. However, the molecular analysis of the resulting transformants can be far from straightforward, especially if ectopic insertions occur.

Perhaps the most reliable way to obtain multiply mutated strains is to target genes singly, and then to combine the mutants by sexual crossing – although this may not be possible if the mutation results in loss of sexual competence. This approach was used to create targeted disruptions of the two *RAD51* genes in *P. patens* (Markmann-Mulisch et al., 2007) and has the benefit that the effects of mutation can be analysed both singly and in combination. In this case, the two genes were very nearly identical, and the characteristic mutant phenotypes of sensitivity to genotoxic treatment and failure to produce viable spores (presumably due to meiotic failure) were only clearly apparent in the double mutant. In this study, crossing lines that had been mutated by the insertion of different selectable marker genes rendered the recognition of spores containing double mutants relatively facile, but this is not a necessary prerequisite for identification of double-mutant progeny.

Simple PCR analyses can easily determine which plants contain multiply disrupted genes, if the same selection cassette is used for each individual gene disruption.

4.4 Targeted gene replacement versus targeted insertion

Not all gene targeting events in *P. patens* result in single-copy TGR. Frequently, TGR involves the integration of multiple copies of the transforming DNA at the site of integration (we have previously found between 40% and 80% of TGRs to involve multicopy integration of the transgene, depending on the locus targeted: Kamisugi et al., 2005). Furthermore, a substantial proportion of gene targeting events (targeted insertions) apparently occur by accurate gene targeting by only one end of the targeting construct. Close analysis reveals targeted insertion to result from two HR events in which both the targeting construct and the targeted locus contribute an invasive strand (Kamisugi et al., 2006).

How might this occur? We have to consider how a gene targeting experiment is performed. Typically, 5×10^5 protoplasts are incubated with 10–15 μg of linear DNA fragment. Assuming a targeting construct of 4 kb is used, this represents 3.8–5.7 picomoles of DNA, corresponding to 2.3×10^{12}–3.4×10^{12} DNA molecules. Thus, for each protoplast, between 4×10^7 and 10^8 DNA molecules are available. It is therefore not unreasonable to expect that any protoplast competent to take up DNA by endocytosis will be likely to take up a very large number of individual fragments. The sudden appearance of a large number of linear DNA fragments in the nucleus of a protoplast is likely to be interpreted by the cell as the appearance of a catastrophic number of DSBs, and it is to be expected that the cell would attempt to repair as many of these as possible. Therefore, we suggest that the first consequence of DNA uptake will be to stimulate the most rapid means of repairing DNA DSBs, namely the NHEJ repair pathway, and that the effect of this will be to generate concatenated DNA molecules. Such a scenario is certainly consistent with the known consequences of stable transformation of most eukaryotic cell types (that in any case favour NHEJ for the repair of DNA DSBs) with naked DNA – the generation of transgenic loci containing multiple concatenated transgenes. In *P. patens*, the generation of such concatemers that did not integrate into the genome would account for the class of unstable transformants that replicate extra-chromosomal concatenated arrays (Ashton et al., 2000). It would also account for the proportion of TGRs that occur, containing multiple concatenated copies of the transforming DNA, following the integration of these arrays by HR between each end of the concatemer and its cognate target sequence.

We believe that the formation of such concatemers also explains the occurrence of targeted insertion events. The process by which this could take place is illustrated in Figure 4.7. We predict such integration events occur following

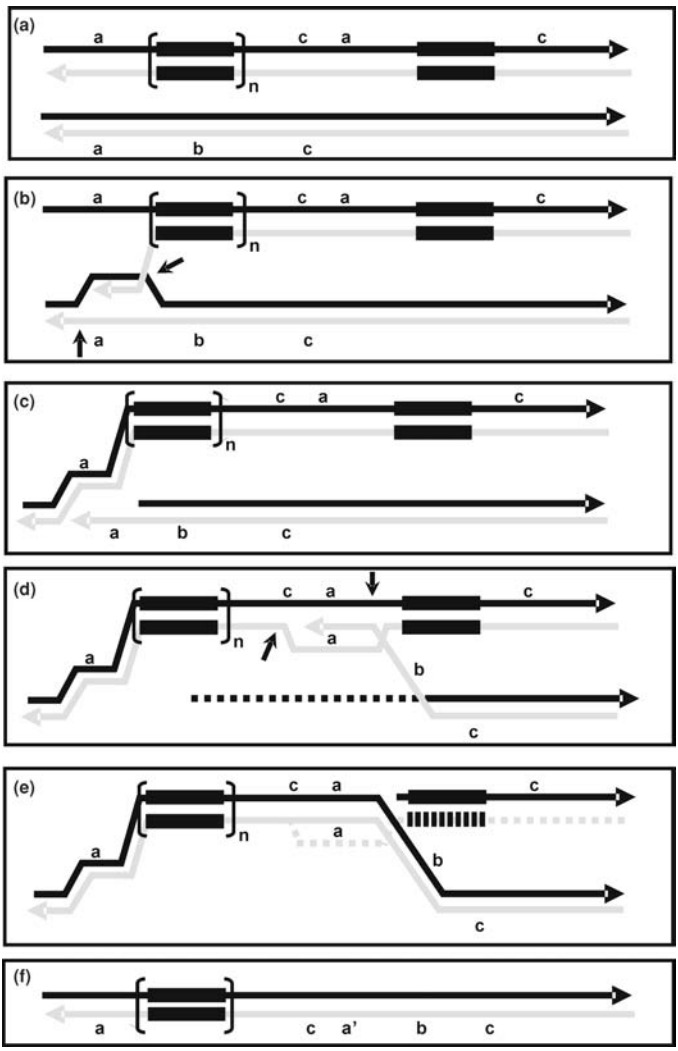

Figure 4.7 The mechanism of targeted insertion. (a) Following transformation with linear DNA, the transforming fragments are concatenated by NHEJ. (b) Homologous recombination is initiated between one end of the concatemer and its cognate target sequence (region 'a'), by 3′-strand invasion. Strand scission of the recombination junction will occur at the arrowed sites. (c) Resolution of the recombination junction by scission and ligation generates a free DS end in the genomic DNA. (d) Resection of this free end occurs to generate a 3′-invasive single strand that can invade a homologous region 'a' sequence elsewhere in the transforming concatemer. (e) Resolution of this second recombination junction results in the integration of multiple copies of the transforming cassette into sequence region 'a'. (f) The presence of multiple copies of the transforming cassette (i) renders PCR amplification of the transgenic locus with primers external to sequences 'a' and 'c' in the genomic DNA impossible and (ii) creates a predictable 'c–a'–b' junction sequence at the insertion site. (*Source*: Adapted from Kamisugi et al., 2006.)

the initiation of gene targeting by one end of a tandem concatemer – sequence 'a' in Figure 4.7a. A strand resection and RAD51-mediated strand exchange would result in the integration of this end of the concatemer at a homologous site, as illustrated in Figure 4.7b. Extension, ligation and resolution of this junction would in turn create a free end at the targeted genomic locus (Figure 4.7c) which, if it were not to be repaired, would result in loss of the acentric DNA segment, with potentially lethal consequences. However, this free end would itself be free to be recognised by the HR-mediated DNA repair machinery, and to generate a resected nucleoprotein strand that could seek out and invade the nearest available homologous sequence – in this case, one of the repeated sequences in the concatemeric transforming DNA, immediately to hand (Figure 4.7d). Resolution of this second recombination event (Figure 4.7e) would result in the generation of a targeted locus where the transgene is inserted at one end of the targeted sequence, without replacing the central region (sequence 'b'), as illustrated in Figure 4.7f. The generation of such a locus would usually not be detectable by a PCR reaction designed to amplify only a short region of sequence using external PCR primers corresponding to either end of the targeted gene, as described earlier (Figure 4.5). Evidence to support this model was obtained by amplifying and sequencing a number of such 'TI junctions' using primers corresponding to sequence 'b' and the selection cassette, and confirming that the structure of the targeted locus was as predicted, and that a concatemerisation between transforming fragments had occurred as a consequence of NHEJ (Kamisugi et al., 2006).

4.5 Mechanisms of gene targeting

The molecular mechanisms by which gene targeting occurs in *P. patens* are not known. However, we may start from the assumption that the mechanisms by which exogenous DNA integrates into the genome of a living organism are most likely based on the existence of endogenous mechanisms for the repair of damaged DNA. Maintaining the integrity of the genetic material is essential for both the short-term survival of an organism and the long-term continuation of the species. Whilst the process of evolution requires that the genetic material be mutable, a high rate of mutation lowers fitness. Consequently, cells have evolved multiple mechanisms for the repair of damaged DNA, which include proof-reading mechanisms inherent in replicative enzymes to prevent the accumulation of replicative misincorporation of bases, mismatch and excision repair mechanisms to replace incorrectly paired or damaged bases, and mechanisms to repair breaks in the backbone of the DNA molecule. It has been estimated that the human body (containing nearly 10^{13} cells) acquires 10^5 DNA lesions per cell, each day. Fortunately, these are repaired with very high efficiency. Potentially, the most catastrophic form of DNA damage that can occur is the incidence of DSBs. Such breaks may occur as a result of exposure to genotoxic chemicals (e.g. free radicals), to ionising

radiation, or as a consequence of the collapse of replication forks during DNA synthesis. If damage of this type is not repaired, it can result in the permanent and ultimately fatal loss of genetic material. DNA DSBs are among the most cytotoxic agents known. To be effective, therefore, the repair of DSBs must necessarily be both rapid and accurate, minimising deleterious reshuffling of sequences that would occur by the rejoining of the 'wrong' ends of DNA molecules.

In eukaryotic organisms, two alternative pathways have been identified that enable the rapid repair of DNA DSBs. These are the rejoining, end-to-end, of broken DNA strands by a process of NHEJ, and the repair of broken molecules by using a homologous copy of the DNA sequence as the template for a replication-associated repair process. Homology-dependent DSB repair is often termed 'homologous recombination' (HR), and it is most likely that the targeted integration of transforming DNA into the *P. patens* genome utilises this process of homology-dependent repair. The frequency with which targeted integration of transgenes is favoured over non-targeted integration suggests that in *P. patens* somatic cells, the homology-dependent DNA repair pathway is favoured over the NHEJ pathway. This is not a common phenomenon. A preference for HR over NHEJ is shared by *P. patens* and the yeast, *S. cerevisiae*, which both show very high targeting efficiencies (up to 100% of stable transformants may be targeted) but is otherwise unusual. Among vertebrates, only a few cell types exhibit high rates of gene targeting when transfected by transgenes containing homology with endogenous sequences. Often, these cells are predisposed to a recombinogenic DNA repair pathway by themselves being specialised for somatic recombination activity. The *Gallus gallus* DT40 cell line is one very well-characterised example. Derived from virally induced lymphoma (Baba et al., 1985), this cell line was found to undergo a continual reshuffling of its immunoglobulin genes – normally, a highly specific application of somatic recombination in immune system cells (Buerstedde et al., 1990). This cell line also utilises this recombinogenic mechanism to incorporate transforming DNA into targeted loci with great facility (Buerstedde and Takeda, 1991). In mammals, gene targeting is also possible in cells with an immune function (e.g. the *Homo sapiens* Nalm-6 cell line: Adachi et al., 2006) and in pluripotent cells such as *Mus musculus* ES cell lines, from which transgenic animals can be regenerated. It is often difficult to compare the efficiencies of gene targeting in vertebrate cell systems with those in *P. patens*, due to different experimental procedures being adopted with these cell lines. Typically, transfection experiments utilise not only a selection step for transformed cells, but also a strong counter-selection to eliminate cells containing randomly integrated DNA. However, targeting efficiencies of between 4% and 8% (of stably transformed cells) are reported for single alleles of target genes in the Nalm-6 cell line, using methodology that is similar to that used in *P. patens* gene targeting experiments. This cell line is described as 'highly proficient' for gene targeting (Iiizumi et al., 2006), but this remains an order of magnitude lower than the 80–100% gene targeting efficiency that

can be achieved in *P. patens* and *S. cerevisiae*. Moreover, as vertebrate cells are diploid (unlike the gametophyte of *P. patens*), it is necessary to target both alleles in order to obtain a gene knockout with a recognisable phenotype, and the efficiency with which both alleles are targeted is concomitantly reduced. An efficiency of 0.85% of stable transformants undergoing targeting at both alleles was reported in Nalm-6 cells (Iiizumi et al., 2006), so it is clear that – as memorably described by Schaefer (2001) – *P. patens* is a 'green yeast'.

We can examine the mechanisms required for DNA repair and gene targeting in *S. cerevisiae* to make some testable predictions about the possible mechanisms involved in gene targeting in *P. patens*. These mechanisms are illustrated in Figure 4.8.

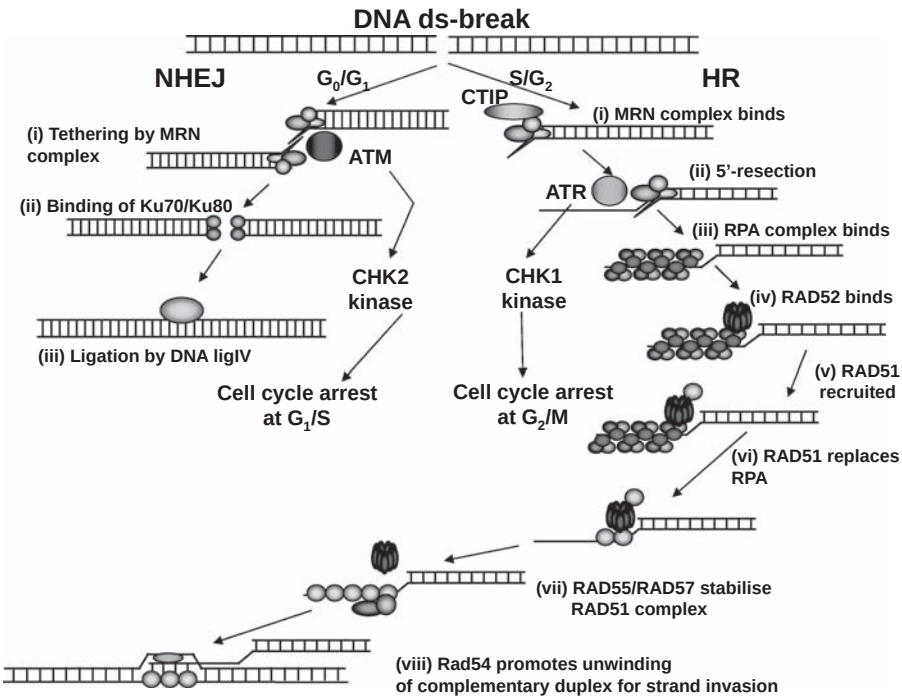

Figure 4.8 Molecular pathways for repair of DNA DSBs. The following model summarises our knowledge of the repair of DNA DSBs based on studies in yeast and mammalian cells. DNA DSBs are detected and bound by the MRN complex (i). If the cell cycle is in G_0 or G_1, then the NHEJ repair pathway followed, in which (i) broken ends are tethered by RAD50, and a CHK2-dependent cell cycle checkpoint is initiated by the ATM kinase. (ii) The broken ends are bound by Ku70/Ku80 and (iii) DNA ligase IV repairs the break. If the cell is in S or G_2, then (i) the CtIP/MRN complex initiates resection to generate (ii) a 3′-ss-DNA strand. ATR signalling via CHK1 imposes a cell cycle checkpoint, and (iii) the RPA complex is recruited to the 3′-ssDNA. (iv) RAD52 binds and (v) recruits RAD51 to form an invasive strand (vi–vii) that is stabilised by additional RAD51 paralogues. (viii) Strand invasion is facilitated by RAD54 to form a homologous recombination junction.

Repair of DSBs in DNA – whether by NHEJ or by HR – has some initial features in common. These features involve the recognition of the broken ends of the DNA, their immobilisation or processing, and the necessity to coordinate DNA repair with the cell division cycle: it would be disadvantageous to the organism to proceed through mitosis before any broken chromosomes are repaired, since this would result in the loss of acentric fragments. The earliest stages of the DNA damage response thus involve the binding of the free ends of broken DNA molecules by a protein complex, and the initiation of a signal transduction cascade that results in the initiation of a cell cycle checkpoint. In *S. cerevisiae*, the broken ends of DNA molecules are recognised and bound by a trimeric protein complex – the 'MRX complex'. The participants in this complex are encoded by the genes *MRE11*, *RAD50* and *XRS2* (Trujillo et al., 2003). These genes, and their products, are highly conserved among all eukaryotes (in *H. sapiens*, the ortholog of *XRS2* is known as *NBS1*, indicating its association with the genetic disorder 'Nijmegen breakage syndrome', and the complex is consequently described as the 'MRN' complex (Stracker et al., 2004). We shall hereafter use the *H. sapiens* notation, since the *A. thaliana* counterparts also use the *H. sapiens* nomenclature. The MRN complex binds to DSBs, and sets in train a series of events. The MRE11 protein has both endo- and exonucleolytic activity that are most likely required for processing the broken ends, by 'polishing' in the event of NHEJ, and in single-strand resection for HR (Lewis et al., 2004). It also has binding sites for the RAD50 and NBS1 proteins, and consequently acts as a 'scaffold' for the complex. The RAD50 protein is distinguished by having a long dimerisation domain. This can be used to tether adjacent broken ends of DNA strands to prevent fragment loss, and to facilitate the rejoining of the correct fragments (Stracker et al., 2004). The NBS1 protein is responsible for recruiting phosphoinositide-3-kinase-related protein kinases (or 'PIKKs') to the broken ends of DNA molecules, through interactions with its C-terminus (Falck et al., 2005). These are instrumental in initiating a DNA damage-mediated cell cycle checkpoint (Jazayeri et al., 2006). These kinases include the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and the products of the '*ATM*' and '*ATR*' genes (in *H. sapiens*, this derives from 'Ataxia telangiectasia-mutated' and 'Ataxia-telangiectasia and Rad3-related', after the genetic neurodegenerative disorder with which they are related. The corresponding *S. cerevisiae* orthologs are named *TEL1* and *MEC1*, respectively.) (Rouse and Jackson, 2002). ATM (and DNA-PKcs) appears preferentially associated with the repair of DSBs, whilst ATR is activated by single-stranded DNA, and also in the repair of breaks that occur as a consequence of replication forms becoming stalled when the polymerase encounters a single-stranded break in its template. (Stalled replication forks probably represent the most common cause of DSBs in DNA under normal growth conditions.) The interaction between NBS1 and ATM results in the phosphorylation of NBS1 by ATM, and also of the histone H2AX, the cohesin complex protein SMC1 ('structural maintenance of chromosomes' – associated with sister chromatid cohesion: Yazdi et al., 2002), and

the cell cycle checkpoint kinase CHK2, to bring about the necessary pause for repair of broken chromosomes before mitosis can proceed.

Following these initial associations, DNA repair may proceed either by NHEJ or by HR. In NHEJ, a heterodimeric protein complex – the 'Ku70/Ku80' complex is recruited to the broken ends of the DNA, displacing the MRN complex. Like NBS1, the Ku80 component also possesses a conserved C-terminal PIKK-binding domain, which recruits the DNA-PKcs and activates PIKK kinase activity. The Ku heterodimer is also itself phosphorylated by DNA-PK and ATM, although whether this is required for DNA repair by NHEJ is disputed (Douglas et al., 2005). Additional components recruited to the site of the DSB include the XRCC4 protein (for x-ray cross-complementing), and DNA ligase IV, which interacts with XRCC4 and other factors (Ahnesorg et al., 2006) and rejoins the broken ends. Another component of the NHEJ machinery is the 'Artemis' protein. Originally identified as an essential component for the processing of V(D)J recombination in the generation of antibody diversity in the mammalian immune system (the protein acts to process hairpin structures), it is thought to be associated with the end processing necessary for the ligation of broken ends. Because DNA DSBs do not necessarily have 'flush ends' that can simply be religated, it is first necessary to process any overhanging single strands. Repair of DSBs by NHEJ is therefore characterised by small DNA deletions, rearrangements and insertions of 'filler' DNA at the site of the DSB, and although a rapid mechanism for DNA repair, it is also error prone.

The repair of DNA DSBs by the use of a homologous sequence shares the initial damage sensing step (MRN/PIKK-mediated), but uses a homologous sequence with which to repair the damaged DNA in a template-dependent manner. It is thus a fully conservative means of repairing DNA damage. For such template-mediated repair to occur, the availability of a template sequence, conveniently to hand, is clearly a prerequisite. This is most likely to happen following the production of a sister chromatid in the S-phase of the cell cycle, and it is notable that whilst NHEJ predominates in the G_0 and G_1 phases of the cell cycle in *S. cerevisiae* and *H. sapiens*, cells undergoing HR-mediated DNA repair experience a CHK1-mediated cell cycle checkpoint at the G_2 to M-phase transition (Weterings and Van Gent, 2004). It has been proposed that the status of the cell cycle is the principal factor in determining the choice of DNA repair pathway favoured by cells. It is therefore highly significant that analysis of *P. patens* protonemal tissue has demonstrated that the majority of chloronemal cells (the predominant cell type from which protoplasts are isolated for transformation) appear arrested in G_2 and contain a 2C quantity of DNA (Schween et al., 2003).

Recruitment of the MRN/PIKK complex at the site of DNA damage occurs following an initial interaction between the MRE11 protein and another component: 'CtIP' (for 'Ctb-interacting protein': in *S. cerevisiae*, the corresponding gene product is designated Sae2). The CtIP protein is specifically recruited to DSBs, but only during the S and G_2 phases of the cell cycle.

(Sartori et al., 2007). The broken ends of the DNA strand are then processed in a manner significantly different to that used in NHEJ-mediated repair. The free 5′-end of the molecule is exonucleolytically processed; this single-strand resection is being mediated by the MRE11-CtIP complex and generating a relatively long 3′-overhanging single-stranded 'tail'. Another heterotrimeric protein complex is recruited to coat this single-stranded DNA. This is the 'Replication Protein A' complex (RPA), comprising subunits of ca. 70, 30 and 14 kDa, respectively (Binz et al., 2004). The 70 and 30 kDa subunits of the RPA complex are highly conserved in eukaryotes; the 14 kDa subunit is less so. The RPA complex is essential for both DNA replication and a variety of DNA damage repair mechanisms, including excision repair, mismatch repair and DSB repair by HR. Phosphorylation of the 30 kDA subunit at an N-terminal location by PIKK activity appears to be required for modulating its activity, resulting in suppression of its replication-associated function, and promotion of its role in DNA-damage repair (Binz et al., 2004).

The loading of the RPA complex onto the 3′-single strand tail appears to be a prerequisite for the CHK1-mediated cell cycle checkpoint, which is initiated following the recruitment of the ATR kinase by the RPA-coated single strand (Zou and Elledge, 2003; Jazayeri et al., 2006), and for the subsequent formation of an invasive nucleoprotein strand that is capable of seeking out and annealing with its homologous sequence in the template DNA. The formation of a heteroduplex is facilitated by the products of a group of genes known in *S. cerevisiae* as the 'RAD52 epistasis group' – a definition of their genetic interrelationship (Krogh and Symington, 2004). Central to the formation of an invasive single strand is the RAD52 protein. This polypeptide forms heptameric ring structures, and becomes rapidly localised to DNA damage repair foci following the initiation of DNA DSBs. In *S. cerevisiae*, such localisation occurs only in S-phase of the cell cycle, and the RAD52 protein physically interacts with both the RPA complex and with the RAD51 protein – the eukaryotic homologue of the bacterial RecA protein (Lisby et al., 2001). The RAD52 protein also forms DNA repair foci in meiotic nuclei. *S. cerevisiae* mutants defective or deleted in the *RAD52* gene are seriously compromised in DNA repair, and are therefore highly susceptible to genotoxic chemicals or ionising radiation. This contrasts with vertebrate cell lines, which remain able to tolerate DNA damage induced by such agents, although they show a marked reduction in their ability to undertake either somatic HR, or the targeted integration of transgenes (Yamaguchi-Iwai et al., 1998). These cells are presumably able to continue to repair DSBs by NHEJ, indicating a central role for the RAD52 protein in directing DNA repair towards the HR pathway and away from the NHEJ pathway. The RAD52 protein is also capable of acting *in vitro* to stimulate DNA strand exchange (Bi et al., 2004).

Interestingly, whilst RAD52 is highly conserved between *S. cerevisiae* and vertebrates, it is not universally conserved in all eukaryotes. Thus, no homologue of the *RAD52* gene has been identified in the genome of *Caenorhabditis elegans*, and nor is there a recognisable *RAD52* homologue in either the

A. thaliana or the *P. patens* genome. Since HR remains an obligatory feature of meiosis in all eukaryotes (and in *S. cerevisiae* and mammals, meiotic recombination is RAD52-dependent), and appears to provide the major pathway for DNA damage repair in *P. patens*, this suggests that alternative mechanisms for coordinating the DNA repair pathway exist. There are clearly other species-specific components to the homology-dependent DNA repair pathway. For example, the breast cancer-susceptibility genes *BRCA1* and *BRCA2* are required for homology-dependent repair in mammalian cells (Moynahan et al., 1999, 2001), in which a direct interaction between the BRCA2 protein and RAD51 occurs (Davies et al., 2001) to stimulate DNA repair focus formation. However, these genes have no homologues in *S. cerevisiae*, whilst in plants, an *A. thaliana* gene similar to *BRCA1* is present, and there are two *BRCA2*-like genes. Functional analysis of these genes indicates that their principal role in *A. thaliana* is in meiotic recombination, the AtBRCA2 protein interacting with the meiosis-specific RAD51-like protein DMC1 (Siaud et al., 2004). In *P. patens*, although some putative genes encode BRCT-like domains, sequence homology searches fail to identify these as obvious homologues of either *BRCA1* or *BRCA2*, and functional characterisation will be necessary to elucidate their roles. Also, both mammalian and plant genomes contain additional paralogues of the *RAD51* gene (designated *RAD51B, C* and *D*). The *P. patens* genome also contains these paralogous genes, as well as (unusually) duplicated *RAD51* genes (Markmann-Mulsich et al., 2002, 2007).

The RAD51 protein interacts directly with the 3′-single-stranded tail. In *S. cerevisiae*, the recruitment of RAD51 results in the displacement of the RPA complex, and this is facilitated by RAD52. The outcome is the formation of a helical nucleoprotein strand that is capable of invading duplex DNA and annealing with a complementary sequence. The formation of heteroduplex DNA as a result of strand invasion is facilitated by other members of the *RAD52* epistasis group. Foremost among these are the Xrcc2 and Xrcc3 proteins (which are designated as RAD55 and RAD57, respectively, in *S. cerevisiae*) that are thought to stabilise the RAD51-single-strand DNA interaction (Masson et al., 2001; Liu, 2002). RAD51 function appears highly conserved in all eukaryotes, including *P. patens*. However, unlike in *A. thaliana*, where the *RAD51* gene is required for progression through meiosis, but not for vegetative growth, the *P. patens* RAD51 proteins are clearly required for repair of somatic DNA damage. Targeted knockout of both copies of the *PpRAD51* genes results in severely reduced protonemal growth, as well as meiotic incompetence. Additionally, these mutant strains are hypersensitive to both ionising (UV) radiation and the genotoxin bleomycin, which causes DNA DSBs (Markmann-Mulisch et al., 2007). This reinforces the view that whereas somatic DNA damage repair in flowering plants occurs primarily through the RAD51-independent NHEJ pathway, the favoured means of DNA DSB repair in *P. patens* is by the homology-dependent pathway. Another important component of the recombinational machinery is the RAD54 gene product. This is a member of the Swi/Snf ATPase superfamily, and it is thought to

facilitate heteroduplex formation by local unwinding of the target DNA and the redistribution of nucleosomes within the chromatin, thereby (presumably) rendering the target sequence more accessible to the invading strand (Alexeev et al., 2003). It has also been shown that the RAD54 protein associates specifically with Holliday junctions to promote branch migration along the DNA. The importance of the RAD54 protein has been highlighted by the demonstration that over-expression of the *S. cerevisiae RAD54* gene in transgenic *A. thaliana* resulted in an elevated frequency of gene targeting, following T-DNA transformation (Shaked et al., 2005), although the frequency of targeting remained two orders of magnitude lower than that achievable in *P. patens*.

Following strand invasion and annealing, extension of the 3′-end by DNA polymerase activity and ligation by DNA ligase I results in the formation of Holliday junctions which are then resolved by cleavage and ligation to produce two intact DNA molecules, in the case of DNA repair. In a gene targeting experiment, transformation with a linear DNA fragment presumably results in the ends of the transfected DNA molecule being recognised as DNA DSBs by the DNA repair machinery. Each end of the transforming DNA would then be able to undergo resection, heteroduplex formation and recombination-mediated integration with its homologous target, to generate a single TGR. Such separate strand-invasion events have been demonstrated to occur in *S. cerevisiae* (Langston and Symington, 2004), and this represents the most likely means of integration in *P. patens*.

4.6 Unanswered questions and future prospects

Gene targeting is a powerful technology for undertaking the genetic modification of an organism. Its very precision recommends it as a means for introducing genetic modifications at specific sites whilst leaving the rest of the genome undisturbed. For this reason, the development of gene targeting technology for the modification of crop species is a desirable strategic objective for the application of biotechnology to agriculture. Whilst recent advances have demonstrated that gene targeting can be achieved in crop species (Terada et al., 2002, 2007; Iida and Terada, 2005), the frequencies achievable remain low. Furthermore, although some enhancement of gene targeting efficiency has been obtained in the model species *A. thaliana* through the transgenic expression of a *S. cerevisiae* recombination gene (Shaked et al., 2005), the mechanisms by which gene targeting is achieved in plants remain opaque. In order to develop gene targeting as a reliable and routine method for the genetic modification of plants, it is first necessary to determine the mechanism by which it is mediated. Only with a comprehensive knowledge of the underlying processes of plant gene targeting can scientifically informed strategies be developed. To this end, it is sensible to study the process of HR, not in organisms that are largely incompetent to carry it out, but in

organisms that exhibit high frequencies of gene targeting. Our understanding of homology-dependent DNA repair in eukaryotes rests largely on a set of paradigms developed from the study of the targeting-proficient yeast, *S. cerevisiae*. It is therefore logical that the best prospects for dissecting the plant-specific aspects of the gene targeting pathway lie in the study of the process in the equally proficient *P. patens*.

How should such studies proceed? Three strategies recommend themselves. First, and most obviously, the roles of the *P. patens* homologues of known recombination proteins can be tested by constructing mutant lines containing targeted mutations of these sequences (Trouiller et al., 2006; Markmann-Mulisch et al., 2007). The availability of the genome sequence enables us to identify many of the *P. patens* homologues of *S. cerevisiae* and mammalian recombination genes, and to construct targeting vectors accordingly. Such a strategy will confirm the necessity for these conserved components in the process, and may reveal some species-specific properties of these candidate genes, but it is unlikely to reveal any novel components required for efficient recombination in *P. patens*. The identification of previously uncharacterised participants in the homology-dependent DNA repair pathway will therefore require experiments that do not rely on *a priori* assumptions. Several strategies spring to mind. The first is a biochemical approach, in which novel interacting partners in HR complexes can be identified through their association with known participants. Such experiments might utilise the yeast 2-hybrid system to identify interactions, *in vivo*, or use affinity-tagged 'pull-down' assays to identify protein complexes *in vitro*. A second approach uses a systems-based approach. Analysis of the transcriptomic responses to DNA damaging agents may reveal novel gene products whose synthesis is required for DNA repair, and which can subsequently be tested by constructing mutant strains. This approach has identified a number of putative damage-repair genes in *A. thaliana* (Doucet-Chabeaud et al., 2001; Lafarge and Montane, 2003). The completion of the genome sequence enables large-scale transcriptional analysis, either through the deployment of a microarray platform (Cuming et al., 2007), or through the application of next-generation sequencing technologies to identify up-regulated transcripts (Weber et al., 2007). Analysis of the DNA-damage-responsive phosphoproteome should identify components of the signal transduction pathway that are activated by phosphorylation in response to DNA damage: little is known about the imposition of DNA-damage-related cell cycle arrest in plants, and homologues of some of the key signalling proteins that act in *S. cerevisiae* or mammalian cells are either difficult to identify (e.g. the CHK1 and CHK2 kinases) or are absent (e.g. the P53 tumour suppressor protein). Finally, a genetic approach should be pursued. Our knowledge of DNA repair pathways derives almost entirely from the analysis of *S. cerevisiae* mutants that were compromised in their ability to survive DNA-damaging radiation (hence their designation as *RAD* – radiation sensitive or *XRS* – x-ray sensitive genes). A corresponding genetic approach in *A. thaliana* has identified several genes

instrumental in plant DNA repair, but these have not been associated with the HR pathway (Jenkins et al., 1995; Jiang et al., 1997; Hefner et al., 2002). This is not surprising, since HR is not the primary mechanism for DNA repair in this species. Since *P. patens* favours homology-dependent DNA repair, it follows that a mutagenic screen for radiation sensitive mutants should identify principally genes required for this process. The development of the *P. patens* genetic linkage map as a tool to support map-based cloning of mutant genes should enable such an experimental approach in the near future.

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Chapter 5

THE SMALL RNAs OF *PHYSCOMITRELLA PATENS*: EXPRESSION, FUNCTION AND EVOLUTION

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Abstract: Information encoded by the sequential arrangement of DNA bases guides the synthesis of functional polypeptides via RNA intermediates. However, the functions of RNA transcend those of a mere messenger. The discovery and characterization of gene regulation orchestrated by short, non-protein-coding RNAs has revealed previously unsuspected but vast systems of gene regulation, especially in metazoans and land plants. Small silencing RNAs, which include short interfering RNAs (siRNAs) and microRNAs (miRNAs), are exceptionally diversified and numerous in plants. Their known roster of functions includes, at a minimum, development, morphogenesis, nutrient acquisition and genome defence. Recent studies of the naturally occurring small silencing RNAs of *Physcomitrella patens* have elucidated fundamental aspects of siRNA biogenesis and miRNA evolution. In this chapter, I describe the biogenesis and expression of miRNAs and siRNAs in *P. patens*, as well as their functions and evolution.

Keywords: Argonaute; Dicer; microRNA; RNAi silencing; siRNA

5.1 Introduction to small RNAs

Most eukaryotes examined to date express at least one type of small silencing RNA. Though the molecular and functional details of these small silencing RNAs are as diverse as are the varied organisms which express them, they are united by three universal characteristics:

1. Small silencing RNAs are produced by the endonucleolytic processing of a longer precursor possessing extensive regions of RNA–RNA base pairing.

2. After production, a single-stranded small silencing RNA is bound to a protein in the Argonaute family to make a functional RNA-induced silencing complex (RISC).
3. The assembled RISC interacts with 'target' RNAs with a specificity conferred by base pairing interactions between the bound small silencing RNA and the target. RISC-target interaction typically leads to the repression of target gene expression through one or more distinct mechanisms.

Many small silencing RNAs can be thought of as *bona fide* gene products: just as for polypeptides, the specific removal or alteration of a single small RNA can have profound biological consequences. However, small RNAs are in one sense fundamentally different than 'traditional' gene products – because their sole molecular mode of action is to direct the repression of one or more specific targets *in trans*, they cannot function in isolation. Rather, it is the specificity and strength of gene regulation conferred by small silencing RNAs that determines their biological functions.

5.2 Classes of small silencing RNAs

5.2.1 siRNAs

Short interfering RNAs (siRNAs) arise from long, perfectly double-stranded RNA (dsRNA). The initiating long dsRNA can come from any number of sources, including the convergent transcription of adjacent genes in the genome, the action of RNA-dependent RNA polymerases (RDRs), which convert single-stranded RNA into dsRNA, and the replication of RNA viruses. Regardless of the source, the long dsRNA is cleaved at 21–24 nt intervals by an endonuclease in the cleverly named Dicer family (Figure 5.1, Plate 3). The sizes of siRNA duplexes are dependent upon the specific Dicer protein which made them, and different size classes often coexist in the same species due to the activities of multiple, functionally diversified Dicers. Because of the catalytic mechanism of Dicer proteins, siRNA duplexes have unique biochemical and structural properties. In contrast to RNA cleavage initiated by hydroxide (as in the spontaneous breakdown of RNA in aqueous solutions), Dicer catalysis leaves siRNA duplexes with a 5′-monophosphate and a 3′-hydroxyl. The siRNA duplexes also contain 2 nt overhangs at the 3′-ends, reflecting the staggered cuts made by Dicers (Figure 5.1, Plate 3). Only one strand from any individual siRNA duplex becomes a functional siRNA – this functional strand is termed the guide strand (to reflect the fact that it guides the selection of targets by base pairing interactions), while the other is termed the passenger strand. The assembly of a functional RISC involves the association of the guide strand with an Argonaute protein coincident with removal of the passenger strand (Figure 5.1, Plate 3). The loaded RISC then selects targets which are complementary to the guide strand and enables their repression

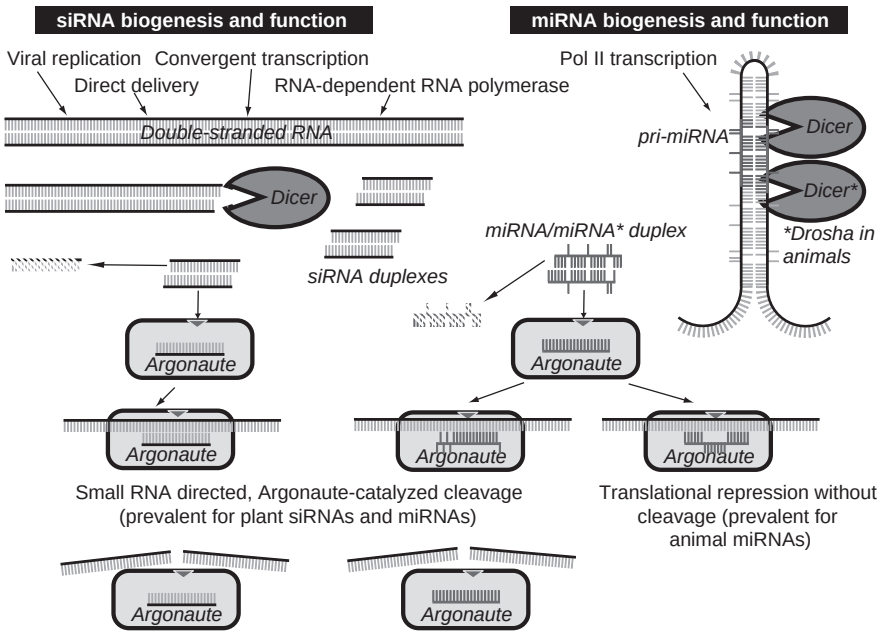

Figure 5.1 Biogenesis and functions of siRNAs (left) and miRNAs (right). (For a color version of this figure, see Plate 3)

either by Argonaute-catalysed target cleavage or by Argonaute-dependent mechanisms independent of target cleavage (Figure 5.1, Plate 3).

The first siRNAs reported were derived from exogenous sources, including viruses and silenced transgenes in plants (Hamilton and Baulcombe, 1999) and directly delivered dsRNA in *Drosophila melanogaster* embryo lysates (Zamore et al., 2000) and intact *Caenorhabditis elegans* (Parrish et al., 2000). The ability to eliminate the expression of specific genes simply by introduction of exogenous dsRNA or siRNAs homologous to the target mRNA has allowed critical advances in molecular genetics, especially in systems such as human cell lines where genetic analyses were previously quite difficult. The importance of this breakthrough was recognized in 2006 when that year's Nobel Prize in Medicine or Physiology was awarded to Andrew Fire and Craig Mello for their pioneering work in silencing genes using exogenous dsRNA – a methodology termed RNA interference (RNAi; Fire et al., 1998). Importantly, RNAi is an effective method of gene silencing in *Physcomitrella patens* as well (Bezanilla et al., 2003, 2005). However, siRNA-mediated gene silencing did not evolve for the benefit of scientific inquiry. The ability to silence the expression of invading nucleic acids, especially RNA viruses that must pass through a dsRNA intermediate to replicate, has likely been the chief selection pressure serving to maintain the siRNA pathway in various eukaryotic lineages. This, however, does not mean that all natural siRNAs are

derived from extra-genomic sources: on the contrary, *C. elegans* and all plants investigated produce abundant siRNAs from multiple endogenous loci dispersed within their respective genomes. The endogenous siRNAs from plants are especially numerous and diversified in their biogenesis and functions.

5.2.2 miRNAs

Both microRNAs (miRNAs) and siRNAs are single-stranded RNAs between 20 and 24 nts in size, which ultimately function to repress the expression of target genes *in trans* based upon Watson–Crick complementarity. What then is the justification for their classification as distinct molecular species? The primary and most definitive distinction between siRNAs and miRNAs is their biogenesis. As opposed to siRNAs which are processed from long, perfectly dsRNAs, miRNAs emanate from single-stranded precursors (termed primary miRNAs or pri-miRNAs) which can adopt an imperfectly base-paired stem-loop secondary structure (Figure 5.1, Plate 3). Certain Dicer family proteins recognize this stem-loop and catalyse the precise excision of a single initial duplex, termed the miRNA/miRNA* duplex. One strand of this transient duplex (the mature miRNA) is incorporated into an Argonaute protein, while the other strand (the miRNA*) is not. The second distinguishing feature between siRNAs and miRNAs is the nature of their targets: most siRNAs act in *cis* – they target single-stranded RNAs identical to the dsRNA which spawn them. In contrast, miRNAs act by targeting in *trans* – they target transcripts *distinct* from the pri-miRNAs from which they arise. However, this second distinguishing criterion between siRNAs and miRNAs is not quite universal: certain plant small RNAs arise as a result of the processing of long dsRNA but function to regulate target RNAs in *trans*. These so-called *trans*-acting siRNAs (ta-siRNAs) combine siRNA-like biogenesis with miRNA-like targeting functions (Peragine et al., 2004; Vazquez et al., 2004), and are discussed in detail below.

As opposed to siRNAs, which in general arise dynamically in response to specific RNA-based threats, miRNA expression is generally guided by highly regulated circuits of transcriptional control. In this respect, miRNAs are similar to typical protein-coding genes. Like protein-coding mRNAs, pri-miRNAs in both animals (Lee et al., 2004) and plants (Xie et al., 2005a) are transcribed by RNA polymerase II and are capped, polyadenylated, and sometimes spliced. Also similar to protein-coding mRNAs, accumulation of miRNAs is often tissue specific or responsive to certain environmental cues. This specificity in miRNA expression likely reflects the necessity of tissue or environment-specific miRNA-mediated target regulation.

5.2.3 piRNAs and 21U RNAs

Other types of small silencing RNAs which are clearly distinct from siRNAs and miRNAs have been described in various animals. The 21U RNAs of

C. elegans emanate from two broad regions of a single chromosome in patterns that suggest that their biogenesis does not resemble the canonical siRNA or miRNA pathways (Ruby et al., 2006); their functional relevance remains unknown. Piwi-interacting RNAs (piRNAs) accumulate specifically in the testes of vertebrates and the germ cells of *D. melanogaster*. piRNAs are substantially longer than siRNAs and miRNAs, with lengths between 28 and 30 nts, and in contrast to siRNAs, their accumulation is not Dicer dependent (Vagin et al., 2006). The piRNAs are found in association with Piwi proteins, which form a distinct, animal-specific sub-clade within the Argonaute protein family (Seto et al., 2007). Though animal piRNAs and the siRNAs are clearly distinct in their biogenesis, they have analogous functions: both play important roles in the silencing of repetitive, likely parasitic, elements of the genome such as transposons. It is interesting to note that piRNA-expressing organisms like *D. melanogaster* and mice also can produce siRNAs against repetitive elements, but their contribution to the overall small RNA repertoire is rather small (Czech et al., 2008; Tam et al., 2008). In contrast, organisms that apparently lack piRNAs, including land plants, instead deploy siRNAs to fulfil this role. Because there are currently no data suggesting the existence of piRNAs or 21U RNAs in *P. patens* or indeed in any other land plant, interested readers are referred to two recent reviews on the subject for more information (Hartig et al., 2007; Seto et al., 2007).

5.2.4 Phylogenetic distribution of small silencing RNAs

Both siRNAs and miRNAs fundamentally depend on two proteins for their biogenesis and functions: the Dicer protein, which excises an initial duplex RNA from a precursor, and the Argonaute protein, which binds the mature small RNA and catalyses repression of target RNAs. Both Dicers and Argonautes have characteristic arrangements of conserved protein domains, which have allowed their identification in many organisms. All Dicers and nearly all Argonaute proteins identified to date are from eukaryotic organisms, suggesting strongly that small silencing RNAs are unique to the eukaryotes. The only exceptions are two archaeal Argonaute homologues. Despite the fact that the crystal structures of these two archaeal proteins were instrumental in the understanding of Argonaute function (Song et al., 2004; Ma et al., 2005; Parker et al., 2005), no small silencing RNAs have yet been reported from any archaeal or eubacterial species, which supports the hypothesis that the Dicer/Argonaute small silencing RNA system is a derived feature of eukaryotic gene expression.

The expression of endogenous small RNAs has been observed in a large diversity of eukaryotic organisms, including vertebrates, invertebrates, fission yeast, land plants, ciliated protozoa and unicellular green algae. While the capacity to express siRNAs is broadly distributed among both unicellular and multicellular organisms, miRNA expression is conspicuously absent from most unicellular organisms and from the fungi. Many animal miRNA

families are conserved between long-diverged animal taxa (Sempere et al., 2006), while many land plant miRNA families are conserved between widely divergent plant species (Axtell and Bartel, 2005; Axtell et al., 2007). However, there is very little evidence for conservation of any homologous miRNAs *between* animals and land plants – while the miRNA families miR854 and miR855 have been reported to be conserved between animals and land plants (Arteaga-Vazquez et al., 2006), closer inspection of these loci reveals that they correspond to retrotransposon fragments which produce siRNAs in multiple species (MJA, unpublished observations).

The separate cohorts of animal and plant miRNAs along with major differences in the biogenesis, processing and dominant modes of target regulation between the two kingdoms suggest that the miRNA system of gene regulation arose independently in each lineage from an ancient siRNA-producing pathway. The demonstration of miRNAs expressed in the single-celled green alga *Chlamydomonas reinhardtii* (Molnar et al., 2007; Zhao et al., 2007) might necessitate the revision of this simple 'two origin' hypothesis of miRNAs. One possibility is that the *C. reinhardtii* miRNAs arose independently of both the land plant miRNAs and the animal miRNAs. The fact that none of the known *C. reinhardtii* miRNAs are homologous to any known plant or animal miRNAs is consistent with this hypothesis. Another possibility is that the miRNA pathway arose in the ancient ancestor of both *C. reinhardtii* and land plants. The fact that both *C. reinhardtii* and land plant miRNAs regulate their targets by directing their cleavage (Molnar et al., 2007; Zhao et al., 2007), as opposed to the translational repression which is typical of animal miRNAs, supports this hypothesis. A third, less parsimonious hypothesis is that the miRNA system was in place long ago in the last common ancestor of all eukaryotes but has diverged considerably in the long-separated lineages leading to today's animals, plants and green algae, and has been lost entirely from many other groups.

5.3 Expression of *P. patens* small RNAs

The expression of small silencing RNAs can be directly observed by sequencing of short cDNAs derived from adapter ligation and PCR (polymerase chain reaction) using standard plasmid cloning and Sanger di-deoxy sequencing (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001), massively parallel signature sequencing (Lu et al., 2005) and pyrosequencing (Rajagopalan et al., 2006). Such small RNA sequencing studies are akin to expressed sequence tag (EST) projects in that they are designed to discover empirically mature RNA transcripts. However, in contrast to ESTs derived from protein-coding mRNAs, meaningful analyses of sequenced small RNAs critically depend upon the availability of a sequenced nuclear genome. A translated protein sequence can be confidently inferred from EST sequences, and database comparisons can often identify homologues of that protein with known functions. In contrast, functional annotation of small RNAs depends

on knowledge of their potential target genes and the biogenesis of the small RNA itself. Most critically, the ability to distinguish a miRNA from an endogenous siRNA depends on analysis of the potential RNA secondary structure flanking the small RNA in question – if the small RNA falls within the stem of a predicted stem-loop structure, it is likely to be miRNA. Genomic sequences flanking small RNAs may occasionally be gleaned from ESTs corresponding to miRNA precursors (Jones-Rhoades and Bartel, 2004), but thorough analyses of any sequenced small RNA data set are greatly enhanced by an available complete genome sequence for the organism under study. The release of an annotated draft genome sequence of the *P. patens* nuclear genome (Rensing et al., 2008) has therefore greatly catalysed small RNA research in this organism.

As of this writing (June 2008), three different groups have reported cDNA sequences derived from *P. patens* small RNAs (Table 5.1). The union of these small RNA data sets yields 215 206 unique small RNAs. Because many small RNAs have been obtained more than once, these small RNAs have been sequenced as an aggregate of 561 491 times (Table 5.1). In total, and in combination with computational predictions of conserved miRNAs (Fattash et al., 2007), these data have formed the basis for the current understanding of *P. patens* small RNAs. Despite the large number of small RNAs that have been observed, it is likely that there are more to be discovered. In particular, the described small RNA libraries have all been derived from readily harvested specimens grown under optimal laboratory conditions. Any small RNAs uniquely expressed in response to alternative environmental cues or in a very small number of cells will therefore be under-represented or absent from these data.

5.3.1 miRNAs

Early evidence for the expression of a *P. patens* miRNA was described by Floyd and Bowman (2004), who demonstrated that homologues of the *Arabidopsis*

Table 5.1 Sequenced small RNA-derived cDNA libraries from *P. patens*

Library source(s)	Uniques	Reads	Citations[a]
Protonemata	124	209	A, B
Mixed protonemata and gametophores	152	180	C
Protonemata	83 726	140 036	D, E
Protonemata with buds and young gametophores	77 823	200 545	D, E
Mature gametophores with sporophytes	71 124	220 521	D, E
	Union[b] 215 206	Sum 561 491	

[a]A: Arazi et al., 2005; B: Talmor-Neiman et al., 2006a; C: Fattash et al., 2007; D: Axtell et al., 2006; E: Axtell et al., 2007.
[b]Union is less than the sum of all of the uniques because many small RNAs were found in more than one library.

thaliana homeodomain leucine-zipper III (*HD-ZIPIII*) genes were expressed in a broad range of land plants, including *P. patens* and other bryophytes. At the time, the *HD-ZIPIII* genes were predicted targets of miR166 in *A. thaliana* (Rhoades et al., 2002), and were known to be cleaved by a presumed miR166 activity in wheat germ extract (Tang et al., 2003). Alignments of the land plant *HD-ZIPIII* genes clearly demonstrated conservation of the presumed miR166 target site among all of the homologues; conservation included the nucleotide identities of the third codon positions within the complementary sites, but not the immediately adjacent third codon positions (Floyd and Bowman, 2004). This observation implied that regulation of *HD-ZIPIII* genes by miR166 was present in the last common ancestor of all land plants and that it has been maintained in all of the lineages leading to today's flora. At about the same time, Jones-Rhoades and Bartel (2004) noted the existence of a *P. patens* EST which appeared to be the primary transcript for a homologue of miR319, which was already known to be conserved between diverse angiosperms (Palatnik et al., 2003). Further descriptions of miRNA expression in non-angiosperm plants were provided by Axtell and Bartel (2005) who used microarrays to detect the expression of miRNAs from gymnosperm, fern, lycopod and bryophyte specimens. This study demonstrated the expression of several conserved miRNAs in various taxa. Importantly, the targets of several miRNAs in diverse species were confirmed and shown to be homologous to the targets of the same miRNAs in different species (Axtell and Bartel, 2005). Thus, these studies together provided a compelling case for the hypothesis that multiple, ancient miRNA families have persisted in diverse plant lineages with their regulatory interactions intact.

The first direct demonstration of *P. patens* miRNA expression was reported by Arazi et al. (2005), who sequenced and analysed 100 unique small RNAs derived from a protonemal sample. These data allowed the identification of three homologues of *A. thaliana* miRNAs (miR156, miR319 and miR390), and the cleavage of a miR156 target, *PpSBP3*, was demonstrated. Similar to earlier observations, *PpSBP3* was shown to be a clear homologue of the *A. thaliana SBP* transcription factors, which are targeted by miR156. Additionally, several other candidate miRNAs were detected, but since at the time no *P. patens* genomic sequence was available, it was not possible to definitively distinguish miRNAs from siRNAs and other sequenced RNAs, which did not derive from Dicer processing. In a subsequent study, sequencing of an additional 24 unique small RNAs from the same protonemata-derived small RNA library, coupled with the use of the whole-genome shotgun (WGS) sequence traces being generated as part of the *P. patens* genome project, allowed the confident annotation of several more *P. patens* miRNAs (Talmor-Neiman et al., 2006a). Most of these miRNAs did not appear to be conserved outside of *P. patens*, suggesting that many moss miRNAs might have arisen after the divergence of the bryophyte lineage from angiosperm lineage. Similar conclusions were reached by Fattash et al. (2007), who sequenced 152 unique *P. patens* small RNAs derived from a mixture of protonemata- and gametophore-derived RNA. Combining these sequences with the results of

computational predictions, several more *P. patens* homologues of angiosperm miRNAs were annotated, as well as several additional, seemingly *P. patens* specific, miRNA families.

The largest reported data set of sequenced *P. patens* small RNAs was derived from pyrosequencing of libraries produced from three wild-type specimens at differing developmental stages (Axtell et al., 2006). In aggregate, this sequencing effort yielded over 200 000 unique small RNAs from over 500 000 reads (Table 5.1). Because these libraries were not normalized, the most abundant small RNA sequences were isolated thousands of times (i.e. one unique small RNA represented by thousands of reads). This over-sampling, which might initially appear to be wasteful, in fact offered quantitative information which was useful. Perhaps most importantly, 'deep' sequencing of small RNA libraries increased the probability that the miRNA* (the unstable strand discarded during the maturation of miRNAs) would be recovered. The identification of two expressed small RNAs which, in the context of a predicted stem-loop secondary structure, form a base-paired duplex with the two nucleotide, 3′ overhangs typical of Dicer-mediated processing is very strong evidence of miRNA-like biogenesis (Figure 5.2a, Plate 4). In contrast, deep sequencing reveals siRNA loci as regions where many distinct small RNAs, corresponding to both strands of the genome, accumulate in a chaotic pattern (Figure 5.2b, Plate 4) – this reflects their origins from long, dsRNA instead of from a precisely processed, single-stranded stem-loop precursor. Using these criteria to separate miRNAs from other types of small RNAs, Axtell et al. (2007) used the pyrosequenced small RNA data to annotate a total of 88 *P. patens* miRNA families which collectively were expressed from 205 distinct loci. These totals included most miRNAs which had been previously discovered.

The collective results of the *P. patens* miRNA discovery efforts described above have yielded the annotation of 220 miRNA-expressing loci, representing 100 sequence-distinct families (miRBase version 10.0; `http://microrna.sanger.ac.uk/sequences/`; Griffiths-Jones et al., 2006). Of these, a subset of 15 have homologues outside of the bryophytes (Table 5.2), while the remaining 85 appear specific to *P. patens.* However, the 15 highly conserved miRNA families generate a disproportionate fraction of the overall miRNA expression – these 15 families had a median of 512 sequence reads in the pyrosequencing data sets compared to a median of 113 reads for the other 85 non-conserved families. Similar observations have been made for the miRNAs of *A. thaliana* (Rajagopalan et al., 2006; Fahlgren et al., 2007) and for the lycopod *Selaginella moellendorffii* (Axtell et al., 2007). Why do the most conserved of plant miRNAs tend to also be those that are most abundant? The answer is unknown, but this observation might be related to the mechanisms involved in miRNA evolution, which will be discussed below.

5.3.2 *Trans*-acting siRNAs

The majority of known plant miRNA targets are cleaved by a miRNA-specifies Argonaute endonuclease. In some cases, one of the two cleavage

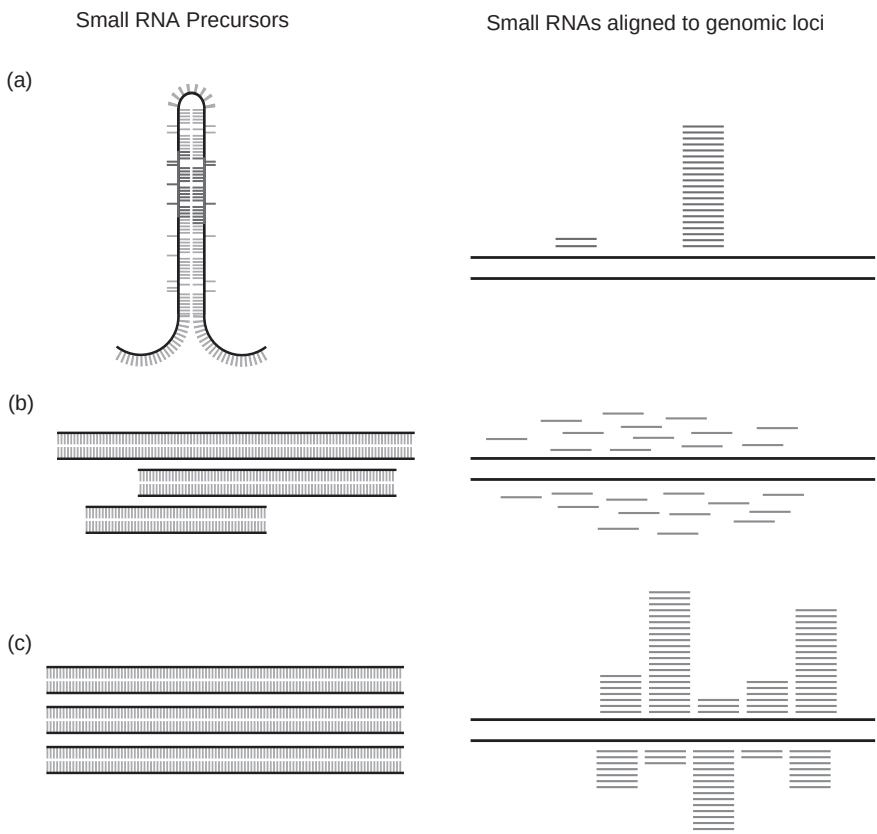

Figure 5.2 Classification of small RNA-producing loci by analysis of sequenced small RNAs. (a) miRNAs are precisely excised from a single-stranded stem-loop precursor as a miRNA/miRNA* duplex (red/blue). When small RNAs derived from miRNA loci are mapped to the genome, they correspond to two discrete positions of the same polarity with respect to the genome. (b) siRNAs are processed from a heterogeneous population of long double-stranded RNAs; thus, when they are mapped to the corresponding genomic loci a chaotic pattern of small RNAs, mapping to both polarities, is observed. (c) ta-siRNAs are processed from a population of long double-stranded RNAs with uniform ends; thus, they correspond to their genomic loci in a regular, phased pattern from both polarities. (For a color version of this figure, see Plate 4)

product becomes a substrate for an RDR, which generates dsRNA using the cleaved mRNA fragment as a template. This dsRNA is subsequently processed by a Dicer enzyme to produce siRNAs, which in some cases can regulate other target mRNAs *in trans*, leading to the term ta-siRNAs. The dsRNA synthesized from a cleaved miRNA target always has one terminus precisely defined by the position of miRNA cleavage. Because Dicer produces siRNAs in a successive manner, beginning with a free dsRNA terminus, the result of this precisely defined dsRNA end is a population of siRNAs which

Table 5.2 Conserved *P. patens* miRNA families, including supporting evidence for the *P. patens* annotation

miRNA family	Prediction by homology	Sequenced	Abundance[a]	miRNA/ miRNA*[b]	RNA blot	Experimentally demonstrated target(s)	Angiosperm homologues[c]	Non-angiosperm homologues[d]
miR156	E	A, E, F	1710	F	A	A, F	*At, Bn, Gm, Mt, Os, Ptc, Sb, So, Zm*	*Sm*
miR160	E	E, F	9160	F	–	–	*At, Gm, Mt, Os, Ptc, Sb, Ta, Zm*	*Sm*
miR166	E	F	350	F	–	F	*At, Gm, Mt, Os, Ptc, Sb, Zm*	*Pta, Sm*
miR167	E	–	–	–	–	–	*At, Gm, Os, Ptc, Sb, So, Ta, Zm*	–
miR171	E	E, F	421	F	E	F	*At, Bn, Mt, Os, Ptc, Sb, Ta, Zm*	*Pta, Sm*
miR319	E	A,E,F	4142	B, E, F	A, E	F	*At, Gm, Mt, Os, Ptc, Sb*	*Pta, Sm*
miR390	E	A, F	1818	B, F	A, C	C, D	*At, Os, Ptc*	*Pta*
miR395	E	–	–	–	E	-	*At, Mt, Os, Ptc, Sb, Zm*	–
miR408	E	F	116	F	E	F	*At, Os, Ptc, Sm, Ta, Zm*	*Pta, Sm*
miR414	E	–	–	–	E	–	*At, Os*	–
miR419	E	–	–	–	E	–	*At, Os*	–
miR477	E	B, F	12 926	F	B, E	E, F	*Ptc*	–
miR529	–	F	512	F	–	F	*Os*	–
miR535	E	A, E, F	7462	F	A	–	*Os*	–
miR536	–	A, E, F	13 137	F	A	F	–	*Sm*

A: Arazi et al., 2005; B: Talmor-Neiman et al., 2006a; C: Talmor-Neiman et al., 2006b; D: Axtell et al., 2006; E: Fattash et al., 2007; F: Axtell et al., 2007.
[a]Number of reads corresponding to the indicated miRNA family in the three cDNA libraries reported by F.
[b]Evidence for accumulation of a miRNA/miRNA* pair which strongly supports a miRNA-like biogenesis pathway.
[c]As reported in miRBase 10.0, *At, Arabidopsis thaliana*; *Bn, Brassica napus*; *Gm, Glycine max*; *Mt, Medicago truncatula*; *Os, Oryza sativa*; *Ptc, Populus trichocarpa*; *Sb, Sorghum bicolor*; *So, Saccharum officinarum*; *Ta, Triticum aestivum*; *Zm, Zea mays.*
[d]As reported in miRBase 10.0, *Pta, Pinus taeda*; *Sm, Selaginella moellendorffii.*

tend to arise in 21 nt increments measured from the end where Dicing began (Allen et al., 2005; Yoshikawa et al., 2005). The 'phasing' of ta-siRNAs becomes especially apparent when examining very large data sets of sequenced small RNAs (Figure 5.2c, Plate 4; Rajagopalan et al., 2006; Howell et al., 2007).

A duo of nearly identical ta-siRNAs are produced from *A. thaliana TAS3* loci which constitute the only conserved siRNA-producing regions between *TAS3* homologues in diverse angiosperms and one gymnosperm (Allen et al., 2005; Williams et al., 2005). These ta-siRNAs direct the cleavage of the *A. thaliana ETTIN/ARF3* and *ARF4 Auxin Response Factor* RNAs. Abrogation of the *TAS3*-derived ta-siRNA-mediated regulation of *ARF3* and *ARF4* by mutations which prevent ta-siRNA biogenesis (Peragine et al., 2004; Xie et al., 2005b; Adenot et al., 2006; Garcia et al., 2006) or by blocking ta-siRNA-mediated regulation of *ETTIN/ARF3* (Fahlgren et al., 2006; Hunter et al., 2006) have effects on both vegetative phase change and upon adaxial/abaxial differentiation in the lateral organs of *A. thaliana*. The importance of *ARF*-targeting ta-siRNAs from *TAS3* loci (ta-si*ARF*s) is likely to extend to monocotyledons: disruption of the maize *leafbladeless1* gene disrupts adaxial/abaxial leaf polarity and is correlated with the disappearance of ta-siARF and consequent upregulation of *ARF3* (Nogueira et al., 2007). Similarly, disruption of three rice homologues of *A. thaliana* genes which are known to play specific roles in ta-siRNA biogenesis and function cause severe disruption of shoot apical meristem formation (Nagasaki et al., 2007).

The angiosperm *TAS3* loci produce single-stranded transcripts which are cleaved by a miR390-directed activity; the 5′ cleavage product is then used to synthesize the dsRNA which is processed to yield the ta-siARFs (Allen et al., 2005). It was therefore quite exciting when the first reports of miRNAs in bryophytes indicated that miR390 was among the most conserved of all plant miRNAs (Arazi et al., 2005; Axtell and Bartel, 2005); this observation immediately suggested that perhaps miR390-dependent ta-siRNA production was an ancient and widely conserved feature of land plant gene regulation. This hypothesis was soon borne out, as two groups independently described *P. patens* ta-siRNA-producing loci whose precursors were cleaved by a miR390-directed activity (Axtell et al., 2006; Talmor-Neiman et al., 2006b). Curiously, there was no apparent homology between the *P. patens* ta-siRNAs and any of the angiosperm *TAS3*-derived ta-siRNAs. The observation that one of the *P. patens* ta-siRNAs directed the cleavage of an *AP2*-domain containing transcript (and not an *ARF*-domain transcript as for the angiosperm ta-siARFs; Talmor-Neiman et al., 2006b) further confounded the simple hypothesis that the miR390-dependent ta-siRNA-producing loci of *P. patens* and angiosperms were homologues descended from a common molecular ancestor. However, alignment of the four loci revealed two conserved, ~21 nt blocks of sequence – one of these blocks corresponded the *AP2*-targeting ta-siRNAs, while the other was shown to direct the cleavage of *P. patens ARF* transcripts similar to *A. thaliana ETTIN/ARF3* and *ARF4* (Axtell et al., 2007). There was no detectable sequence similarity between the *P. patens* ta-siARFs and the angiosperm

ta-siARFs despite the fact that they had analogous molecular functions and were both produced by miR390-dependent ta-siRNA formation. This suggests either that the *P. patens* ta-siRNA loci and angiosperm *TAS3* loci have greatly diverged from a common molecular ancestor or arisen through convergent evolution.

A. thaliana ta-siRNAs require the activity of the *RDR6* gene for the production of ta-siRNAs, including the ta-siARFs (Peragine et al., 2004; Vazquez et al., 2004; Allen et al., 2005). Talmor-Neiman et al. (2006b) cloned a *P. patens RDR6* homologue (*PpRDR6*), and generated transgenic lines in which this locus was disrupted. Two independent disruptions of *PpRDR6* resulted in the elimination of detectable ta-siRNAs and the upregulation of the validated *AP2*-domain containing ta-siRNA target. The two independent *Pprdr6* disruptants also showed accelerated differentiation of leafy gametophores relative to wild-type controls, demonstrating that the *PpRDR6* regulates this process. Although it seems likely that this developmental phenotype is due to loss of the miR390-dependent ta-siRNAs, the available data cannot formally rule out the possibility that other, as yet undiscovered, *PpRDR6*-dependent small RNAs contribute to the phenotype. These seminal experiments were the first to report a *P. patens* mutant phenotype due to disruption of small RNA function.

Why do the cleaved fragments of some miRNA targets stimulate dsRNA formation and subsequent generation of phased siRNAs, while most do not? An important clue came from the observation that the *P. patens* ta-siRNA precursors had two miR390 complementary sites, which were both cleaved *in vivo* (Axtell et al., 2006; Talmor-Neiman et al., 2006b). The ta-siRNAs arose only from the regions flanked by the complementary sites, indicating that only this portion of the precursor was efficiently converted to dsRNA. This led to the hypothesis that the doubly cut RNA strongly stimulated dsRNA formation because of its lack of both a 5′-cap and a 3′ poly-A tail (Figure 5.3; Axtell et al., 2006). This hypothesis predicted that genes with two or more target sites were highly likely to produce secondary siRNAs 'in-phase' with one or the other site. Data from *A. thaliana* support this hypothesis – most genes with two or more small RNA complementary sites produce siRNAs from within, but not outside, the bounded region (Axtell et al., 2006; Howell et al., 2007). In contrast, most genes with only one cleaved small RNA complementary site produced little or no siRNAs. This 'two-hit' hypothesis for siRNA formation has important implications; for instance, it may explain the propensity for very highly transcribed plant transgenes to be silenced via siRNA formation. Assuming that very rare, spontaneous RNA cleavage events occur at a very low frequency, the chances of any individual mRNA sustaining two such spontaneous cleavages is very low. However, as the population of identical mRNAs increases in the cell, as it would for a highly expressed transgene, the probability that one member of that population sustains two spontaneous cleavage events increases. If the two-hit hypothesis is correct, the middle fragment, lacking both a cap and a poly-A tail, would

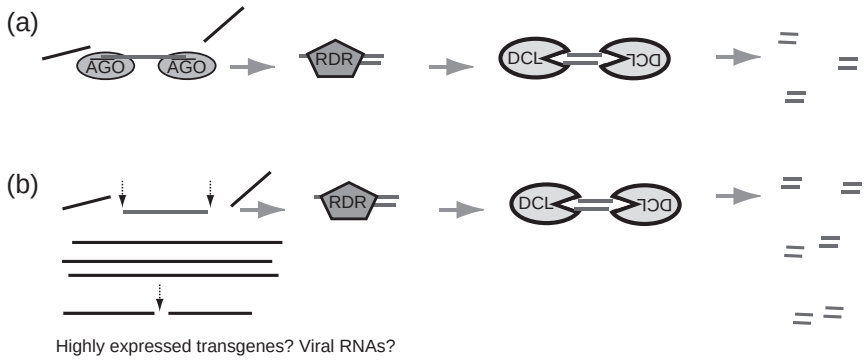

Figure 5.3 A 'two-hit' trigger for siRNA biogenesis. (a) Transcripts which are cleaved twice (or more) by small RNA-directed AGO activities spawn secondary siRNAs from the region between the cleavage sites. This may be due to the lack of both a 5′ cap and a 3′ poly-A tail on the internal fragment. (b) The segment of a very abundant RNA defined by two rare, random cleavage events becomes recognized as a substrate for RNA-dependent RNA polymerase (RDR) activity and subsequent production of secondary siRNAs. This mechanism may contribute to transgene and virus silencing. (Reprinted from A Two-Hit Trigger for siRNA Biogenesis in *Plants*, **127**, Axtell, Jan, Rajagopalan and Bartel, p. 13, copyright 2006 with permission from Elsevier.)

stimulate the formation of dsRNA which would then be processed to form siRNAs. These siRNAs would be likely to encounter additional members of this highly abundant transcript population, cleave them at multiple locations and producing more cap-less and tail-less fragments, and thus initiate a feed-forward cycle of repression ultimately resulting in the silencing of expression. This model is especially attractive for explaining the 'threshold effect' observed in plant transgene silencing, where high amounts of transcription correlate positively with initiation of silencing (Lindbo et al., 1993; Smith et al., 1994; Elmayan and Vaucheret, 1996). Despite the explanatory power of this hypothesis, it is also clear that the 'two-hit' model does not account for all instances of known ta-siRNA formation: The *A. thaliana TAS1a-c*, *TAS2* and *TAS4* loci all initiate efficient ta-siRNA production dependent upon only a single miRNA-mediated cleavage event (Allen et al., 2005; Yoshikawa et al., 2005; Rajagopalan et al., 2006), as do a small number of other *A. thaliana* miRNA targets with only one cleavage site (Lu et al., 2005; Axtell et al., 2006; Ronemus et al., 2006).

5.3.3 Other *P. patens* small RNAs

Despite the clear successes of efforts to describe *P. patens* miRNAs and ta-siRNAs, the majority of the sequenced small RNAs listed in Table 5.1 are not yet understood. For instance, only about 31% of the *P. patens* small RNAs discovered in the three pyrosequencing libraries emanate from the currently known miRNAs and ta-siRNAs (Axtell et al., 2007). A striking 47% of the

pyrosequenced small RNAs do not have any matches in the version 1.1 draft genome assembly. Many of these sequences are likely to be the result of sequencing errors introduced by the error-prone pyrosequencing methodology or library artefacts of some sort, but some of them also are likely to have emanated from genomic regions which are difficult to sequence or assemble. Without knowing the genomic context from which they arose, it will be quite difficult to reach an understanding of this subset of the small RNAs. The remaining 22% of the pyrosequenced small RNAs (over 124 000 reads) do match the *P. patens* draft genome assembly in one or more places but have yet to be annotated or understood. The size distribution of this population is distinct from that of the annotated miRNA loci and ta-siRNA loci, perhaps suggestive of the contributions of different Dicer proteins to their collective biogenesis (Axtell et al., 2007). Future analyses will undoubtedly focus on understanding this largely unexplored component of *P. patens* small RNA expression.

5.4 Biogenesis of *P. patens* small RNAs: Dicers, Slicers and other utensils

As discussed, the Dicer and Argonaute endonucleases constitute the fundamental components required for small silencing RNA biogenesis and function. Proteins in both of these families have readily recognizable arrangements of conserved domains across the eukaryotes, which allows for relatively easy identification by computational searches. The release of the *P. patens* draft genome assembly and especially an associated set of predicted full-length cDNA transcripts has facilitated the identification and preliminary classification of Dicers and Argonautes, as well as the identification of homologues of other known components of small RNA pathways, such as RDRs. At the present time, the only component of the *P. patens* small RNA machinery with an experimentally defined function is the *PpRDR6* gene, which is required for the accumulation of ta-siRNAs and for the regulation of the gametophore differentiation (Talmor-Neiman et al., 2006b). Given the potent combination of the draft genome sequence and the ease of gene disruption in *P. patens*, it seems likely that the experimentally defined functions of more potential components of small silencing RNA pathways will soon be reported.

5.4.1 *P. patens* Dicer-like genes

Dicer homologues in plants are referred to as *Dicer-like* (*DCL*) loci. DCL proteins, like the Dicers from other eukaryotes, are large multi-domain polypeptides containing N-terminal helicase domains, a central conserved domain of unknown function (DUF283), a PAZ domain (which probably functions to bind single-stranded RNA; Ma et al., 2004; Song et al., 2004), two tandem RNAseIII domains and two C-terminal dsRNA-binding domains (Schauer

et al., 2002). The *A. thaliana* genome encodes four distinct *DCL* genes, *DCL1-4*. In the wild type, the four DCL proteins manufacture distinct cohorts of small RNAs: DCL1 is specialized for miRNA production and produces mostly 21 nt small RNAs (Park et al., 2002; Reinhart et al., 2002; Qi et al., 2005), while DCL2 produces 22–23 nt siRNAs and contributes to small RNA-mediated viral resistance (Xie et al., 2004; Gasciolli et al., 2005; Deleris et al., 2006). DCL3 produces endogenous 24 nt siRNAs which are often associated with chromatin modifications (5-methyl cytosine and methylation of histone H4 at lysine 9) at homologous genomic loci (Xie et al., 2004; Qi et al., 2005). Finally, DCL4 produces 21 nt ta-siRNAs and also contributes to 21 nt siRNA formation in response to viral infections (Gasciolli et al., 2005; Xie et al., 2005b; Yoshikawa et al., 2005; Deleris et al., 2006). While each DCL protein is largely specialized for a given function in the wild type, analyses of mutants where one or more *DCL* genes are inactivated demonstrate partial redundancies in function (Gasciolli et al., 2005; Kasschau et al., 2007). Phylogenetic analysis of *Populus trichocarpa* and *Oryzae sativa* DCL proteins indicated that diversification of these four DCL types occurred before the divergence of the monocotyledonous and dicotyledonous lineages (Margis et al., 2006).

Inspection of the *P. patens* draft genome sequence revealed four potential *DCL* homologues (Axtell et al., 2007). However, phylogenetic analysis of the predicted DCL protein sequences revealed that these four homologues corresponded to only three distinct clades: two DCL1 homologues (*Pp* DCL1a and *Pp* DCL1b), a DCL3 homologue (*Pp* DCL3) and a DCL4 homologue (*Pp* DCL4; Table 5.3). By analogy with the known functions of *A. thaliana* DCL proteins, these data suggested that the production of *P. patens* miRNAs may be directed redundantly by the two DCL1 homologues. The green alga *C. reinhardtii* appears to encode a single DCL protein which is distinct from the land plant DCLs (Axtell et al., 2007); these data demonstrated that the diversification of the plant *DCL* gene family into the *DCL1*, *DCL3* and *DCL4* clades occurred very early in land plant evolution.

Table 5.3 *P. patens* Dicer-like (DCL) and Argonaute (AGO) homologues

Clade	Function in *A. thaliana*	Size of associated small RNAs (*A. thaliana*)	Number of *P. patens* homologues
DCL1	miRNA biogenesis	21	2
DCL2	Viral siRNA biogenesis and some endogenous siRNAs	22–23	0
DCL3	Endogenous siRNA biogenesis and some viral siRNAs	24	1
DCL4	ta-siRNA biogenesis and viral siRNAs	21	1
AGO1	miRNA and siRNA-directed silencing	21	3
AGO4	siRNA-directed chromatin modifications	24	3
AGO7	*TAS3* ta-siRNA function	?	0

5.4.2 *P. patens* Argonaute genes

Eukaryotic Argonaute proteins contain a central PAZ domain followed by a large PIWI domain. The PIWI domain is a structural relative of RNAseH, and contains the catalytic centre which serves to cleave small RNA-targeted proteins (Song et al., 2004). Just as for the *DCL* genes, *A. thaliana* and other plant genomes express multiple Argonaute-encoding *AGO* genes with specialized functions. There are ten *AGO* genes in the *A. thaliana* genome: *AGO1–9* and *ZWILLE/PINHEAD* (Fagard et al., 2000). Unlike the *DCL* genes, not every *A. thaliana AGO* gene has an experimentally defined function in small RNA biogenesis. However, it is well established that AGO1 is especially critical for both the functions of miRNAs and in the siRNA-mediated process of post-transcriptional gene silencing (Fagard et al., 2000; Vaucheret et al., 2004; Baumberger and Baulcombe, 2005; Qi et al., 2005). On the basis of close protein similarities and the synergistic effect with *ago1* mutants, the developmental defects observed upon loss of the Argonaute protein ZWILLE/PINHEAD are also likely to be due to a disruption of miRNA functions, though the specific effects of *zwille/pinhead* mutations upon small RNA functions have not been described (Lynn et al., 1999). In contrast, *A. thaliana* AGO4 and AGO6 are required for mediating chromatin modifications directed by endogenous 24 nt siRNAs (Zilberman et al., 2003; Xie et al., 2004; Qi et al., 2006; Zheng et al., 2007). The AGO7 protein, also known as ZIPPY, seems to have a very specific role in the function of ta-siRNAs derived from the *TAS3* loci, but not other ta-siRNAs (Hunter et al., 2003, 2006; Adenot et al., 2006). To date, biological functions have not been ascribed to the other members of the *A. thaliana AGO* gene family.

Six potential *AGO* genes were apparent in the version 1.1 draft genome assembly of *P. patens* (Axtell et al., 2007). Phylogenetic reconstruction demonstrated that their protein products could be grouped into two distinct groups of three. The first group, consisting of *Pp* AGO1a, *Pp* AGO1b and *Pp* AGO1c was related to a clade of AGO proteins anchored by *A. thaliana* AGO1 (Table 5.3). One or more of these three predicted proteins are thus likely to be the AGOs responsible for miRNA-directed target cleavage in *P. patens*. The other three *P. patens* AGO family members are most closely related to *A. thaliana* AGO4 and AGO6. Curiously absent from the currently recognized *P. patens AGO* genes were homologues of *AGO7* (Table 5.3). Nonetheless, the available data demonstrate that, as for the *DCL* gene family, diversification of plant *AGO* genes occurred early in or prior to land plant evolution.

5.4.3 RDRs and other factors

By synthesizing dsRNA from a variety of RNA templates, endogenously expressed RDRs are known to be critical components of several plant siRNA pathways. As discussed earlier, the *P. patens PpRDR6* gene has been shown to be necessary for ta-siRNA formation and for the normal regulation of

gametophore initiation (Talmor-Neiman et al., 2006b). Based on BLAST homology searches against the transcript models accompanying the *P. patens* draft genome release, there appear to be three other moss *RDR* homologues (MJA, unpublished observations). In phylogenetic reconstructions performed using alignments with the known *A. thaliana* RDR proteins, two of the *P. patens* RDRs are quite divergent, while a third is closely related to *At* RDR1 and *At* RDR2 (MJA, unpublished observations). This similarity is intriguing in light of the fact that *A. thaliana* RDR2 plays an indispensable role in the production of the DCL3 and AGO4-dependent 24 nt endogenous siRNAs (Xie et al., 2004; Lu et al., 2006; Kasschau et al., 2007). *P. patens* homologues of other factors known to be required for small silencing RNA biogenesis or functions have not yet been reported. However, given that the 3′-most nucleotide of a *P. patens* miRNA does not possess both 2′- and 3′-hydroxyls (Axtell et al., 2007), it is likely that a homologue of the HUA Enhancer 1 (HEN1) methyltransferase is functional (Yu et al., 2005).

5.4.4 Implications

The overall patterns of conservation of *P. patens DCL*, *AGO* and *RDR* genes may be instructive as to the functions of the 'orphan' *P. patens* small RNAs whose biogenesis and functions have not yet been annotated. Arguably the most intriguing observation is that *P. patens* appears to possess homologues of *DCL3*, *AGO4* and *RDR2*, which in *A. thaliana* conspire to manufacture and use 24 nt siRNAs to direct chromatin modifications at dispersed repetitive and non-repetitive sites in the genome. Initial observations of bryophyte small RNA populations showed that they were dominated by 21-mers (Arazi et al., 2005; Axtell and Bartel, 2005). However, after subtracting the sequences contributed by known miRNAs, ta-siRNAs and those which did not match the *P. patens* draft genome assembly, the remaining orphan small RNAs had a distinct size profile with clear peaks at 23 and 24 nts (Axtell et al., 2007). It is therefore tempting to speculate that the *P. patens DCL3*, *AGO4* and *RDR2* homologues are involved in the production of these 23–24 small RNAs. To test this hypothesis, it will be imperative to describe precisely the genomic loci from which the orphan small RNAs arise as well as to define experimentally the biological functions of all the *P. patens DCL*, *AGO* and *RDR* family members.

5.5 Targets of *P. patens* small RNAs

Biological information is not directly encoded within small silencing RNAs; it is only through regulation of target genes that they contribute to phenotype. Therefore, accurate predictions and validations of small RNA targets are indispensable for the understanding of small RNA functions. This is especially true for miRNAs and ta-siRNAs, which regulate target RNAs distinct from

their precursors. Repression of target expression by miRNAs has been especially well studied and operates either at the post-transcriptional level via Argonaute-catalysed target cleavage or at the translational level independent of Argonaute-catalysed cleavage. The former process is typical of most known plant miRNA-target interactions, while the latter typifies most interactions between animal miRNAs and their targets. Cleavage-independent target repression requires only a short region of complementarity between miRNA and target, centred upon positions 2–8 of the miRNA (Lewis et al., 2005; Lim et al., 2005). This limited complementarity, coupled with the relatively modest repression conferred by such interactions, has made the discovery of animal miRNA targets a difficult and sometimes contentious challenge. In contrast, miRNA-directed target cleavage requires a greater degree of complementarity between miRNA and target, with base pairing to the 5′ and central regions of the miRNA being especially critical (Mallory et al., 2004; Parizotto et al., 2004). This has allowed extensive identification of cleavage-type miRNA interactions, especially in the land plants.

5.5.1 Prediction of *P. patens* small RNA targets

The high degree of complementarity between miRNAs and their cleavage targets enables the use of relatively simple protocols for target prediction. For instance, Arazi et al. (2005) predicted targets of *P. patens* miRNAs by simply looking for EST-based transcript contigs (Nishiyama et al., 2003) with potential complementary sites containing three or fewer mismatches. This level of stringency had earlier been shown to result in a favourable signal to noise ratio when applied to miRNA-target predictions in *A. thaliana* (Rhoades et al., 2002). The same group later adopted the target prediction scheme of Schwab et al. (2005), which involved position-specific scoring reflective of the differential importance of base paring at different regions of a potential miRNA-target interaction; this approach was used to predict targets of *P. patens* miRNAs and ta-siRNAs using both EST data and raw WGS traces (Talmor-Neiman et al., 2006a, b). Fattash et al. (2007) also used the Schwab method to predict miRNA targets using assembled EST data. Finally, Axtell et al. (2007) used a slight modification of the target prediction scheme of Allen et al. (2005) coupled with the annotated transcripts derived from analysis of the draft genome assembly to predict a large number of miRNA and ta-siRNA targets. This target prediction methodology is similar to the Schwab methodology in the use of position-specific scoring reflective of mismatches between small RNAs and potential targets.

5.5.2 Validation of *P. patens* small RNA targets

Even the most rigorously controlled target prediction protocols result in mere hypotheses about possible small RNA targets – these hypotheses must then be tested by experimentation to demonstrate the postulated interactions.

Table 5.4 Experimentally confirmed *P. patens* small RNA targets

Small RNA	Citation(s)	Predicted target function(s)
miR156	A, B	SBP-box transcription factors (2)
miR166	B	HD-ZIPIII transcription factors (5)
miR171	B	GRAS-domain transcription factors (2)
miR319	B	MYB-domain transcription factors (2), cyclin-domain protein (1)
miR390	C, D	*PpTAS3* ta-siRNA precursors (4)
miR408	B	Plastocyanin-domain protein (1)
miR477	B, E	HLH-domain transcription factor (1), *CONSTANS*-like transcription factor (1)
miR529(5′)	B	AP2-domain transcription factor (1)
miR534	B	Ankyrin-domain proteins (2)
miR536	B	F-box protein (1)
miR537	E	Unknown (1)
miR538	B	MADS-box transcription factors (3)
miR902(5′)	B	HLH-domain transcription factors (2)
miR904	B	AGO1 proteins (3)
miR1029	B	AP2-domain transcription factor (1)
miR1215	F	Protein kinase (1)
miR1218	F	NAM/CUC domain transcription factor (1)
miR1219	B	ARF-domain transcription factors (2)
miR1221	F	Histidine kinase protein (1)
miR1223	F	NAC domain transcription factor (1)
PpTAS3 ta-siRNAs	B, D	AP2-domain transcription factor (1), ARF-domain transcription factors (2)

A: Arazi et al., 2005; B: Axtell et al., 2007; C: Talmor-Neiman et al., 2006b; D: Axtell et al., 2006; E: Fattash et al., 2007; F: Talmor-Neiman et al., 2006a.

Fortunately, a simple methodology based upon 5′ rapid amplification of cDNA ends (5′-RACE) is available to test miRNA-target predictions in vivo. The 3′ fragment resulting from small RNA-mediated cleavage possesses both a 5′-monophosphate and a 3′ poly-A tail. Because Argonaute-catalysed cleavage always hydrolyses the phosphodiester backbone between the bases complementary to the 10th and 11th nucleotide of the guiding small RNA, products derived from 5′-RACE of uncapped, polyadenylated RNAs that terminate at this position of a predicted complementary site offer convincing evidence for small RNA-directed cleavage (Llave et al., 2002; Kasschau et al., 2003). All three groups who have reported the isolation of *P. patens* small RNAs have also provided evidence for multiple cleaved miRNA and ta-siRNA targets using the 5′-RACE technique (Arazi et al., 2005; Axtell et al., 2006, 2007; Talmor-Neiman et al., 2006a, b; Fattash et al., 2007) (Table 5.4). In total, 41 targets of 20 different miRNAs and ta-siRNAs have been confirmed *in vivo*.

The ultimate test of the biological function of a small RNA is to examine the phenotypic consequences of disrupting it. This may be done by

over-expressing the small RNA, isolating mutants specifically lacking small RNA expression, or by rendering a target recalcitrant to small RNA-mediated regulation. To date, there have been no reports trying to assess directly *P. patens* small RNA functions in this manner. Given the potent combination of the complete nuclear genome sequence, efficient methodologies for gene disruption, and the intense interest in small RNA biology, we are unlikely to be left in the dark about *P. patens* small RNA functions for much longer.

5.6 Evolution of plant miRNAs

As discussed in Chapter 1, the last common ancestor of both *P. patens* and modern angiosperms is likely to have existed no earlier than 450 million years before the present. In the time since the divergence of these two lineages, the available evidence suggests that the bryophytes have changed little in morphology and life cycle, while the lineage leading to today's angiosperms has undergone major alterations in both. Thus, comparisons between *P. patens* and angiosperms are particularly informative for understanding the evolution of land plants. Certainly, a chief impetus for *P. patens* small RNA research has been curiosity about ancestral and derived miRNAs within the land plants. Comparisons of miRNA functions, conservation and divergence between the deeply branching angiosperm *A. thaliana* and *P. patens* have been particularly important in framing hypotheses for plant miRNA functions.

5.6.1 Ancient miRNA–target interactions

A total of 15 *P. patens* miRNA families have homologues outside of bryophytes (Table 5.2). Targets for nine of these families have been experimentally verified using the 5′-RACE technique described previously (Table 5.4). Many of these experimentally verified targets encode proteins containing putative DNA-binding domains, implying that they function as sequence-specific transcription factors. Strikingly, in almost every instance, the corresponding angiosperm miRNAs have been shown to regulate homologous transcription factor-encoding mRNAs. The unavoidable conclusion is that these miRNA-target relationships not only originated very early in land plant evolution but also persisted unchanged in multiple diverse lineages.

Several conserved miRNA–target regulatory interactions involving transcription factors have been shown experimentally to be important for development of angiosperm sporophytes. For instance, miR156-mediated regulation of multiple *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes is required for the proper timing of vegetative phase transitions in both *A. thaliana* (Wu and Poethig, 2006) and *Zea mays* (Chuck et al., 2007). miR156 clearly regulates homologous *SPL* genes in *P. patens* protonemata (Arazi et al., 2005; Axtell et al., 2007), but cannot be responsible for developmental processes homologous to those in *A. thaliana* or *Z. mays* because this regulation

occurs during the gametophytic, rather than the sporophytic phase of the life cycle. Several other conserved miRNA–target interactions which have been demonstrated to occur in the gametophytic phase of *P. patens* development (Axtell et al., 2007) are known to be critical for the sporophytic development of angiosperms, including miR166-mediated regulation of *HD-ZIPIII* transcription factors (McConnell et al., 2001; Mallory et al., 2004), miR160-mediated regulation of *Auxin Response Factors* (Mallory et al., 2005; Wang et al., 2005) and miR159/319-mediated regulation of *MYB* transcription factors (Millar and Gubler, 2005). It is commonly assumed that the gametophyte-dominated life cycle of *P. patens* represents the ancestral state of land plants, and that a transition to a sporophyte-dominated life cycle occurred in the ancestors of modern angiosperms but not in those of modern bryophytes. It follows then, that ancient miRNA interactions with transcription factors were transferred from the gametophytic to the sporophytic phase during the diversification of the angiosperms. As transcription factors, each of these miRNA targets is likely to regulate a cohort of downstream genes at the transcriptional level. Were similar cohorts of downstream regulatory genes retained during the gametophyte to sporophyte switch, or did this switch involve a 're-wiring' of miRNA-regulated transcription factor functions? The answers await the experimental determination of *P. patens* miRNA functions, and the determination of the downstream genes regulated by the conserved transcription factors in multiple species.

5.6.2 Plant miRNAs: live fast, die young

Of the 100 *P. patens* miRNA families annotated to date, 85 have no known homologues outside of the bryophytes. For convenience, these miRNAs will be referred to as 'non-conserved' keeping in mind that future experiments could overturn this designation for some of them. In general, the non-conserved miRNAs are sequenced less frequently in small RNA libraries prepared from wild-type tissues compared to conserved miRNAs (Figure 5.4), implying that they are generally less abundant. As a group, the non-conserved miRNAs are also far less likely to be encoded by multiple paralogous genes in the *P. patens* genome. Standard methods of miRNA–target identification have yielded very little for non-conserved miRNAs compared to the harvest of predicted and validated targets for the conserved miRNAs (Talmor-Neiman et al., 2006a; Axtell et al., 2007). The targets of predicted or experimentally validated non-conserved miRNAs have a diverse array of functions, and are not dominated by transcription factors, as are the targets of more conserved miRNAs. These trends are not unique to the miRNAs of *P. patens*: *A. thaliana* also expresses many low-abundance, non-conserved miRNAs which tend to be encoded by single loci, and for which the predicted targets are fewer in number and more functionally diverse relative to conserved miRNAs (Figure 5.4; Rajagopalan et al., 2006; Fahlgren et al., 2007). A similar situation seems likely to exist in

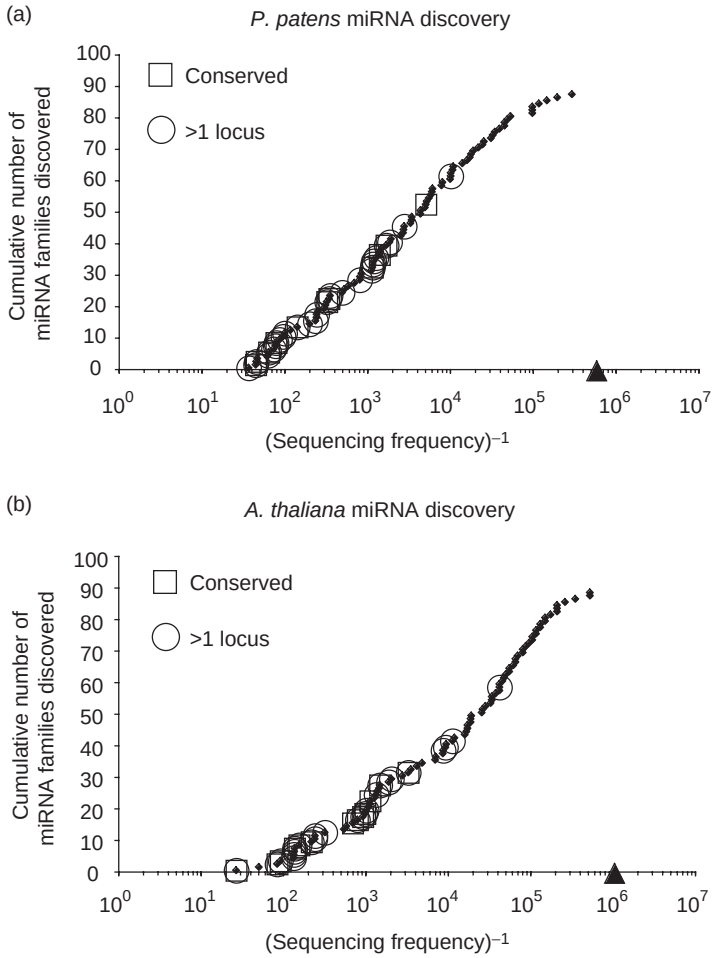

Figure 5.4 miRNA abundance is correlated with conservation. (a) Cumulative number of miRNA families discovered as a function of abundance in sequenced small RNA libraries from *P. patens*. Conserved miRNA families are indicated by squares and miRNA families encoded by more than one locus are indicated by circles. (b) As in (a) for *A. thaliana*.

S. moellendorffii, where again most of the known miRNA diversity is accounted for by low-abundance, single-copy genes; however, for most of these, the targets remain obscure (Axtell et al., 2007).

What processes might account for these striking differences between conserved and non-conserved miRNAs? One possible answer was suggested by an elegant study by Allen et al. (2004). This study provided evidence which suggested a simple mechanism for the birth of new miRNAs. Tandem duplication of one member of a multigene family can easily be envisioned to form an inverted repeat – should that inverted repeat be transcribed, the resulting

RNA would form a perfect self-complementary hairpin which a Dicer protein would recognize and process, resulting in a population of small RNAs which would have complementarity to the other intact members of the originating multigene family. Over time, mutational drift obscures the relationship between the originating gene family and the small RNA-producing locus except for a single 21 nt stretch – thus were miRNAs and the relationships to their target genes suggested to be born (Allen et al., 2004). This theory predicts that the stem-loop precursors of 'young' miRNAs would possess extended complementarity to regions of their target genes surrounding the miRNA complementary site. Indeed, several 'young' miRNA–target relationships have been described in *A. thaliana* which fulfil this prediction (Rajagopalan et al., 2006; Fahlgren et al., 2007). In principle, this theory of miRNA origins need not be confined to duplications of protein-coding genes: any local genome duplication resulting in an inverted repeat could conceivably come under the control of a neighbouring or cryptic promoter, resulting in production of a self-complementary stem-loop RNA which a Dicer protein would recognize as a substrate. Such proto-miRNAs would be unlikely to possess complementarity to any potential target. Without any selection pressure, such loci would rapidly accumulate mutations which destroy the self-complementarity of the stem-loop, rendering it incapable of being processed by Dicer. When seen through the prism of the inverted repeat hypothesis for miRNA origins, the properties of the non-conserved miRNAs can be rationalized: their low abundance and tendency to have only a single locus stems from their recent origins as an inverted duplication and the rudimentary state of their transcriptional regulation. The functional diversity of the predicted targets of non-conserved miRNAs could be a reflection of the diversity of genes encoded by multigene families, while the seeming absence of targets for other non-conserved miRNAs could reflect origins from the inverted duplication of non-protein-coding areas of the genome. Thus, as a group, the non-conserved miRNAs may represent one variety of transcriptional 'noise' – transcripts which have no biological role and which will rapidly perish in the fires of mutational drift. There are certainly known exceptions to this generalization, including *A. thaliana* miR824, which is restricted to the Brassicaceae but plays a biological role in stomatal development (Kutter et al., 2007), and *P. patens* miR904 which despite being non-conserved in sequence shares a conserved molecular function in target gene regulation (Axtell et al., 2007). However, the available data suggest that at least some non-conserved miRNAs may have limited or no biological relevance.

If miRNAs do indeed exhibit 'frequent birth and death' as Fahlgren et al. (2007) suggest, then over evolutionary time miRNA-mediated regulation of many types of genes has been 'sampled' by the transcriptome. The most ancient miRNA–target relationships would represent those rare instances where a 'sample' miRNA–target interaction has been stabilized due to strong selection pressures. Why might these highly conserved miRNAs be so obviously biased towards the control of mRNAs encoding transcription factors as

opposed to other functional classes of targets? Perhaps post-transcriptional control is especially beneficial to control the expression levels of mRNAs encoding particularly 'potent' gene products. It may be that subtle changes in the dosage of transcription factors can have huge effects on the morphology and development of the organism. Thus, regulating potent gene products at the post-transcriptional level, in addition to the more common-place regulation at the transcriptional and post-translational levels, may provide an important additional level of control with strong selective advantages.

5.7 Conclusions

Spectacular progress has been made in identifying, classifying and annotating the small RNAs expressed by *P. patens*. This success is due almost entirely to the sequencing and assembly of the nuclear genome. The discovery of both ancient and less conserved small RNAs in *P. patens* has helped to frame our understanding of the evolution of gene expression and post-transcriptional control in land plants. Future experiments will undoubtedly focus upon the functional characterization of *P. patens* miRNAs and the enzymatic machinery responsible for their production. It will be particularly exciting to dissect the functions of the most ancient miRNA–target interactions in *P. patens* in parallel with identical analyses in phylogenetically diverse land plants – such studies promise to open a new chapter in the understanding of the evolution of gene regulation in the land plants.

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Chapter 6

TIP GROWTH IN THE MOSS *PHYSCOMITRELLA PATENS*

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Abstract: Albeit restricted to a subset of cell types, polarized cell tip growth is a fundamental development mechanism in the plant kingdom from algae to angiosperms. We are focusing in this chapter on the current understanding of such a growth mechanism in mosses, with *Physcomitrella patens* as a model. Spore germination and the first stage of the haploid developmental phase, the protonemata, rely completely on tip growth for their development. Simultaneously to this purely developmental aspect, protonemal tip cells integrate environmental cues (e.g. light, gravity) and respond in a coordinated and polar manner. Different physiological and pharmacological studies have implicated directly and indirectly the actin cytoskeleton as a central component in both aspects of tip growth. With the recent development of the reverse genetic tool kit in *P. patens*, the role of the actin cytoskeleton in regulating tip growth can now be finely dissected. So far, genetic disruption of four components of the conserved eukaryotic actin cytoskeleton regulation machinery, Arp2/3 complex and its regulatory WAVE complex, profilins and ADF/cofilin confirmed a direct implication of the actin cytoskeleton in polar tip growth, allowing the establishment of a functional model of cell tip elongation in *P. patens*.

Keywords: actin; cytoskeleton; moss; protonemata; tip growth

6.1 Introduction

Plant cell morphogenesis can be driven by a diverse array of growth mechanisms (Mathur, 2004; Fleming, 2006). Amongst them, polar tip growth is defined as an anisotropic growth mechanism in which cell expansion is restricted to a single region of the cell, the tip. Such a growth pattern is present in plant and fungal organisms as well as prokaryotes (Heath, 1990) and leads to the formation of an oblong cell body. While gymnosperms and angiosperms rely on the tip-growing pollen tube for sexual reproduction, no

other group of land plants rely more on tip growth than mosses for their development: the haploid phase of mosses develops with such a growth mechanism during spore germination, protonemata development and rhizoid development. Several recent reviews have presented advances in moss biology using *Physcomitrella patens* as a model system (Cove et al., 2006; Quatrano et al., 2007) due to the discovery of efficient gene targeting in *P. patens* (Schaefer and Zryd, 1997). Moreover, the recent publication of the *P. patens* genome sequence (Rensing et al., 2008 and Chapter 3) positions this moss at the forefront of genomic studies in plants (see Chapters 3–5 and 8). This chapter is more specifically aimed to describe data on tip growth in mosses while emphasizing specifically the use of *P. patens* in the study on polar tip growth.

The best introduction to this chapter is the recent demonstration of strict polar tip growth in protonemata of *P. patens*. Menand et al. (2007a), using time-lapse video coupled to tagging of the cell wall with fluorescent microspheres, showed that both protonemal cell types elongated only at the apical tip of the cell and that diffuse growth along the cell body did not play a significant role in cell shape formation. This observation allows for a direct comparison of *P. patens* growth with the most recent tip growth model developed for plant and hyphal cells (Dumais et al., 2006), and will potentially allow inter-phylum growth model comparison.

6.2 Morphology and structure of a tip cell

Three different cell tip types can be observed in *P. patens*. Chloronemal and caulonemal cells form the protonemata (Figure 6.1a–6.1c, Plate 5). Rhizoids are found at the base of gametophores (Figure 6.1d and 6.1e, Plate 5) as well as along the stem part of the adult gametophore (Sakakibara et al., 2003). They all share the characteristic tubular shape with a growing tip at one end as the other end connects to the next cell of the filament. Growth of these filaments is hence characterized by two main mechanisms: cell elongation at the tip and unequal cell division. Most studies have focused on the two protonemal cell types.

The chloronemal cell is the first cell type to emerge from a spore or a protoplast. It is full of well-developed chloroplasts, with a large central vacuole. Intercalary cell walls are perpendicular to the axis of development, its position being defined during cell division (Figure 6.1c, Plate 5). Sub-apical cells are 75–80 μm long (Perroud and Quatrano, 2006), but the apical cell can be twice as long before cell division. Its tip is spherical, and its growth rate has been measured at 5.8 ± 0.51 μm h^{-1} (Menand et al., 2007a). Secondary chloronemal cells with the same characteristics are produced as side branches from both chloronemal and caulonemal sub-apical cells. In *Daowsonia superba*, where protonemal tissue is composed of chloronemal-like cells,

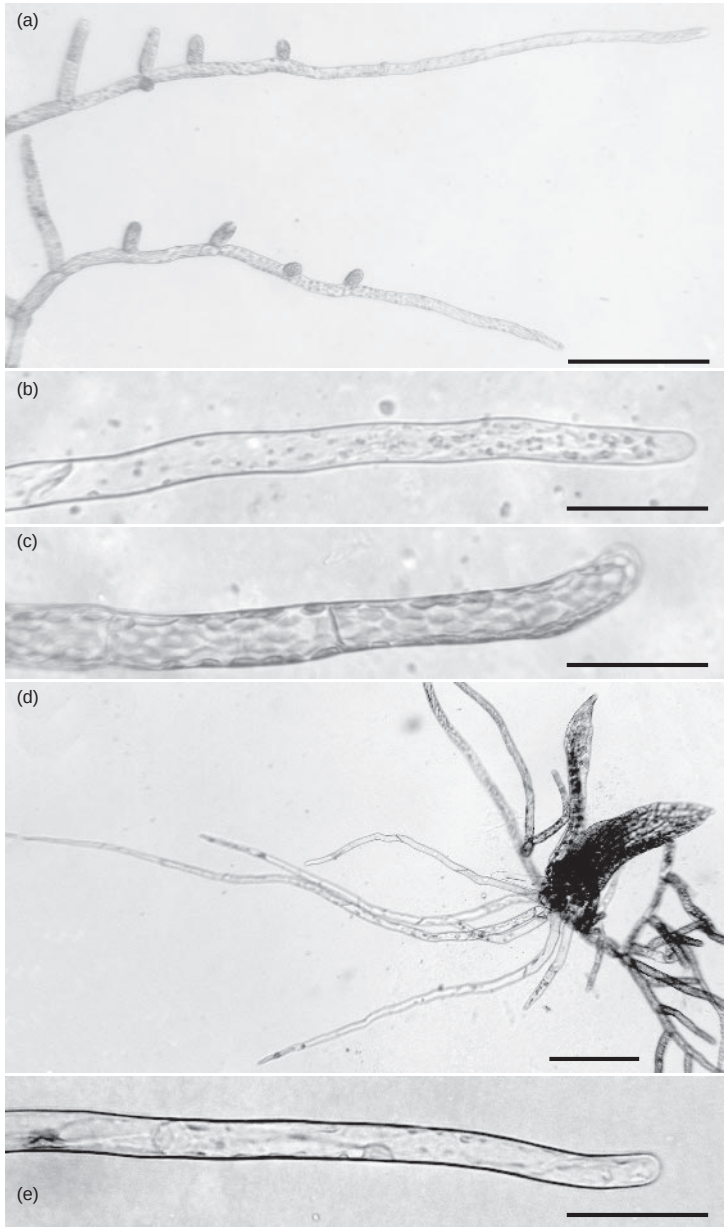

Figure 6.1 Tip-growing cells of *P. patens*. (a) Protonemal filaments formed of two cell types, chloronema and caulonema. (b) Caulonemal tip cell, (c) chloronemal tip cell, (d) early gametophore formed of leafy shoot and basal rhizoids, (e) rhizoid tip cell. Scale bar: (a) and (d) 200 μm. (b), (c) and (e) 50 μm. (For a color version of this figure, see Plate 5)

electron microscopy showed a homogenous organelle distribution in the cell body without a visible tip organization (DeMaggio and Stetler, 1977).

Caulonemal cells, by contrast, are longer; 180–200 μm for a sub-apical cell (Perroud and Quatrano, 2006), with a maximum of 400 μm for a pre-mitotic tip cell. Chloroplasts, albeit present, have reduced size and are less numerous than in chloronemata. Intercalary cell walls are oblique to the axis of development, following a rearrangement of the cell plate at mitosis (Figure 6.1b, Plate 5). Menand et al. (2007a) report that caulonemal cells grow at 19.87 ± 0.51 μm h^{-1}, which is significantly faster than chloronemata.

In contrast with chloronemal cells, ultrastucture analyses performed on caulonemal cells from different moss species, *Funaria hygrometica* (Schmiedel and Schnepf, 1980; McCauley and Hepler, 1992), *Physcomitrium turbinatum* (Jensen and Jensen, 1984), *Ceratodon purpureus* (Walker and Sack, 1995) and *P. patens* (Tucker et al., 2005) show clear differentiated sub-cellular organization. The most apical part of the tip is devoid of large organelles and appears full of small electron dense vesicles as well as smooth endoplasmic reticulum. Just below this layer, a second area still devoid of plastids is observed containing a large amount of Golgi stacks, rough and smooth endoplasmic reticulum and mitochondria. These two layers are 30–60 μm long and form the elongating part of the tip cell. Next to this first section, sits a zone of variable depth containing plastids (chloroplasts in light grown protonemata, amyloplasts in dark grown protonemata), mitochondria, Golgi stacks, endoplasmic reticulum of both types, small vacuoles and the nucleus. The base of the apical cell forms the last distinguishable zone: it is filled with a large vacuole with a fringe of cytoplasm adjacent to the cell wall. This basal zone is the most variable in size. During the elongation of the cell, it can vary from almost nothing after cytokinesis to 300 μm just before the next mitosis.

Finally, particle rosette complexes, sites of microfibril biosynthesis, have been detected in caulonemal cell tips of *F. hygrometica* (Reiss et al., 1984; Schnepf et al., 1985). Specifically, the analysis of their distribution along the plasma membrane revealed a specific accumulation in the tip of the cell. These complexes provide cell wall growth components. More recently, a publication detailed the cellulose synthase gene superfamily in *P. patens* (Roberts and Bushoven, 2007). An interesting point was the over-representation of the CLSD gene family, which is thought to represent tip growth-specific cellulose-like synthases.

In their most recent publication, Menand et al. (2007b) demonstrated how tip cell growth can be modulated to generate the different cell types present in *P. patens*. They investigated the RH6-like transcription factor family relationship between *Arabidopsis thaliana* and *P. patens* and demonstrated the functional conservation between the two *A. thaliana* genes AtRH6 and AtRSL1 and the pair of *P. patens* genes PpRSL1 and PpRSL2 in, respectively, root hair formation and rapid cell elongation observed in caulonemata and rhizoids. In *P. patens*, the double mutant *Pprsl1* and *Pprsl2* is viable and can go through a normal life cycle. Spores germinate and chloronemata develop normally,

showing that polar tip growth occurs normally in this mutant, but caulonemata and rhizoids fail to develop. Hence, the rapid elongation characteristic of these two-cell types is abolished, but without affecting polar tip growth itself and normal chloronemal development.

6.3 Environmental signals affecting polar cell elongation

Tip growth polarity can be modified by at least two major environmental cues, light and gravity (Cove et al., 1978). Studies focused on these two cues and the responses in mosses show the ability of mosses to direct tip growth in response to environmental factors. These studies have not, as yet, provided details about the tip growth process itself.

6.3.1 Light and polar tip growth

Light is a major environmental cue that affects plant growth, morphology and physiology at all developmental stages (Fankhauser and Chory, 1997) through energy flow and spatiotemporal signaling. We are focusing here on the light effects on tip-growing cells of *P. patens*, but the impact of light on chloroplast movement (Kadota et al., 2000; Sato et al., 2001; 2004; Kasahara et al., 2004, Uenaka et al., 2005) or gametophore morphology has been studied as well (for recent photomorphogenesis review in mosses, see Lamparter, 2006).

The light requirement for moss spore germination is species-specific (Meyer, 1948). While certain species can germinate without any light if water and nutrients are available, *P. patens* does require light to germinate (Ashton and Cove, 1977). Moreover, *P. patens* protoplasts require light to divide: left in the dark after protoplasting, protoplasts produce cell walls but do not initiate cell elongation or division (Jenkins and Cove, 1983a). Presumably, both observations reflect the light-dependent cell division requirement found in chloronemal cells. However, tip growth can occur in *P. patens* in the absence of light, providing a carbon source is provided (Cove et al., 1978), indicating that light itself is not required for all tip growth.

Nevertheless, light intensity and quality affect tip growth through different photo-morphological processes: orientation of the cell (phototropism *sensus stricto*) and modification of protonemal architecture through cell branching. Initial studies in *P. patens* used white light to assess plant responses, however, *P. patens* has genes for the defined three sets of known plant photoreceptors: phytochromes, cryptochromes and phototropins that respond to a spectrum of wavelengths. In seed plants, these photoreceptors are the primary integrator of light signaling that results in an integrated response to light by the whole plant (Jiao et al., 2007). Phytochromes and cryptochromes have been shown to affect, at some level, polar tip growth in *P. patens*.

The photoreversible red/far-red phytochrome photoreceptors have been the most studied in *P. patens* (for review, see Hughes et al., 2005) using

specific light direction (phototropism) and polarization (photopolarotropism). Protonemal responses seem to be dependent on the cell type; chloronemata show a different response than caulonemata, and the intensity of the light signal. Jenkins and Cove (1983b) established a clear phytochrome-related response of a growing tip from a regenerating sporeling under a red light stimulus. Below 5 $\mu molem^{-2s-1}$ cells grow toward the light, but above this, value growth is reoriented perpendicular to the light source. Using polarized red light a similar switch is observed, with a low-light response leading to growth parallel to the E vector and a high-light response perpendicular to the E vector. Working with dark grown caulonemal cells submitted to red light treatment, Mittman et al. (2004) observed a distinct pattern: between 0.015 and 0.15 $\mu molem^{-2s-1}$, cells are negatively phototropic, switching to positive phototropism between 1 and 5 $\mu molem^{-2s-1}$ and finally displaying light avoidance behavior above 15 $\mu molem^{-2s-1}$. The first genetic evidence of phytochrome involvement in such phenomena in mosses comes from analyses of aphototropic mutants in the moss, *C. purpureus*. The mutants were defective in phytochrome chromophore biosynthesis (Lamparter et al. 1996). More specifically, the loss of phototropism observed in the disruption of *PHY4* (Mittman et al., 2004), one of the four *P. patens* phytochromes, demonstrated the direct role of this class of photoreceptors in phototropism. However, in *P. patens*, the light response is a more complex story: mutants display differential responses to light intensity with the wild-type responding at low intensity and mutants responding at high intensity (Jenkins and Cove, 1983c). Unfortunately, genes for these mutants have not been yet identified.

The two blue-light photoreceptors (PpCRY1a and PpCRY1b) have been investigated in *P. patens* (Imaizumi et al., 2002). Disruption of both genes exhibited defects in diverse aspects of moss development, suggesting that cryptochromes are involved throughout the life cycle. Focusing on tip growth, chloronemal branching from a caulonemal cell and side-branch chloronemal elongation are inhibited specifically in blue-light growth conditions, but caulonemal tip cells continue to grow normally. The explanation seems to lie with the interaction between auxin signaling and cryptochrome. The double mutant is hypersensitive to auxin treatment under blue light, specifically leading to rapid caulonemal cell differentiation of all tip cells, as auxin is required for this developmental transition. The proposed explanation is the repressive action of cryptochrome on auxin-regulated genes that leads to caulonemal cell differentiation. Additionally, but without a direct observation of tip growth, cryptochromes have been linked to calcium ion movement in the apical cell (Tucker et al., 2005).

6.3.2 Gravity and polar tip growth

Dark grown caulonemata are negatively gravitropic (Cove et al., 1978), but as in other mosses (e.g. *C. purpureus*, Hartmann, 1984), the tip growth response

to gravity is only observed in the dark and any light input masks the gravity effect (Cove et al., 1978). In *C. purpureus*, mutations in phytochrome chromophore biosynthesis, necessary for red-light perception, render caulonemal cells sensitive to gravity in white light indicating that the phytochrome-based light perception inhibits gravity perception under light in the wild type (Lamparter et al., 1996). Physiological experiments and mutant analyses performed on both *C. purpureus* and *P. patens* established a model probably common to the two species. The gravity response observed in both species is rapid and follows a similar pattern: 2–30 min after reorientation of the filaments relative to the gravity vector, tip growth is observed toward the gravity gradient for a short period of time, and then its growth reorients against gravity (Knight and Cove, 1991; Young and Sack, 1992).

During mitosis in *P. patens*, the direction of gravitropic response is reversed (Knight and Cove, 1991). In *C. purpureus*, interphasic negative gravitropic growth requires an intact microtubule cytoskeleton (Schwuchow et al., 1990; Schwuchow and Sack, 1994), a fact that may explain why *P. patens* growth orientation is modified as the microtubule cytoskeleton is totally reorganized around the mitotic spindle during mitosis. Amyloplast sedimentation has been proposed as the actual sensor of graviperception in *C. purpureus* (Hartmann, 1984, Schwuchow and Sack, 1993; Kuznetsov et al., 1999; Kern et al., 2001; Kern et al., 2005) and more generally in mosses (Schwuchow et al., 2002), but other organelles may play a role in this phenomenon in *P. patens*, since the same relocation of amyloplasts is not observed in this species (Knight and Cove, 1991).

Finally, mutagenesis studies in both *P. patens* (Jenkins et al., 1986; Knight et al., 1991) and *C. purpureus* (Wagner et al., 1997; Cove and Quatrano, 2006) lead to a description of two major classes of defects in tip cell response: total loss of gravitropism and misorientation with respect to the gravity vector. Additionally, complementation studies on these mutants show that at least three independent loci are responsible. These classes reflect the potential for assersing perception and transduction mechanisms as well as the integration of the signal to tip growth. To date, none of these mutants have a molecular identification. It is interesting to note that these mutants specifically affect perception of gravity in tip growth and none of them affect the negative gravitropic growth of the multicellular gametophore.

6.4 Cellular structural components involved in polar cell elongation

6.4.1 Cell wall components

Specific cell wall biosynthesis, deposition and organization are key in shaping the cell. Using protoplasts as non-polarized starting cells, Burgess and

Linstead (1981, 1982) investigated cell wall formation during their regeneration by scanning electron microscopy. Their observations defined three different patterns of cell wall appearance. The first 24 h of regeneration showed a rapid initial accumulation of a uniform meshwork of fibers without any detectable polarized structure (Burgess and Linstead, 1981). Then, a thickening and a modification of the cell wall composition marked the future position of outgrowth. This cell wall labeling disappears after the outburst of growth leaving a scarring at the base of the cell. Finally, the growing tip presented a seemingly non-organized mesh of fibrils, contrasting with the complex and organized lateral and basal cell wall (Burgess and Linstead, 1982). Although the specific nature of the different cell wall compounds detected in these studies has not been elucidated, their specific localization and sequential deposition are indicative of a role in tip growth.

Arabinogalactan proteins (AGPs), localized at the interface of plasma membrane and cell wall, have been proposed to play a role in this process. AGPs form a large group of glycoproteins characterized by the presence of a type II arabinogalactan glycan decoration. Using compounds that specifically interact with such decoration, that is, β-Glycosyl Yariv reagent and an antibody raised against (1–5) arabinan (LM6), Lee et al. (2005) demonstrated the presence of AGPs in *P. patens* and localized them predominantly at the tips of elongating protonemal cells. Moreover, application of β-Glycosyl Yariv reagent inhibited tip growth in a reversible manner. Taken together, these observations suggest that AGPs are involved in tip growth. The authors further analyzed polypeptides co-precipitated with β-Glycosyl Yariv reagent, and showed that canonical AGP proteins were present in *P. patens*. Finally, they deleted one AGP, PpAGP1. The mutant was viable, but showed a 25% reduction in protonemal cell size (Lee et al., 2005) suggesting the specific importance of PpAGP1 in cell elongation.

6.4.2 Calcium (Ca^{2+})

Ca^{2+} has long been recognized as a key molecule in almost all aspects of plant cellular physiology. We will focus here on evidence of its implication in moss tip growth. Ca^{2+} gradients in protonemal cell tips were first detected in *F. hygrometica* using chlorotetracycline (CTC) fluorescence (Reiss and Herth, 1979). This method, which allows the detection of membrane-bound Ca^{2+}, showed a steep tip to base gradient in the tip cell. In *C. purpureus*, inhibition of growth by the monovalent cation ionophore monensin led to a quick disappearance of the CTC signal (Hartmann and Weber, 1988). After washing out the ionophore, the CTC gradient recovered as growth restarted. Unfortunately, the observation of such gradients was always concomitant with growth itself, and no direct causal link between the two phenomena could be established. High extracellular Ca^{2+} concentration inhibits growth altogether in *C. purpureus* (Herth et al., 1990). Similarly, the calcium-specific ionophore A23187 and calcium channel blocker Diltiazem can stop growth. These effects

on tip growth probably reflect alteration of the very tightly controlled, and low (10^{-7} M), intracellular concentration of free Ca^{2+} measured in protonemal cells (Herth et al., 1990).

In *P. patens*, the use of a calcium-sensitive aequorin protein (Russell et al., 1996) enabled cytosolic Ca^{2+} measurements. Reconstitution of this calcium-sensitive photoprotein *in vivo* has been achieved by over-expressing the apoaequorin cDNA in *P. patens* and subsequently complementing the media with the luminophore coelenterazine. A transient increase in cytosolic Ca^{2+} has been detected in response to cold shock, mechanical stress and pH change, but the overall cytosolic Ca^{2+} concentration seems to be tightly regulated. Using the same reporting method, Russell et al. (1998) showed that blue light elicited the same type of transient increase (wave) of Ca^{2+}. This result was confirmed and extended to the UV-A response by Tucker et al. (2005) using a fura-2-dextran-based visualization method. This later study showed that these Ca^{2+} variations were dependent upon both cryptochrome and phototropin photoreceptors and detected two different Ca^{2+} waves initiated in the tip region and the basal vacuolar region of the tip cell, respectively. Interestingly, these Ca^{2+} waves seem to be independent of extracellular calcium input but rather rely on organellar pools of Ca^{2+}. Additionally, extracellular calcium has been shown to participate in the phytochrome-mediated protonemal side-branch initiation (Ermolayeva et al., 1996 and 1997; Johannes et al., 1997).

Recently, a functional comparative study between the mammalian and *P. patens* protein presinilin (PpPS) sheds new light on the implication of Ca^{2+} in tip growth (Khandelwal et al., 2007). The authors demonstrated that the canonical mammalian β-secretase activity of animal presinilin is not conserved in the moss protein, but provide evidence that PpPS can rescue a β-secretase independent proliferation defect in mouse embryonic fibroblasts. Conversely, the mammalian presinilin can rescue the phenotype observed by disruption of *PpPS*. The disruption of *PpPS* leads to several distinct effects on protonemata and gametophores, including protonemata lacking wild-type curvature.

Moreover, FM4-64 staining of such tissue revealed a strong reduction of membrane recycling. Finally, phytochrome-dependent chloroplast relocation was abolished in this mutant. Although all these observations could be attributed to defects in membrane or cytoskeleton organization, a modification of Ca^{2+} homeostasis can explain them as well. A recent publication demonstrated that some isoforms of presinilin have an ER-Ca^{2+} channel activity (Tu et al., 2006).

6.4.3 Cytoskeleton

In the 1980s, several groups investigated the effects of cytoskeletal inhibitors on tip growth in moss protonemata, including *P. patens* and *F. hygrometica*. One of the first studies in *F. hygrometica* discovered that both colchicine and

cytochalasin B inhibited caulonemal tip growth (Schmiedel and Schnepf, 1980). Tip growth stops 10 min after application of colchicine and loss of microtubules is apparent after 30 min. Interestingly, microtubules appear to recover within 24 h even in the continued presence of colchicine. Growth resumes, but is altered and irregular formations appear ballooning off the tip. Cytochalasin B appears to inhibit growth, but no aberrant tip morphology was reported. Nuclear position was altered in both cases, with the nucleus migrating away from the tip in the presence of colchicine and toward the tip in the presence of cytochalasin B.

A later study employed oryzalin as a microtubule inhibitor instead of colchicine. At concentrations ranging from 0.5 to 1 μM oryzalin, tip growth was inhibited from the normal 48 to 7–10 μm/h. Nuclear migration was inhibited, which was shown to be critical for determining the site of side-branch formation (Doonan et al., 1986; Wacker et al., 1988). Within 2–5 h of inhibitor addition, tip swellings and sub-apical protrusions were evident. Organelles, such as the vacuole and plastids, were found in the tip zone, which is usually devoid of these organelles. At these concentrations, mitotic spindles and phragmoplasts were observed. Using immunofluorescence, the authors show that the cytoplasmic microtubules are more sensitive to oryzalin, being depolymerized at 0.5–1 μM oryzalin. At concentrations of 5–10 μM oryzalin, tip growth is further inhibited and does not exceed 5 μm/h. At these concentrations, no spindles or phragmoplasts are observed. Longer exposures at this concentration result in considerable damage to tip cells leading in many cases to cell death. The lower concentration of oryzalin depolymerizes the cytoplasmic microtubules and inhibits nuclear migration as well as plastid movement. The higher concentrations also affect mitotic spindle and phragmoplast assembly.

Both the actin and microtubule cytoskeletons appear to be predominately associated with the cortex. Microtubules and microfilaments have been visualized by immunofluorescence and microfilaments have also been visualized by fluorescent phalloidin in protonemal tissue (Doonan et al., 1985, 1988).

Using immunofluorescence, Doonan et al. show that microtubules are oriented along the long axis of the cell with an accumulation at the apex of the tip cell. 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, as is observed in other plant cells (Doonan et al., 1985). In the sub-apical 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 sub-apical cell. When a sub-apical cell branches, the nucleus migrates toward the site of branch formation. Bundles of microtubules exist between the nucleus and the prospective division site and these become progressively thicker and shorter as the nucleus migrates. Treatment with Cremart, a microtubule-destabilizing drug inhibits nuclear migration and subsequent treatment with Taxol, a microtubule

stabilizer, relieves this inhibition (Doonan et al., 1986). These studies demonstrate a clear role for microtubules during nuclear migration.

Doonan et al. (1986) showed in *P. patens* that the microtubule depolymerizing drug, Cremart, caused tip swelling similar to that observed with colchicine and oryzalin. 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 (Doonan et al., 1988). Under these conditions, actin filaments were still present near the tip of the cell. Interestingly, the actin depolymerizing drug cytochalasin D not only inhibited the tip swelling induced by Cremart but also inhibited tip growth. At lower concentrations of Cremart that caused disorganization of the microtubule cytoskeleton as opposed to complete depolymerization, new swollen growing points were initiated along the cell. These could be inhibited by the presence of the microtubule-stabilizing drug Taxol. Thus, proper organization of the microtubule cytoskeleton is required for growth to occur at the apex. From these studies, a consensus emerged that actin filaments appear to be critical for outgrowth, while microtubules appear to be important for organizing the cytoplasm and thus for polarity of growth.

Actin filaments appear to be axially oriented along the cortex and concentrated at the apex of the tip cell (Doonan et al., 1988) (for further discussion of actin filament localization, see below). Depolymerization of these apical actin filaments by cytochalasin D inhibits tip growth (Doonan et al., 1988). More recent studies using the actin depolymerizing drug, Latrunculin B, show that depolymerization of the actin cytoskeleton results in small rounded cells (Harries et al., 2005), suggesting that the actin cytoskeleton is essential for polar outgrowth of *P. patens* protonemal cells.

6.4.3.1 Microtubule cytoskeleton and associated proteins

P. patens contains similar numbers of alpha, beta (6 beta tubulin (Jost et al., 2004)) and gamma tubulin (Wagner et al., 1995) family members as in seed plants. Additionally, the kinesin family is just as large in *P. patens* as in seed plants, with about 60 members. As in seed plants, one-third of the kinesins belong to the Kin14 family, which are minus-end directed kinesin motors. The large expansion of the Kin14 family in plants compared with other kingdoms presumably reflects the loss of the minus-end directed cytoplasmic dynein, which appears to have been lost in all land plant lineages.

6.4.3.2 Actin cytoskeleton and associated proteins

As a general trend, the gene families associated with the actin cytoskeleton are smaller in *P. patens* than those of seed plants. As in seed plants, the myosin motors found in *P. patens* belong to the class VIII and XI myosin gene families, but there is a striking difference in gene content. For the class XI family, *P. patens* has two members whereas *A. thaliana* has 13. The same trend holds for a subset of genes associated with regulation of the actin cytoskeleton. The actin depolymerizing factor (ADF)/cofilin gene family which is involved in

mediating actin filament turnover by severing and/or depolymerizing actin filaments has only one gene in *P. patens* compared with 12 in *A. thaliana*. The formins, nucleators of actin filament polymerization, separate into three distinct phylogenetic groups, two of which are present in all plants. The third family seems to be specific for plants containing flagellate sperm (Grunt et al., 2008). Again the gene content is significantly smaller in *P. patens* compared with *A. thaliana*, with 6 class I and 2 class II formins in *P. patens* against 11 class I and 10 class II in *A. thaliana*. There is a single member of the class III formins. The small GTPases belonging to the plant-specific RAC-like family known as ROPs are also reduced in size with only 4 members compared with 11 in *A. thaliana*. Even actin has only four members in *P. patens* compared with ten in *A. thaliana*. Mosses have a simpler developmental pattern and reduced numbers of cell types compared with seed plants, thus fewer members of these gene families may be required for function. In seed plants, it is likely that these gene families have evolved tissue-specific functions.

Interestingly, other actin-associated cytoskeletal gene families including profilin (Vidali et al., 2007), capping protein, cyclase-associated protein, actin-interacting protein, fimbrin, villin, the Arp2/3 complex members and class VIII myosins (Bezanilla et al., 2003) have similar numbers of members in *P. patens* compared with seed plants. Perhaps, these gene families have retained evolutionarily conserved functions from mosses to seed plants. Notably, the myosins have significantly reduced gene numbers for the class XI family (see above), while the class VIII family has similar, with five members in *P. patens* compared with four in *A. thaliana*. This suggests that class VIII myosins may perform conserved functions from mosses to seed plants, while class XI myosins may have evolved tissue and/or developmental-specific functions in seed plants.

6.4.3.2.1 Actin filament organization in tip-growing cells. In the 1980s, several reports were published describing actin filament localization in moss protonemata. Both immunofluorescence and fluorescent phalloidin were employed in these studies and showed an abundance of axially aligned filaments, with a concentration at or near the apex of the tip cell. Since these studies, localization of actin filaments in plant cells has been analyzed more and more using green fluorescent protein (GFP) fusions of actin-binding domains, such as GFP-mouse-Talin (Kost et al., 1998), GFP-ABD2 (fimbrin) (Kovar et al., 2001; Sheahan et al., 2004; Wang et al., 2004), GFP-ADF (Chen et al., 2002) and GFP-villin (Klahre et al., 2000). This allows imaging of actin in live cells. A recent report by Finka et al. (2007) used GFP-mouse-Talin to image actin filaments in *P. patens*. As in other plant cells (Kost et al., 1998, Kovar et al., 2001; Timmers et al., 2002; Ketelaar et al., 2004; Sheahan et al., 2004), high levels of GFP-Talin expression inhibits growth in moss, specifically protonemal growth. The authors were able to isolate a stable line containing GFP-Talin under a constitutive promoter. However, they report that actin filaments were not observed in the apical cells of this line; fluorescent filamentous actin was

notably only observed in fully grown sub-apical cells that had completed their differentiation. To detect actin filaments with GFP-Talin in growing apical cells, Finka et al. (2007) regulated the expression of GFP-Talin using a heat shock-inducible promoter. After controlled heat shock conditions, low levels of GFP-Talin expression permitted wild-type cell growth and actin filaments were detected in apical cells. The actin filament organization using this marker generally reflected older studies that had used immunofluorescence or fluorescent phalloidin. Tip-growing cells have a dense cortical network of axially organized actin filaments. The actin filaments are tightly associated with the cortex; very few filaments are found in the medial section of the cell. Interestingly, a perinuclear actin filament 'cage-like' structure is observed, particularly in older cells. Near the tip of apical cells, there is a higher concentration of GFP fluorescence, but without a clear filamentous organization (Figure 6.2b and 6.2d). Thus, it is unclear whether the GFP fluorescence present at the apex is associated with actin filaments. Additionally, this study notes that overproduction of GFP-Talin inhibits tip growth and causes deformation of the tip of the apical cell. Therefore, it is critical to carefully control GFP-Talin levels in the cell to avoid any negative effects on growth.

Talin is an actin-bundling protein. Thus, it is possible that GFP-Talin may alter actin dynamics in the cell, possibly stabilizing actin filaments.

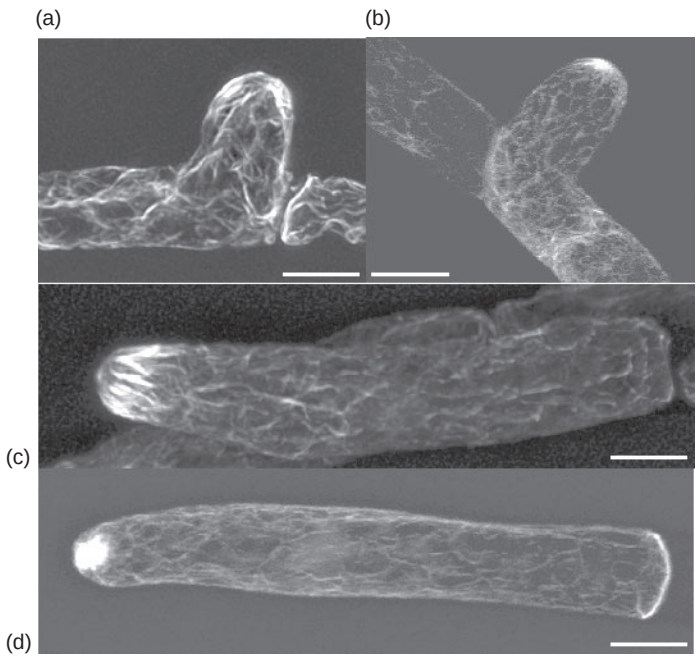

Figure 6.2 Actin labeling of protonemal tip cell. Rhodamine phalloidin staining (a, c) and GFP-Talin labeling (b, d) of chloronemal tip cell as they branch (a, b) and as elongated tip cells (c, d). Scale bar: 20 μm.

Additionally, GFP-Talin may not label all populations of actin filaments in *P. patens*. In lily pollen tubes, another tip-growing plant cell, there is a highly dynamic population of sub-apical cortical actin filaments that does not label with the GFP-Talin marker (Wilsen et al., 2006). A recent study by Vidali et al. (2007) revisited fluorescent phalloidin staining of actin filaments in *P. patens* protonemal cells. In this study, *P. patens* was fixed *in situ*, under active growing conditions, with two chemical crosslinkers, EGS and MBS. After a subsequent fixation with formaldehyde and membrane permeabilization with saponin, protonemal cells were incubated in fluorescent Alexa488-phalloidin. Using these conditions, Vidali et al. (2007) observed prominent axially organized actin filaments that were tightly associated with the cortex, similar to previous observations both in fixed and live cells. In addition, a cortical structure was observed near the tip of the apical cell. (Figure 6.2a and 6.2c). The apical cortical structure is very labile. Only protonemal cells that are actively growing exhibit this structure. Any manipulation of protonemal tissue, such as moving tissue from one growth surface to another, appears to disrupt the apical F-actin. Therefore, it is critical to ensure that the cells under observation are growing when determining sub-cellular localization patterns.

6.4.3.2.2 Regulators of actin dynamics are critical for tip growth. Several recent studies have provided further evidence that the actin cytoskeleton is required for tip growth. These studies have used reverse genetics to reduce the function of key regulators of actin dynamics, thereby examining their roles during protonemal growth. Harries et al. (2005) and Perroud and Quatrano (2006) examined the role of the actin filament nucleating Arp2/3 complex using two distinct approaches. The Arp2/3 complex is required for nucleation of actin filaments in animals and fungi. It is composed of seven subunits, two of which, Arp 2 and Arp 3, resemble an actin dimer. Upon activation of the Arp2/3 complex by activators such as SCAR/WASP, an actin monomer is recruited to the complex. Arp2 and Arp3, together with the recruited actin monomer, resemble a nucleus of an actin filament and thus nucleate polymerization of actin. The Arp2/3 complex has been shown in many systems to be essential for proper organization of actin filaments and many actin-mediated processes. Interestingly, in plants, Arp2/3 complex function is not essential for plant viability. Mutations in individual subunits lead to overall mild phenotypes. In *A. thaliana*, the cell type that is most affected by the absence of the Arp2/3 complex is the trichomes (Szymanski, 2005), which are distorted. In some Arp2/3 complex mutants, tip-growing cells such as root hairs are significantly shorter than wild type (Mathur et al., 2003).

In *P. patens*, Harries et al. (2005) used RNA interference (RNAi) to reduce the function of the ARPC1 subunit and Perroud and Quatrano (2006) used homologous recombination to knock out the only copy of the ARPC4 subunit. Stable *P. patens* lines containing an RNAi construct targeting degradation of ARPC1 have multiple defects. During protoplast regeneration, *arpc1-rnai* plants are unable to establish a polar axis, although they eventually begin

to grow chloronemal filaments of reduced size and abnormal shape from an unpolarized group of cells. These phenotypes can be mimicked by treatment with the actin depolymerization drug, latrunculin B. Interestingly, the protonemal filaments that do grow remain chloronemal in character. There are no apparent caulonemal cells and thus no gametophore formation in *arpc1-rnai* plants, even in response to cytokinin treatment. This study clearly demonstrates the importance of an intact actin cytoskeleton for proper cell growth and morphogenesis in *P. patens*.

Deletion of the ARPC4 subunit (Perroud and Quatrano, 2006) resulted in viable plants able to undergo protonemal growth and gametophore formation. However, the protonemal cells were significantly smaller than wild-type cells. The *arpc4* mutant only had one type of protonemal cell, which was four times smaller than caulonemal cells and two times smaller than chloronemal cells present in wild type. As a result, *arpc4* plants were small and compact compared with wild type. Rhizoids, another tip-growing cell type, emanate from the base of the gametophore and were significantly affected in *arpc4* plants. The rhizoids fail to elongate and their development aborts after four to six cell divisions. The gametophores of *arpc4* plants were also significantly stunted compared to wild type. A functional YFP-fusion to ARPC4 localized to the tip of the apical cell, suggesting that the Arp2/3 complex function is required at the site of growth. However, *arpc4* plants do not respond properly to polarized light, suggesting that the Arp2/3 complex may be required to mediate tip growth in response to polarizing cues. In comparison to the ARPC1 RNAi plants, loss of ARPC4 resulted in a significantly milder phenotype, suggesting that subunits of the Arp2/3 complex in moss may have distinct functions. Although the Arp2/3 complex is essential for viability in many other systems, in plants it is likely that formins, another class of actin-nucleating proteins, either act redundantly with the Arp2/3 complex or are the essential nucleators and organizers of actin polymerization in plant cells. This remains to be tested and has been difficult to address in *A. thaliana*, which has 21 formin genes. *P. patens* is an excellent system to now address formin function since there are only nine putative formin genes.

The activity of the Arp2/3 complex is tightly regulated in the cell. The Wave/SCAR complex acts upstream by directly binding and activating Arp2/3 complex function. In a recent study, Perroud and Quatrano (2008) show that BRICK1, one of the subunits of the Wave/SCAR complex is also critical for tip growth in moss. Deletion of the only BRICK1 gene leads to a drastic reduction in overall plant size, due to a dramatic reduction in cell size and an apparent delay in cell division. Overall, the *brk*1 cells are small and somewhat round in appearance. Additionally, the division planes are not uniform along the protonemal filament. Thus, the activity of Brick is required for proper growth and possibly placement of the cell division plane. As in *Arabidopsis*, loss of BRICK1 function leads to a stronger phenotype than loss of subunits of the Arp2/3 complex. This is intriguing and suggests that Brick may be required to activate additional components critical for proper

organization of the actin cytoskeleton. BRICK1 localizes to the apex of the tip-growing cell, which is similar to the ARPC4 subunit of the Arp2/3 complex. Perroud and Quatrano show that *brk1* cells have cortically associated filamentous actin as detected by GFP-Talin. However, the accumulation of the GFP-Talin signal observed in tip cells is disrupted in *brk1* cells. *brk*1 cells have multiple foci of GFP-Talin fluorescence at the apex in contrast to the uniform cap present in wild-type cells. Additionally, BRICK1 is required for the tip localization of ARPC4, demonstrating that BRICK1 functions upstream of ARPC4 for proper maintenance of the actin cytoskeleton.

The Arp2/3 and Wave/SCAR complex studies have suggested that proper control of actin dynamics is important for optimal tip growth. To address whether actin dynamics are essential for tip growth, recent studies by Vidali et al. (2007) and Augustine et al. (2008) have analyzed the function of actin-binding proteins critical for actin dynamics. Vidali et al. analyzed the role of profilin, a small actin monomer-binding protein. In many cell types, profilin acts to promote barbed-end directed actin polymerization. In animals and fungi, profilin also enhances the exchange of ADP (adenosine diphosphate) for ATP (adenosine triphosphate) on the actin monomer, further promoting actin dynamics. In plants, however, profilins do not exhibit this activity, which may be in part compensated by plant actin's intrinsically higher rate of nucleotide exchange. In all systems studied, profilin has been found to be essential for viability and for proper organization and control of the actin cytoskeleton. However, in plants, isolation of complete loss of function mutants for profilin has been complicated by the presence of multiple profilin genes.

Vidali et al. (2007) used RNAi to silence all three profilin genes in moss. Transformation of an RNAi construct that targets the coding sequence of PrfA, the most highly expressed profilin gene in protonemal tissue, results in dwarfed plants compared with wild type. These plants are composed of small, rounded cells. Since the three moss profilin genes are highly similar at the nucleotide level, it is likely that the PrfA RNAi construct silences all three profilin genes. To test this, Vidali et al. used another RNAi construct, which contains sequences from the 3′ untranslated regions (3′-UTR) of PrfB and C. Transformation of this construct resulted in exactly the same phenotype as obtained with the PrfA RNAi construct. PrfA sequences were not included in the 3′-UTR construct, since PrfA and B are highly similar, even in their untranslated regions. Using an antibody raised to lily pollen profilin that cross-reacts with moss profilin, immunofluorescence of RNAi plants showed that profilin levels are dramatically reduced in silenced plants. Thus, profilin is required for polarized extension of protonemal cells.

In contrast to the RNAi studies with ARPC1, it was not possible to obtain stable profilin-RNAi lines, suggesting that profilin is essential for tip growth and viability. Using the transient RNAi assay, Vidali et al. (2007) investigated the molecular interactions required for proper profilin function in *P. patens*. Profilin binds numerous ligands in the cell. Most notably besides actin, profilin binds poly-L-proline containing proteins such

as the formins. Mutations in profilin have been identified that specifically affect either actin or poly-L-proline binding without affecting the other binding activity. Using the analogous mutations in moss profilin, Vidali et al. used complementation studies to show that actin binding is essential for profilin function. Interestingly, poly-L-proline binding is important for polarized extension of protonemal filaments, but loss of poly-L-proline binding does not phenocopy loss of profilin function. Plants expressing the poly-L-proline-binding mutant were able to grow larger than profilin-RNAi plants but were compact and highly unpolarized compared with wild type.

Vidali et al. (2007) used fluorescent phalloidin staining of actin filaments to investigate the localization of actin filaments in plants lacking profilin function. Plants lacking profilin still had filamentous actin, suggesting that profilin is not required for polymerization *per se*. However, the actin filaments were significantly disorganized compared with wild type, in which the filaments are aligned along the length of the cell. Vidali et al. quantified the degree of disorganization using a fast Fourier transform analysis. This revealed that while filamentous actin is highly ordered in wild-type cells, the profilin-RNAi plants contain filamentous actin in randomized orientations. Analysis of the actin and poly-L-proline-binding mutants revealed that the actin filaments were equally disorganized in these plants as well. Thus, profilin appears to be important for the proper organization of the actin cytoskeleton. Profilin RNAi plants have cortical patches of filamentous actin. Interestingly, these patches are tightly associated with the cortex and are always on the pole of the cell away from the previous cell division. This suggests that profilin may not be required to obtain a polarized site of actin polymerization at the cortex, but rather is required for proper organization of the filaments.

Augustine et al. (2008) analyzed the role of ADF in tip growth. Similar to profilin, ADF is critical for regulating actin turnover and binds to both actin filaments and monomers. ADF has a strong preference for ADP-bound actin, thus generally associating with older actin filaments. Once bound, ADF severs and stimulates depolymerization of filaments regenerating a pool of actin monomers and exposing barbed-ends for rapid polymerization in the presence of profilin. Interestingly ADF is a single-copy gene in *P. patens*. Using transient RNAi, Augustine et al. demonstrated that ADF is essential for plant viability. Loss of ADF results in tiny plants that within 8 days of expression of the RNAi construct exhibit cell senescence as demonstrated by loss of chlorophyll fluorescence. Silencing of ADF was verified by immunofluorescence of individually silenced plants with an antibody that specifically recognizes *P. patens* ADF. The ADF-RNAi phenotype was significantly stronger than that observed with profilin-RNAi, suggesting an absolute requirement for ADF function for plant viability.

The role of ADF in tip growth was investigated by generating a mutant of ADF that has reduced interaction with actin. ADF's actin binding is known to be dependent on phosphorylation of ADF at an N-terminal serine residue whereby phosphorylation of ADF inhibits interaction with actin.

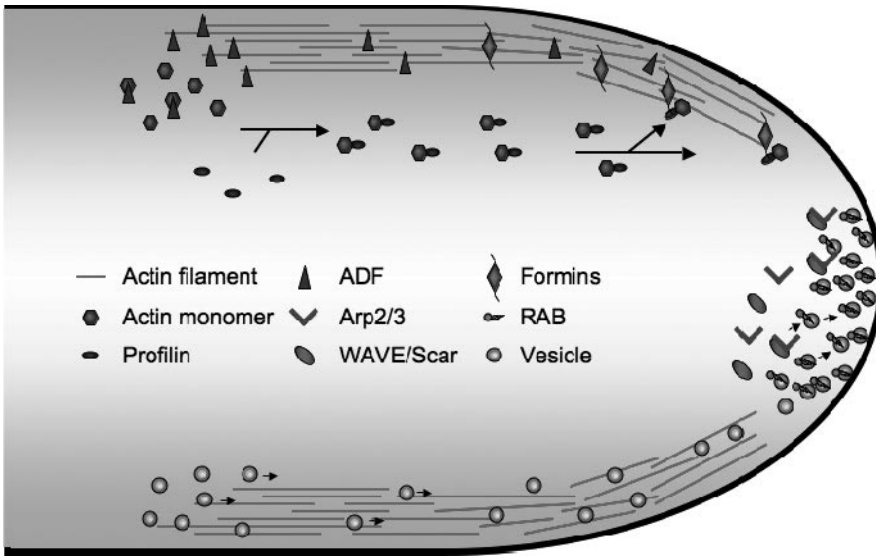

Figure 6.3 A model for the organization of the actin cytoskeleton at the tip of a growing cell. At the cell tip, the Arp2/3 complex and its activator, the WAVE/SCAR complex, are necessary for proper actin-mediated cell elongation and are both required for accumulation of RAB-associated vesicles. The apical F-actin organization is also necessary for cell elongation and is initiated by profilin and formin-mediated polymerization of actin monomers. ADF controls the integrity of the actin filament network by regulating actin turnover. ADF, actin depolymerizing factor; Arp2/3 complex, actin-related protein 2/3 complex; RAB, Ras-related in brain protein (small GTPase protein); WAVE/SCAR complex, Wiskott–Aldrich Verprolin homologous protein/SCAR complex. (For a color version of this figure, see Plate 6)

The phospho-mimetic ADF mutant (ADF-S6D) was partially able to rescue ADF-RNAi plants. ADF-S6D plants never exhibited cell death, but were very small and unpolarized compared with control plants, suggesting that ADF's interaction with actin is absolutely required for tip growth. In contrast, the unphosphorylatable mutant, ADF-S6A, rescued polarized growth in ADF-RNAi plants. ADF-S6A plants were not as big as control plants but were highly polarized, suggesting that proper control of ADF function is important for optimal rates of tip growth.

Similar to profilin-RNAi cells, ADF-RNAi cells contained actin filaments and appeared to be able to polarize cortical F-actin structures. In ADF-RNAi cells, the polarized F-actin structures in many cases resembled 'star-like' foci, whereas profilin-RNAi cells had aggregated patches of F-actin. The more organized foci present in ADF-RNAi cells may perhaps represent nascent formation of apical F-actin structures, which in the absence of proper actin dynamics is not able to fully assemble. Furthermore, ADF-S6A plants which were able to undergo tip growth exhibited normal F-actin apical structures, while ADF-S6D plants more closely resembled ADF-RNAi plants. This

suggests that proper actin turnover is essential for establishing and maintaining the cortical F-actin apical structure.

These recent studies have suggested that apical F-actin is crucial for carrying out tip growth and allow us to establish the following model (Figure 6.3, Plate 6). The Wave/SCAR and Arp2/3 complexes appear to localize to the tip of apical cells and be required for localization of apically associated factors, such as a tip localized Rab11-like GTPase from *Arabidopsis* (Perroud and Quatrano, 2008). Profilin and ADF appear to be required for formation of the F-actin cortical apical structure. Since profilin is known to interact with formins by binding poly-L-prolines, and the poly-L-proline-binding site is critical for polarization of growth, this suggests a model whereby apical F-actin formation is directed by profilin-formin-mediated actin polymerization. The apical F-actin structure is then maintained by proper rates of actin turnover, which is critically controlled by ADF activity. Future studies will need to address the role of formins in tip growth as well as the possible functional redundancies between formins and Arp2/3 complex.

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Chapter 7

GAMETANGIA DEVELOPMENT IN THE MOSS *PHYSCOMITRELLA PATENS*

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Abstract: Eggs and sperm are reproductive cells produced, respectively, in archegonia and antheridia in all land plants except seed plants, in which gametangia are highly reduced. The moss *Physcomitrella patens* is a suitable model plant to study the molecular mechanisms underlying the development of archegonia and antheridia and to gain an insight into their evolutionary origin. We have described the development of gametangia and the sporophyte in *P. patens*. We have screened 4825 gene-trap lines, in which a promoterless GUS reporter gene has been integrated, and have obtained 38 lines with GUS expression in gametangia. Expression patterns of GUS reporters have been examined during the development of gametangia and classified into several categories. We have discussed possible future approaches for the study of gametangia development and evolution.

Keywords: antheridium; archegonium; development; gametangium; gene-trap; sporophyte

7.1 Introduction

A significant difference between metazoa and land plants is the origin of their reproductive cells. In metazoa, eggs and sperm are formed by meiosis from diploid cells, whereas in land plants, a multicellular haploid generation occurs between the formation of reproductive cells and diploid cells. Spores are formed via meiosis in land plants. In bryophytes, these germinate to form multicellular gametophytes via subsequent mitotic cell divisions. After a period of vegetative growth, the gametophyte develops reproductive organs, which subsequently produce eggs and sperm mitotically.

Archegonia and antheridia, the organs in which, respectively, eggs and sperm are produced are found in all land plants except seed plants (Gifford and Foster, 1989; Kenrick and Crane, 1997). They are recognized as homologous structures and support the monophyly of the lineage, although a substantial amount of morphological and developmental diversity exists (Kenrick and Crane, 1997). In contrast, the origin of the gametangia is enigmatic. Charophycean algae, including Zygnematales, Coleochaetales and Charales, are the sisters of land plants, although the phylogenetic relationships among the three algal lineages are unclear (reviewed in Turmel et al., 2007). Eggs and sperm are not covered with gametophytic tissue in Zygnematales, as in other green algae, and most coleochaetaleans also form naked gametes (Graham and Wilcox, 2000). In contrast, multicellular oogonia and antheridia are produced in charophyceans, although their developmental processes are different from those in land plants. Thus, charophycean and land plant gametangia are not likely to be homologous (Kenrick and Crane, 1997), and the evolution of land plant gametangia cannot be traced based on comparative morphology. Morphological and developmental characters are sometimes discontinuous between extant lineages because of the discontinuous nature of such characters or the extinction of intermediate taxa. Each of these characters is likely governed by networks composed of hundreds of genes, which may be partly conserved despite discontinuous phenotypic divergence. The evolutionary history of morphological characters may therefore be traced based on the evolution of gene networks. However, no studies have identified the gene networks involved in gametangia development in land plants because of the reduction in gametangia in flowering plants and because of a lack of model non-flowering plants in which genetic manipulation is feasible.

Gene targeting, which may be used to study the gene networks involved in gametangia development, is feasible in the moss *Physcomitrella patens* (Cove, 2000; Schaefer and Zrÿd, 2001). Most of the genome of this moss has been sequenced (Rensing et al., 2008), and comparative studies of the genes involved in the moss's development are now possible. A forward genetic approach using mutants defective in gametangia formation is difficult in vascular plants because such mutants are often sterile and diploid plants are not formed. In contrast, it is feasible to study such mutants in bryophytes as their

high vegetative regeneration rates allow them to be propagated vegetatively in the haploid generation. However, although *Agrobacterium tumefaciens*-mediated transformation, particle bombardment and shuttle mutagenesis (Nishiyama et al., 2000) have been used, useful forward genetic methods are not well established. Hiwatashi et al. (2001) established approximately 20 000 gene- or enhancer-trap lines, which may be used to identify organ-specific gene expression. Here, we describe the isolation of gene-trap lines with specific expression during gametangia development and the characterization of their expression patterns.

7.2 Development of the gametangia

Gametangia formation is environmentally regulated in bryophytes (Chopra and Bhatla, 1983). *P. patens* is a short-day plant, and gametangia are induced when the growth conditions are shifted from 25°C to 15°C and enhanced in an 8-h light/16-h dark regime (Engel, 1968; Hohe et al., 2002). The position and number of gametangia vary among gametophores in clonally derived plants (Ashton and Raju, 2000) and may depend on differences in the external microenvironment. However, antheridia are usually formed at the gametophore apex earlier than are archegonia (Figure 7.1a), and archegonia tend to be formed at the apex of a gametophore branch (Figure 7.1b).

The development of antheridia and archegonia in *P. patens* is similar to that in *Physcomitrium cyathicarpum* (Lal and Bhandari, 1968). The primordial cells of the gametangia are derived by cell division at the surface of the gametophore apex, although the exact lineage of the antheridial primordial cells that give rise to a gametophore apical cell and the positional relationship with the filiform and clavate paraphyses have not been examined. Archegonia and antheridia cannot be distinguished during the early developmental stages (Figures 7.1c and 7.1d). The primordial cell tends to swell, followed by division in the transverse direction (Figure 7.1c). The upper cell is thus designated as an apical cell, and it subsequently undergoes several oblique divisions with two cutting faces to form regular rows of segments on either side (Figure 7.1d). Periclinal divisions of the cells in each row, which are specific to antheridium development, form outer jacket cells and inner primary spermatogonial cells (Figure 7.1e). Subsequent cell divisions form an antheridial jacket containing numerous spermatocytes (Figure 7.1f), that then mature into crescent-shaped sperm with two flagella at one end (Figures 7.1g and 7.1h).

After the formation of two rows of cells in the archegonial primordium, the pattern of division in the apical cell changes, and a central cell covered with peripheral cells is formed. The central cell then divides further into an upper primary canal cell and a lower ventral cell (Figure 7.1i). Series of transverse cell divisions in the primary canal cell and its surrounding cells form neck canal cells and neck cells, whereas the ventral cell expands without

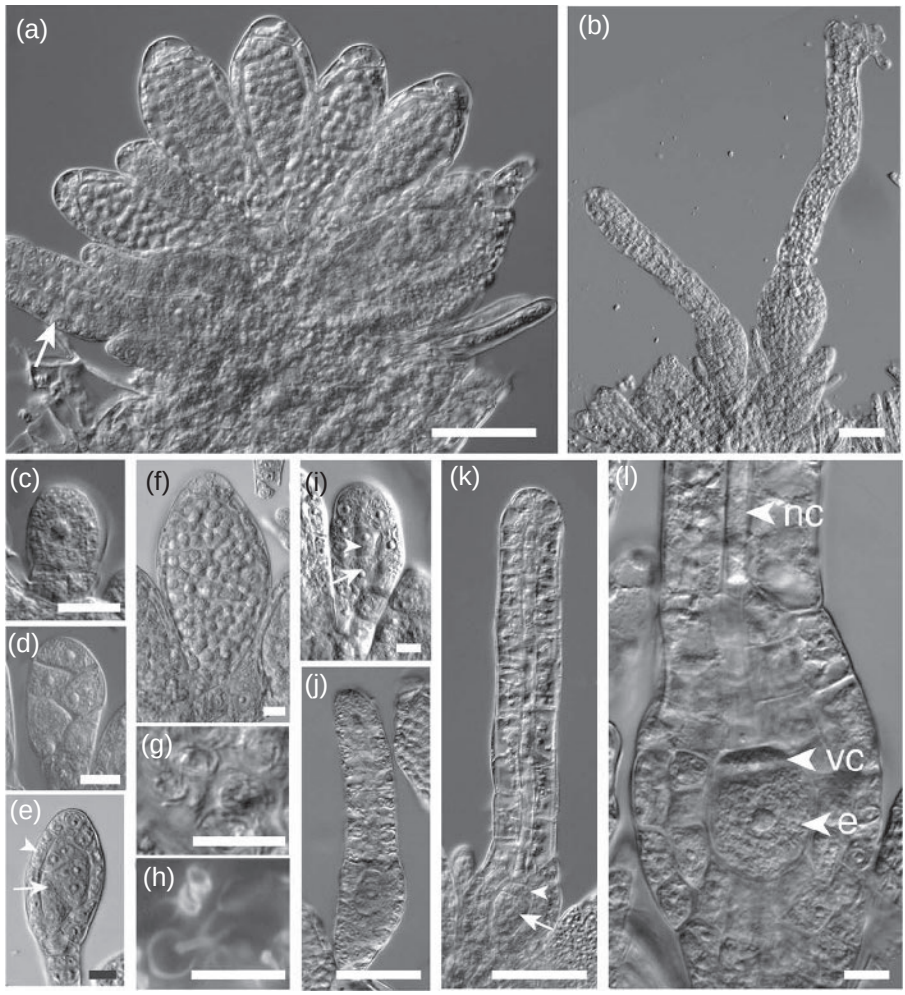


Figure 7.1 Gametangia development. (a) A cluster of antheridia with a developing archegonium (arrow). (b) An immature (left) and mature (right) archegonium. The tip of the mature archegonium is open. Two- (c) and six-cell stage (d) primordia of gametangia. We were unable to distinguish the archegonial and antheridial primordia at this stage. (e) An antheridium following periclinal division, with outer jacket cells (arrowhead) and inner spermatogonial cells (arrow). (f) An antheridium with spermatocyte. (g) Magnified image of a mature antheridium showing differentiated flagella on each sperm. (h) DAPI signal of a sperm nucleus. (i) A developing archegonium with a ventral cell (arrow), a primary canal cell (arrowhead) and surrounding peripheral cells. (j) A developing archegonium in which the ventral cell is expanding. (k) A developing archegonium containing a ventral canal cell (arrowhead) and an egg cell (arrow). (l) An archegonium showing neck canal cells (nc), a ventral canal cell (vc) and an egg cell (e). Scale bars = 50 μm (a, b, j and k); 10 μm (c–i, and l).

dividing (Figure 7.1j). Asymmetric division of the ventral cell then forms a smaller ventral canal cell and an egg cell (Figures 7.1k and 7.1l). The neck canal cells and ventral canal cells are subsequently degraded, and the sperm moves to a cavity in the archegonia where the egg cell floats. The egg cell is always located in the center of the cavity, where it is surrounded by materials, although the compounds have not been studied.

7.3 Development of the gametangia after fertilization is accompanied by growth of the sporophyte

The morphology of the egg changes dramatically after fertilization (Tanahashi et al., 2005). In glutaraldehyde-fixed tissue, the autofluorescence of the nucleus is weaker than that of the cytosol before fertilization. After fertilization, however, the situation is reversed. The surface of the zygote becomes wavy in appearance and expands to fill the archegonial cavity because of vacuolar expansion (Figure 7.2a). The subsequent development of the sporophyte is similar to that in *P. cyathicarpum* (Lal and Bhandari, 1968), although the elongation of a seta and the differentiation of operculum and peristome are not observed in *P. patens*.

The angle of the first cell division of the sporophyte is usually transverse, forming an apical cell and a basal cell (Figure 7.2b). The apical cell continues to divide obliquely at two faces (Figure 7.2c), producing two lines of wedge-shaped cells (Figure 7.2d). The apical cell forms approximately 12 cells, whereas the basal cell divides two or three times to form the basal-most cells of the foot (Figure 7.2d). After a series of oblique divisions in the apical cell, the cells expand, forming the longitudinal axis of the embryo (Figure 7.2e). At the same time, the cells in the upper half of the embryo divide periclinally to form the endothecium and amphithecium (Figures 7.2e and 7.2f), which then differentiate into the collumella, with its surrounding sporogenous tissue, and the capsule wall, respectively (Figures 7.2g–7.2i). Finally, the wedge-shaped cells derived from the apical cell form the upper part of a foot, a seta and a sporangium. After fertilization, the cells of the archegonium divide and elongate, particularly at the base (Figures 7.2e and 7.2f). The upper part of the archegonium then differentiates into a calyptra, which detaches during sporophyte development.

7.4 Gene-trap lines with GUS expression in the gametangia

Gene trapping in plants and animals is a powerful tool for the identification of novel genes and unidentified regulatory elements based on the expression of a reporter gene (Springer, 2000; Lee et al., 2007). Gene trapping, which involves the insertion of a promoterless reporter gene at random points in the

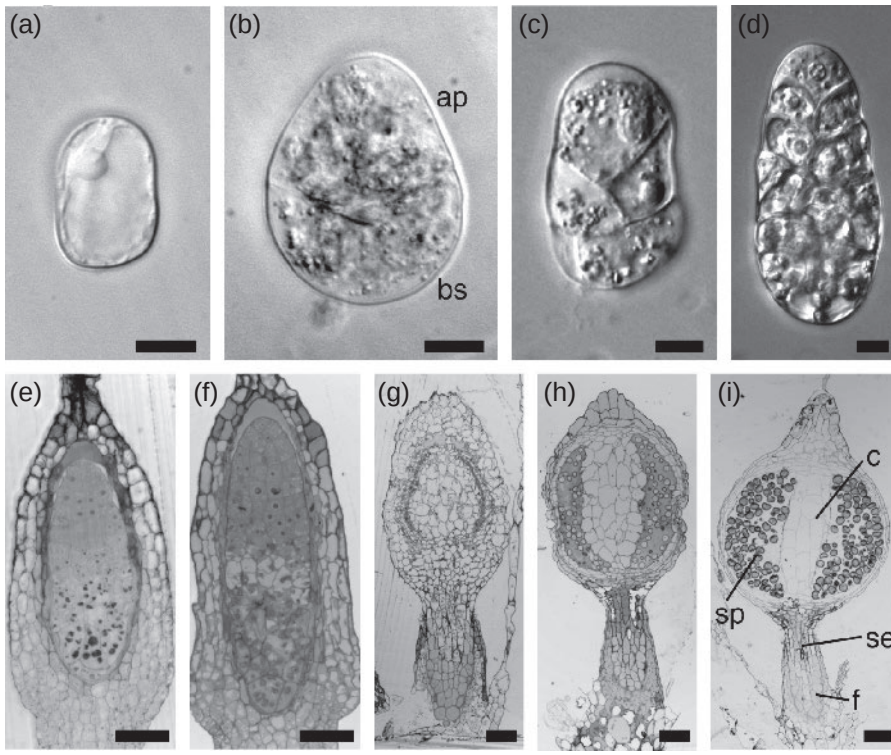


Figure 7.2 Development of a sporophyte. A zygote or sporophyte was extruded from an archegonium in (a–d). (a) A zygote. (b) A two-cell sporophyte with an apical (ap) and a basal (bs) cells. (c) A four-cell sporophyte. (d) An eight-cell sporophyte. (e) A sporophyte undergoing periclinal divisions. (f) A sporophyte with an elongating seta. (g–h) Sporophytes with developing sporangia. (i) A mature sporophyte with spores (sp), the columella (c), the seta (se) and the foot (f). Scale bars = 10 μm (a–d); 50 μm (e–f); 100 μm (g–i).

genome, allows the detection of genes expressed in a tissue-specific manner. Shuttle mutagenesis is effective for the delivery of gene-trap elements into the genome of *P. patens* (Nishiyama et al., 2000). Shuttle mutagenesis was originally developed in the budding yeast *Saccharomyces cerevisiae*, which has a high rate of gene targeting (Seifert et al., 1986; Ross-Macdonald et al., 1997). This method is also well suited to other organisms that have high rates of gene targeting such as *P. patens*.

Gene-trap lines of *P. patens* were generated using a construct that is a variant of the mini-transposon used in shuttle mutagenesis (Hiwatashi et al., 2001). It included a gene-trap element (HI-GT) containing a NPTII expression cassette as a selection marker and *uidA*, which encodes β-glucuronidase (GUS), as a reporter cassette (Jefferson, 1987). The fourth intron of *GPA1* from *Arabidopsis thaliana*, which encodes the alpha subunit of the G-protein gene, was fused to the 5′ end of the reporter gene in the gene-trap element to enable the detection of gene expression upon insertion of the element into introns or exons

(Sundaresan et al., 1995). Approximately 5400 transformants containing HI-GT were screened for GUS activity, and over 400 transformants were histochemically stained (Hiwatashi et al., 2001). GUS activity was detected in various portions of the transformants, including the protonemata, gametophores, antheridia, archegonia and sporophytes, indicating that the genes trapped by this method function in many cell types and tissues. DNA gel-blot analysis was used to estimate the number of copies of mini-transposons inserted into each trap line. Most of the lines contained multiple mini-transposons integrated into the genome. Thus, the isolation of a trapped gene using a genomic sequence-based approach such as TAIL-PCR (thermal asymmetric interlaced polymerase chain reaction) (Liu and Whittier, 1995) will probably be difficult. However, the isolation of a trapped gene from a gene-trap line can be easily accomplished using RACE (rapid amplification of cDNA ends) because the transcript of the trapped gene is fused to that of the reporter gene.

To obtain gene-trap lines with specific expression during gametangia development, the GUS expression patterns were re-examined in 4825 lines of the 5400 stable transformant lines containing HI-GT. From that analysis, 38 lines with expression in the gametangia were selected for detailed analysis (see Figures 7.3–7.6, Plates 7–10). We detected GUS expression in the antheridia or archegonia in six and two lines, respectively, whereas the other lines showed GUS expression in both the antheridia and archegonia. GUS expression was also detected at specific stage(s) of gametangia development in most lines. GUS expression was detected in the spermatogonial cells and sperm in lines Gt3, Gt6, Gt8, Gt16, Gt22 and Gt31. Prominent expression was detected in the antheridial stalk in lines Gt2 and Gt17. Stained sperm cells were observed in two lines (Gt32 and Gt35). In the archegonium, GUS expression in some lines was detected mainly in the egg cell (Gt1 and Gt20), in both the ventral canal and egg cells (Gt12 and Gt36), or in other tissues.

7.5 Future prospects

The isolation of a trapped gene from a gene-trap line is feasible using 5′-RACE (Hiwatashi et al., 2001). The detailed procedure is accessed on PHYSCOmanual (`http://moss.nibb.ac.jp`). The outline of the procedure for identifying a trapped gene is as follows:

1. Estimate the number of HI-GT constructs integrated into the trapped line using DNA gel-blot analysis.
2. Estimate the size and number of each candidate transcript containing both the trapped gene and the GUS reporter gene using Northern blotting.
3. Isolate all *uid*A-fused cDNA fragments from the trap line by 5′-RACE using *uid*A-specific primers.
4. Isolate the corresponding HI-GT-tagged DNA fragment from the original tagged genomic DNA library.

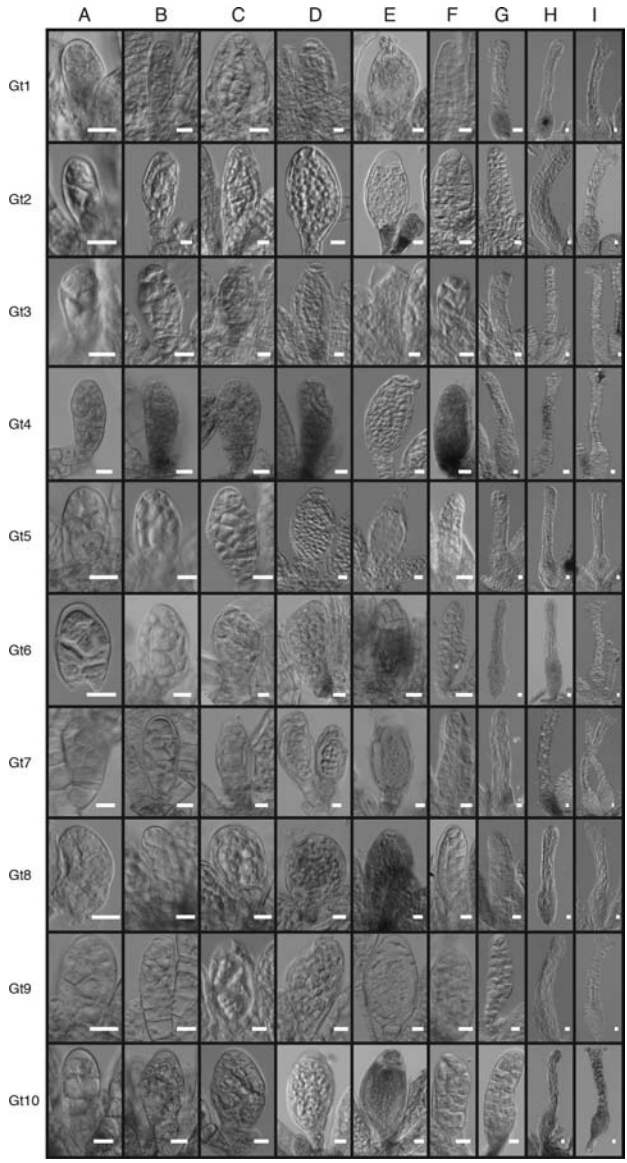


Figure 7.3 Detection of GUS expression in gametangia. Lines Gt1-Gt10. Each row corresponds to a line whose name is given on the left side. Columns (A)–(I) correspond to various developmental stages. (A) The primordium of a gametangium with an apical cell with two cutting faces. (B) The primordium of an antheridium just after periclinal cell division, showing outer jacket cells and inner primary spermatogonial cells. (C) An antheridium with dividing spermatogonial cells. (D) An antheridium at the stage between (C) and (E). (E) A mature antheridium with mature spermatozoids. (F) The primordium of an archegonium with an apical cell with three cutting faces. (G) An archegonium with an expanded ventral cell. (H) An archegonium containing a mature egg without an open neck. (I) An open archegonium around the time of fertilization. (For a color version of this figure, see Plate 7)

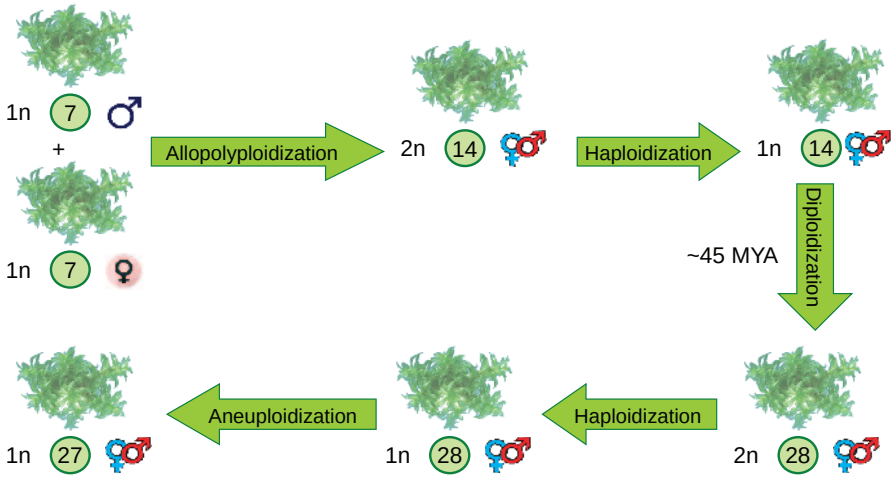


Plate 1 Hypothesized genome evolution of *P. patens* 1n/2n depicts a functional haploid/diploid; chromosome numbers are shown in circles; the male/female symbol shows dioicous plants, the hermaphrodite symbol shows monoicous plants. The age of the most recent genome duplication (~45 MYA) is derived by Rensing et al. (2007).

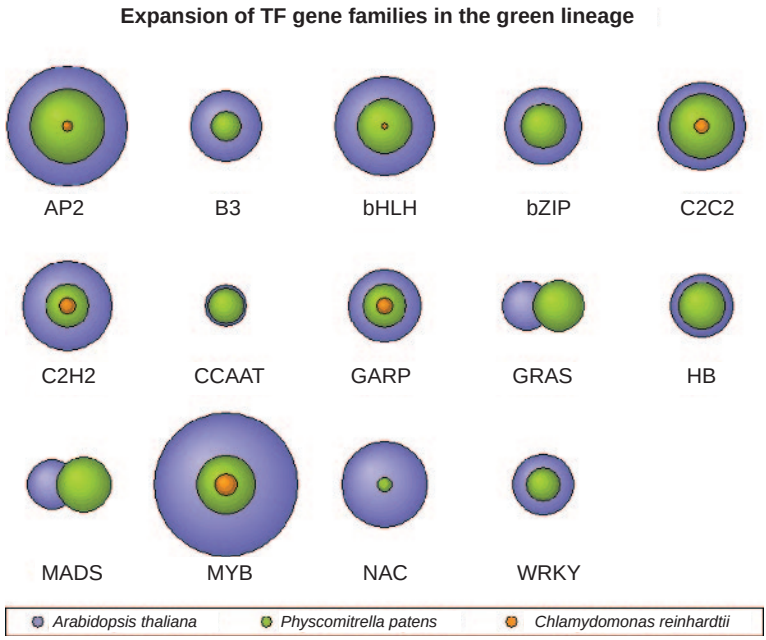


Plate 2 Comparison of transcription factor family sizes between *A. thaliana*, *P. patens* and *C. reinhardtii*. (*Source*: Courtesy of S. Richardt.)

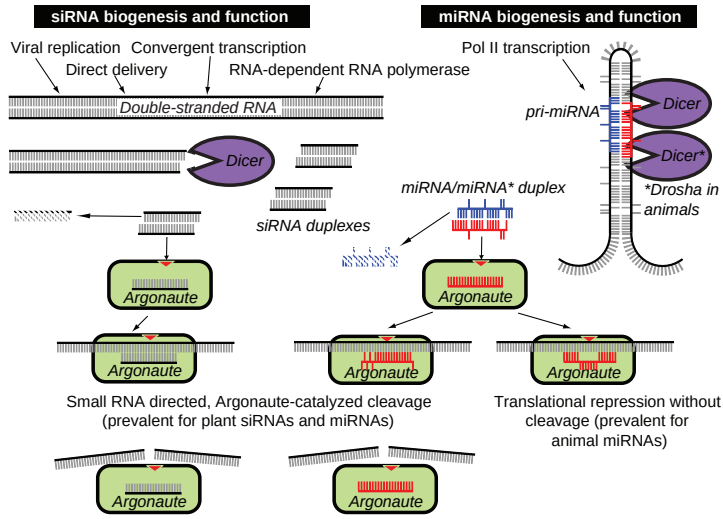


Plate 3 Biogenesis and functions of siRNAs (left) and miRNAs (right).

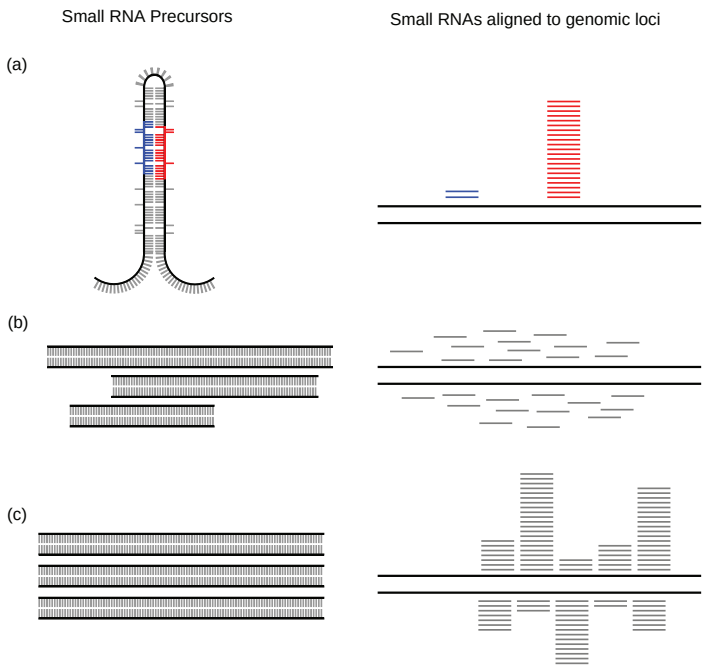


Plate 4 Classification of small RNA-producing loci by analysis of sequenced small RNAs. (a) miRNAs are precisely excised from a single-stranded stem-loop precursor as a miRNA/miRNA* duplex (red/blue). When small RNAs derived from miRNA loci are mapped to the genome, they correspond to two discrete positions of the same polarity with respect to the genome. (b) siRNAs are processed from a heterogeneous population of long double-stranded RNAs; thus, when they are mapped to the corresponding genomic loci, a chaotic pattern of small RNAs, mapping to both polarities, is observed. (c) ta-siRNAs are processed from a population of long double-stranded RNAs with uniform ends; thus, they correspond to their genomic loci in a regular, phased pattern from both polarities.

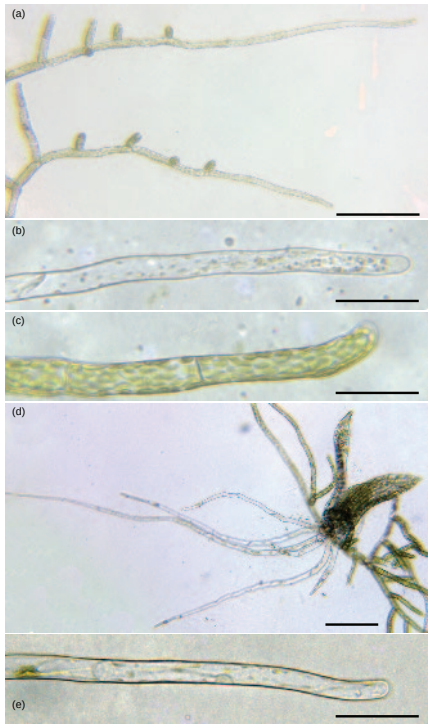


Plate 5 Tip-growing cells of *P. patens*. (a) Protonemal filaments formed of two cell types, chloronema and caulonema. (b) Caulonemal tip cell, (c) chloronemal tip cell, (d) early gametophore formed of leafy shoot and basal rhizoids, (e) rhizoid tip cell. Scale bar: (a) and (d) 200 μm. (b), (c) and (e) 50 μm.

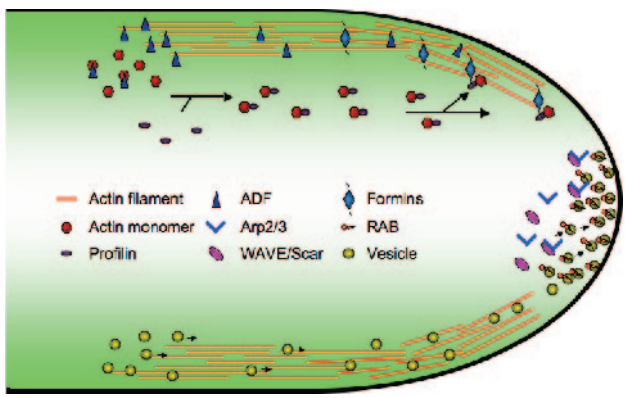


Plate 6 A model for the organization of the actin cytoskeleton at the tip of a growing cell. At the cell tip, the Arp2/3 complex and its activator, the WAVE/SCAR complex, are necessary for proper actin-mediated cell elongation and are both required for accumulation of RAB-associated vesicles. The apical F-actin organization is also necessary for cell elongation and is initiated by profilin and formin-mediated polymerization of actin monomers. ADF controls the integrity of the actin filament network by regulating actin turnover. ADF, actin depolymerizing factor; Arp2/3 complex, actin-related protein 2/3 complex; RAB, Ras-related in brain protein (small GTPase protein); WAVE/SCAR complex, Wiskott–Aldrich Verprolin homologous protein/SCAR complex.

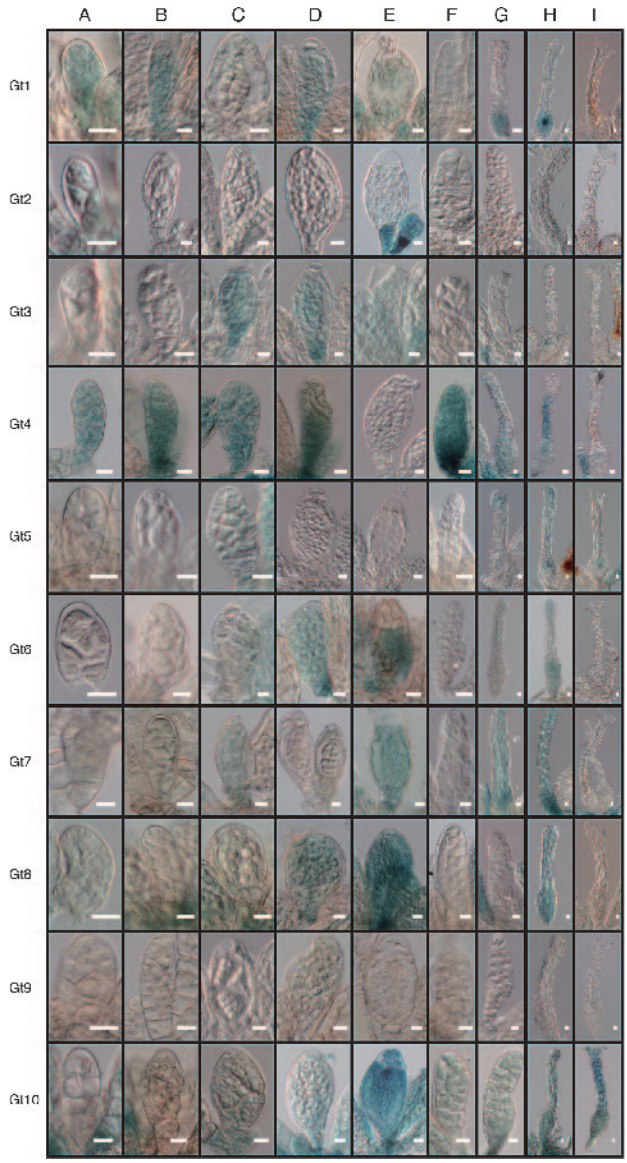


Plate 7 Detection of GUS expression in gametangia. Lines Gt1-Gt10. Each row corresponds to a line whose name is given on the left side. Columns (A)–(I) correspond to various developmental stages. (A) The primordium of a gametangium with an apical cell with two cutting faces. (B) The primordium of an antheridium just after periclinal cell division, showing outer jacket cells and inner primary spermatogonial cells. (C) An antheridium with dividing spermatogonial cells. (D) An antheridium at the stage between (C) and (E). (E) A mature antheridium with mature spermatozoids. (F) The primordium of an archegonium with an apical cell with three cutting faces. (G) An archegonium with an expanded ventral cell. (H) An archegonium containing a mature egg without an open neck. (I) An open archegonium around the time of fertilization.

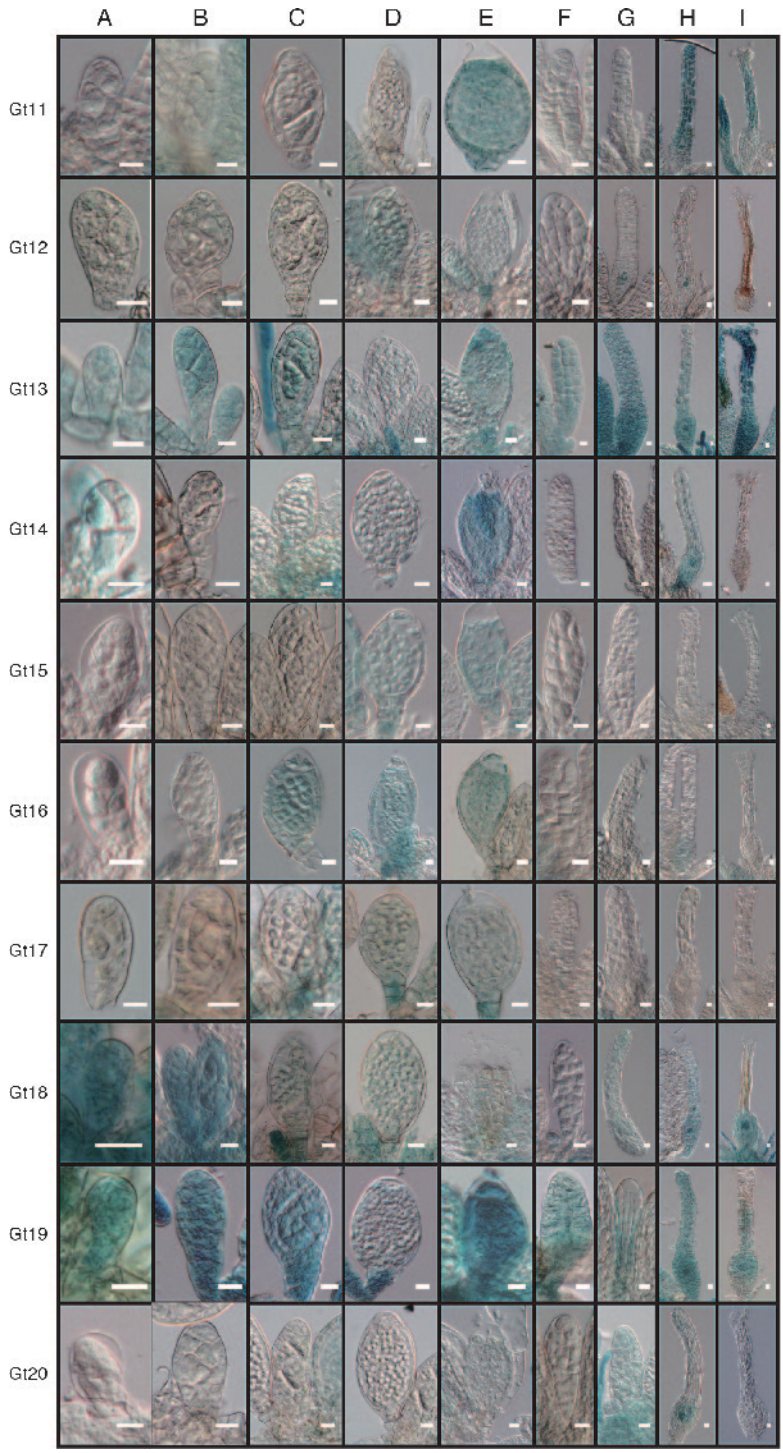


Plate 8 Detection of GUS expression in gametangia. Lines Gt11-Gt20. See Figure 7.3 for full legend.

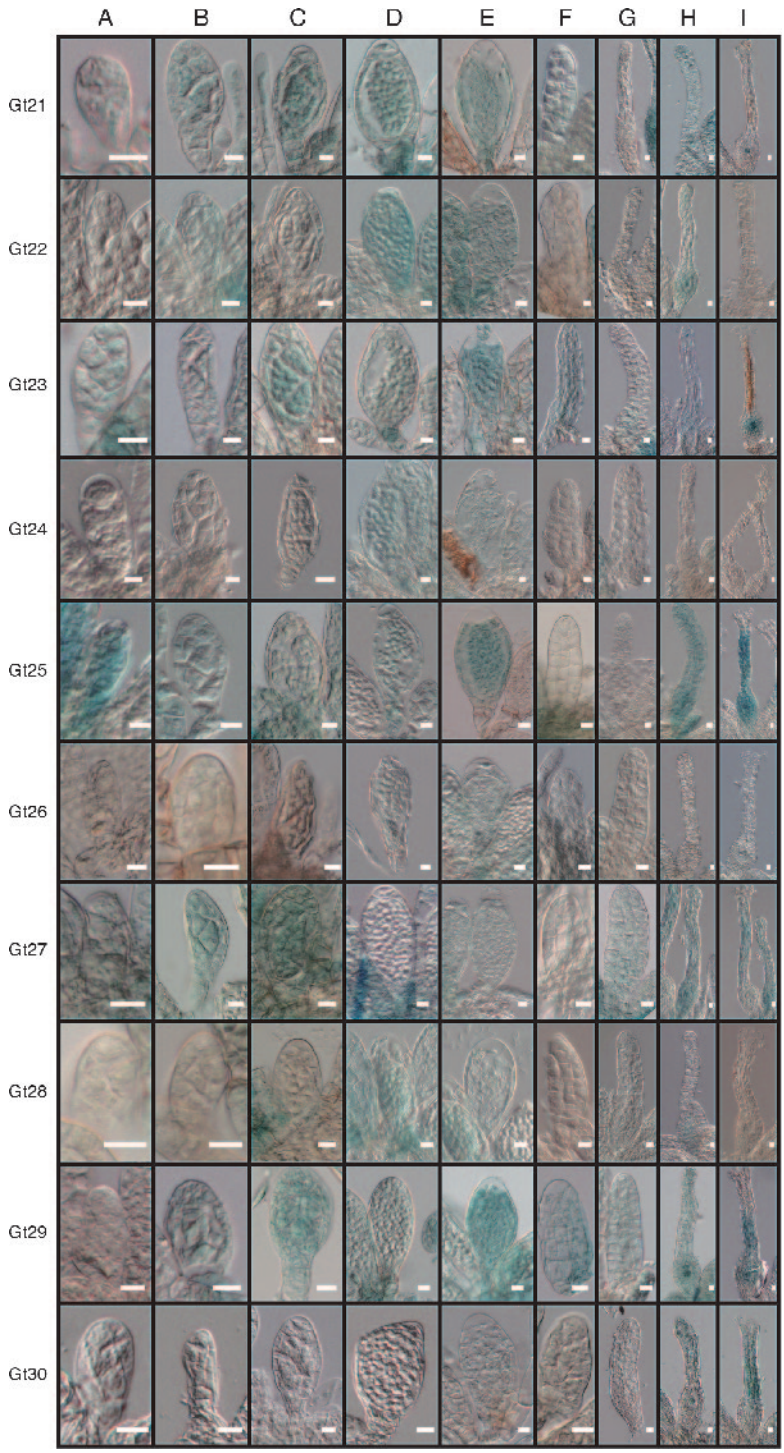


Plate 9 Detection of GUS expression in gametangia. Lines Gt21-Gt30. See Figure 7.3 for full legend.

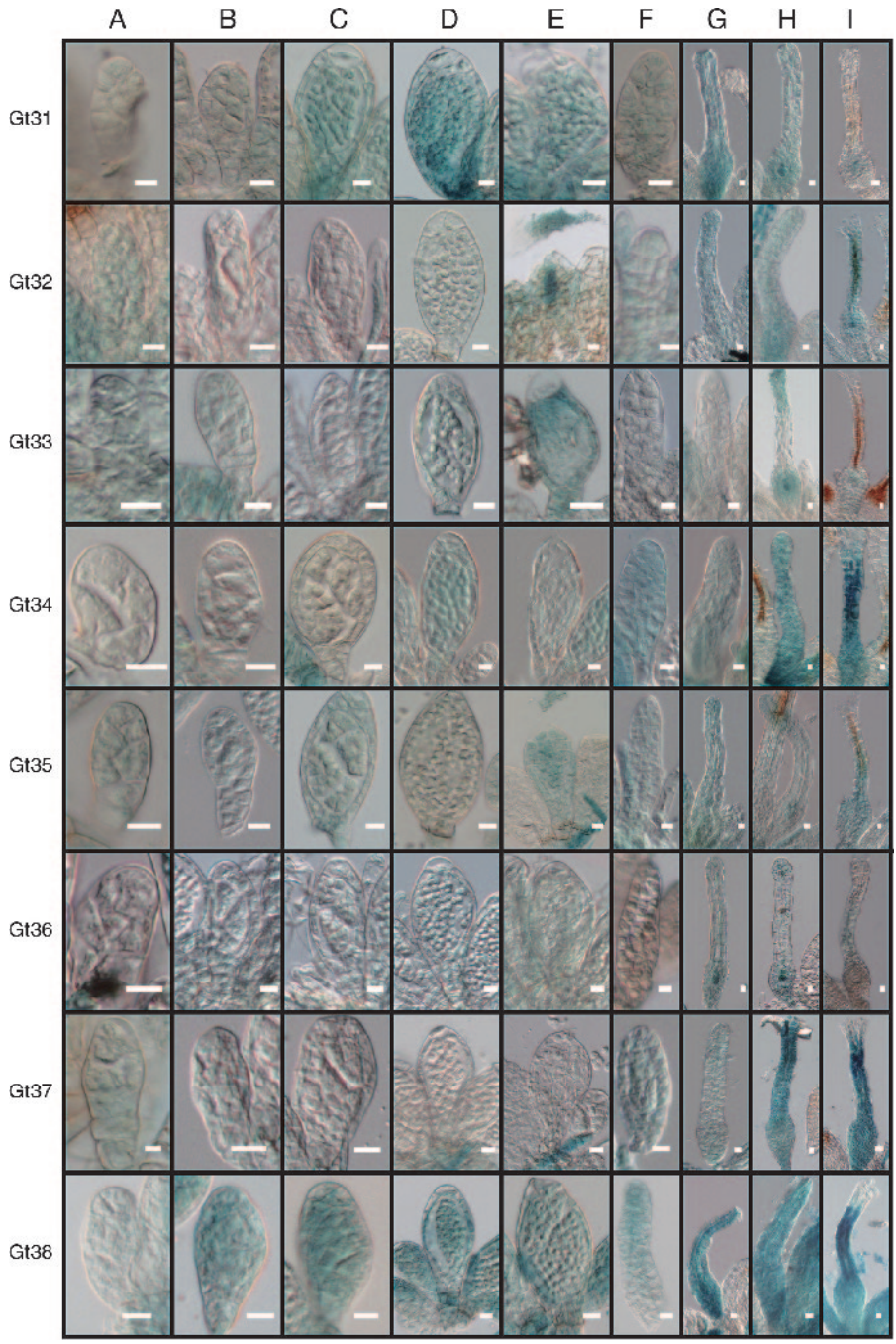
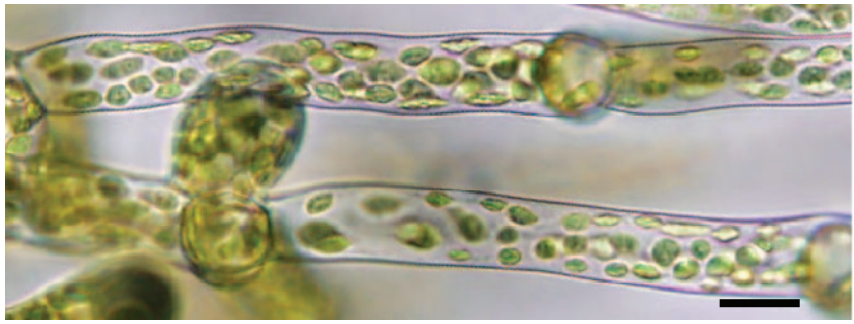
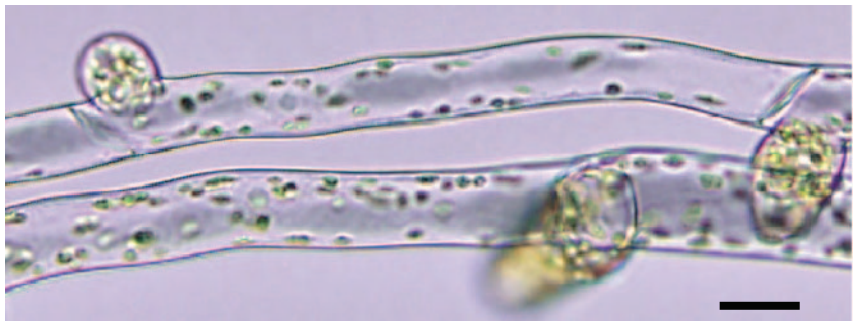


Plate 10 Detection of GUS expression in gametangia. Lines Gt31-Gt38. See Figure 7.3 for full legend.

(a) Chloronema cells



(b) Caulonema cells



(c) Leafy cells

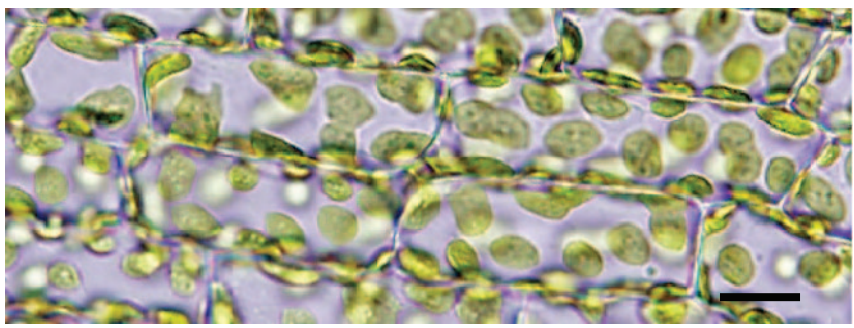


Plate 11 Light micrographs of chloroplasts in *P. patens* gametophyte cells. (a) Chloronemal cells. (b) Caulonemal cells. (c) Gametophore leaf cells. Bars = 20 μm (*Source*: Courtesy of Dr Mitsuru Hattori.)

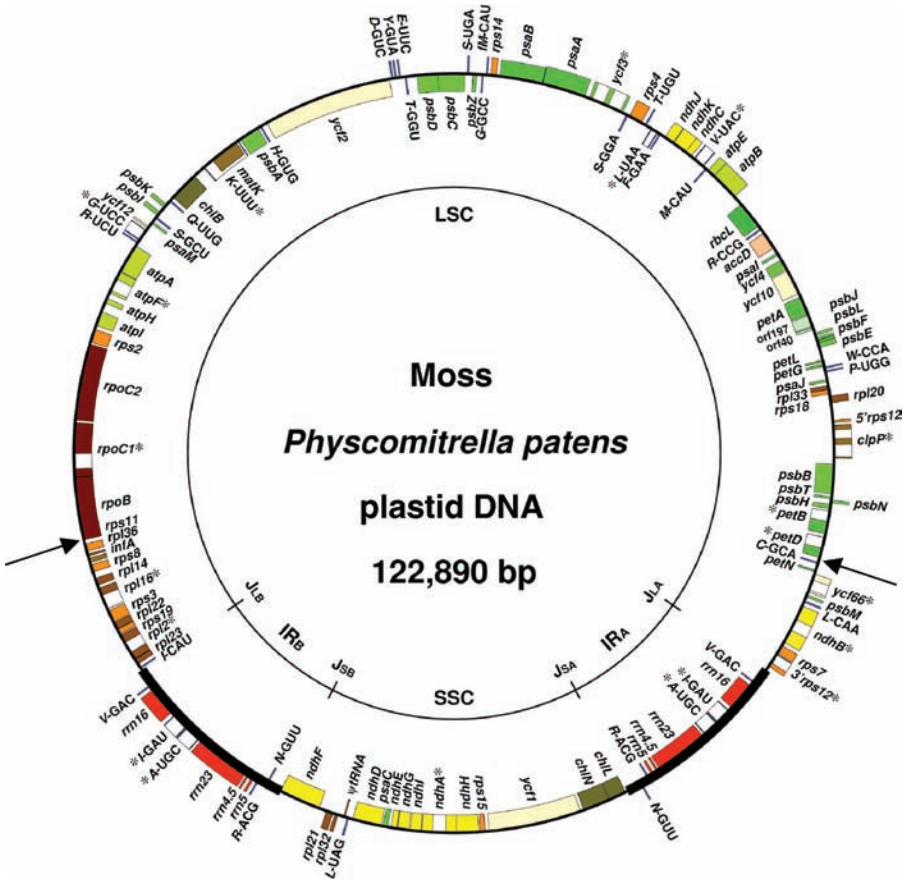


Plate 12 Gene map of the moss *P. patens* plastid genome. IR sequences (IR_A and IR_B) are separated by the SSC and LSC regions. Genes on the inside of the map are transcribed clockwise and genes on the outside are transcribed counter-clockwise. Genes with related functions are shown in the same color. Asterisks denote intron-containing genes or split genes. Arrows indicate positions of endpoint of a 71-kb large inversion relative to liverwort and hornwort plastid DNA. (*Source*: Reprinted and modified from Sugiura et al., 2003.)

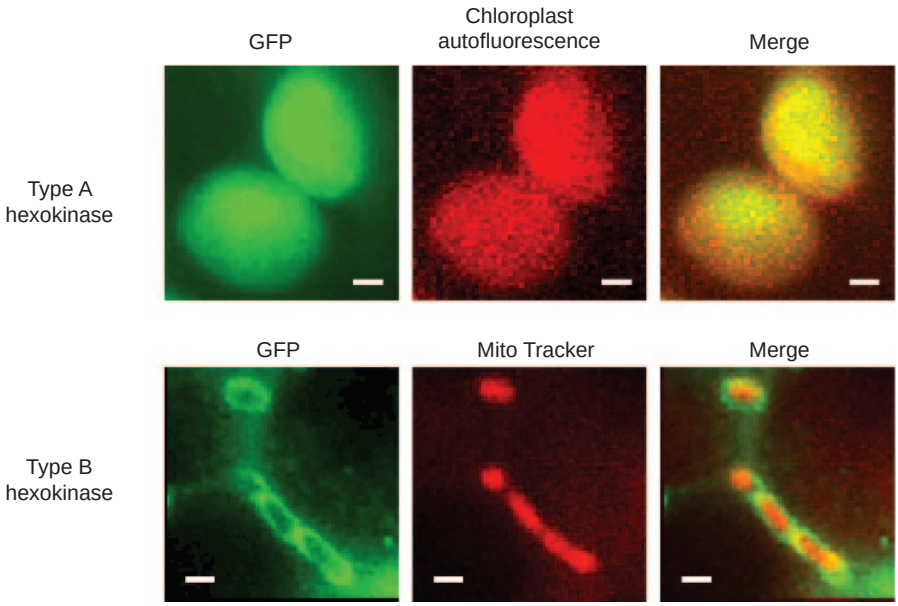


Plate 13 The intracellular localization of different hexokinases in *P. patens* visualized by transient expression of fusions of hexokinases to the green fluorescent protein (GFP). Type A hexokinases as shown in the upper panels have N-terminal chloroplast transit peptides, which target them to the chloroplast stroma as is evident from the GFP signal being localized inside the chloroplasts. The red signal shows chloroplast autofluorescence. The merge picture is an overlay of the signal from the hexokinase-GFP fusion protein and the chloroplast autofluorescence channel. Type B hexokinases as shown in the lower panels have N-terminal membrane anchors, which target them to organelle membranes. The GFP signal in this case shows that the hexokinase is localized to mitochondrial membranes. Mitochondria are visualized in red using the mitochondrion-specific dye MitoTracker Orange. The merge picture is an overlay of the signal from the hexokinase-GFP fusion protein and the MitoTracker Orange channel. The bars represent 1 μm.

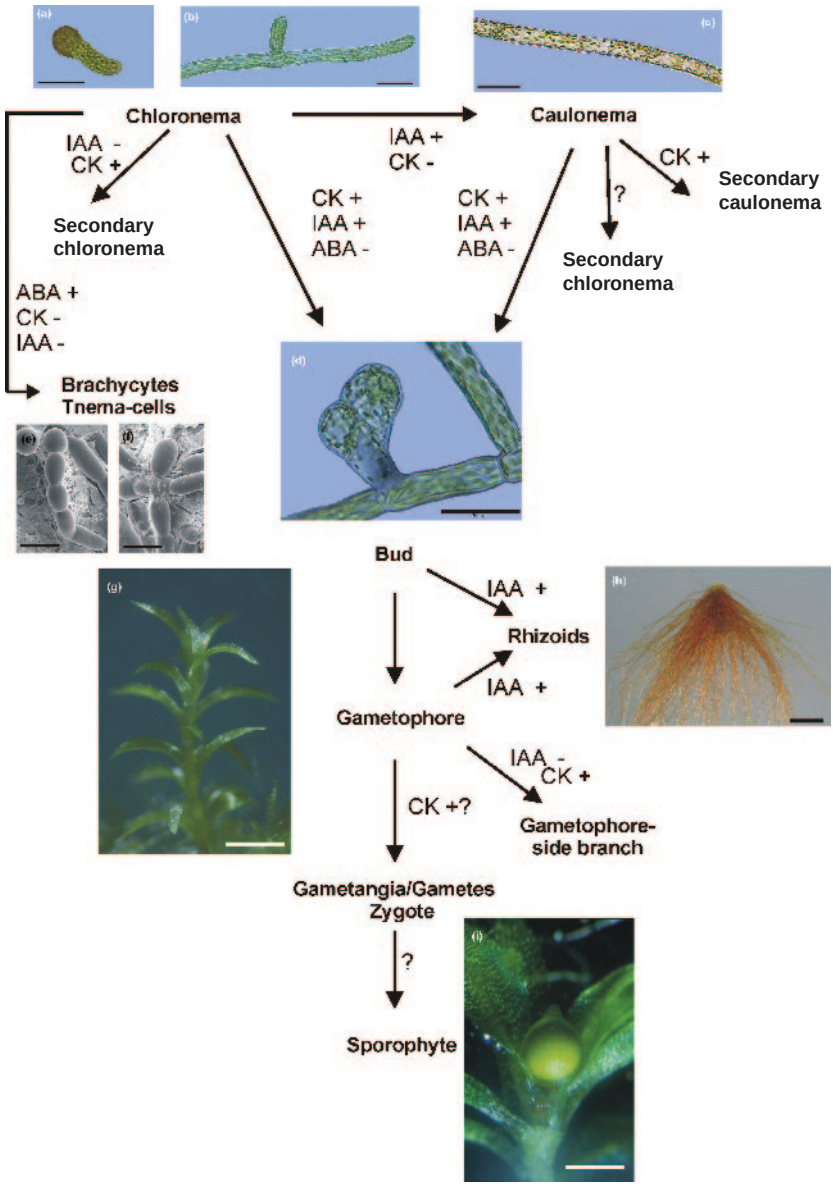


Plate 14 Simplified scheme summarising known morphogenetic actions of the plant hormones ABA, auxin (IAA) and cytokinins (CK) in mosses. '+' promoting effect, '−' inhibiting effect. For references, see Tables 10.1 and 10.2, and for ABA, see Table 11.1. Brachycytes (e) and tnema-cell (f) were visualised by scanning electron microscopy (*Source:* From Decker et al. (2006), printed with kind permission of University Freiburg). Rhizoids on gametophore (h) were induced by growth on 1 μM NAA during 6 weeks, picture was obtained by courtesy of K. Sakakibara (Monash Univ. Australia) and M. Hasebe (NIBB, Okazaki, Japan). Pictures (a)–(d), (g) and (i) were taken by S. Bringe, N. Wegner and K.v.S. (University Hamburg). Scale bars correspond to 50 μm in (a)–(f), to 350 μm in (g), (h) and to 150 μm in (i).

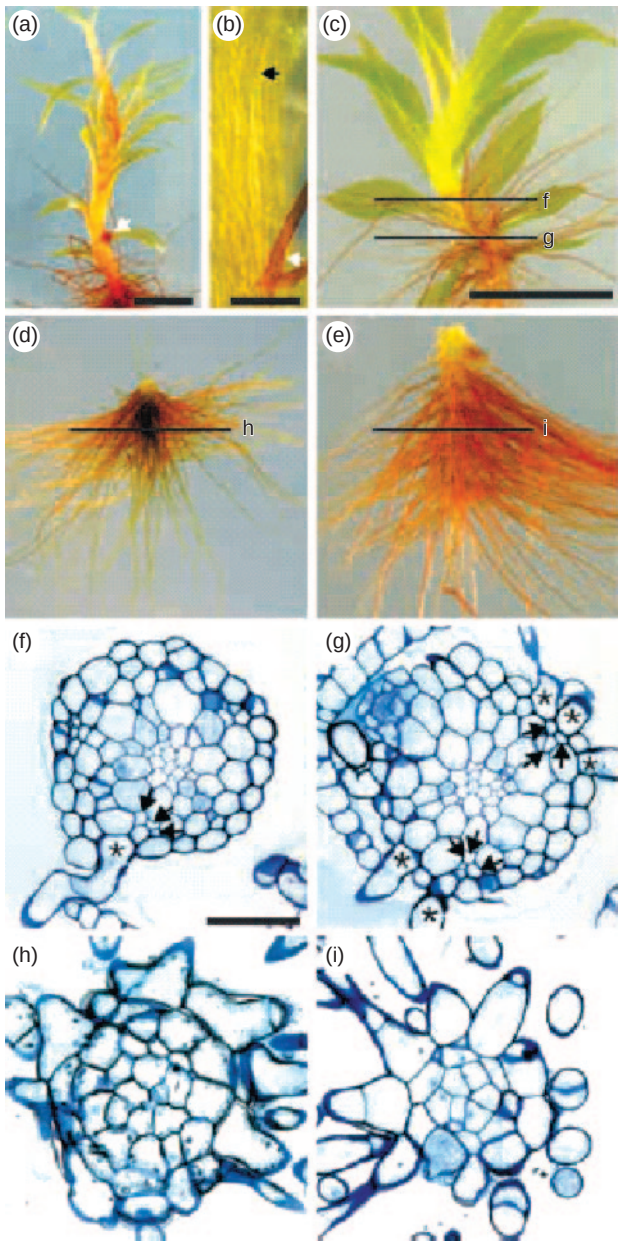


Plate 15 The effects of exogenous auxin on wild-type *P. patens*. (a) Gametophore cultured in 1 μM NAA for a week with adventitious gametophore (white arrow). (b) The uppermost mid-stem rhizoid of the gametophore (white arrow) in (a). The black arrow indicates a midrib. (c–e) Gametophores grown with 0.1 (c), 1.0 (d), and 10 (e) μM NAA for 6 weeks. The positions of the transverse sections in (f–i) are indicated by the lines. The asterisks and arrows in (f) and (g) indicate rhizoids and cells of the leaf traces, respectively. Scale bars: (a) 1 mm; (b) 100 μm; (c–e) 1 mm; (f–i) 100 μm. (*Source*: From Sakakibara et al. (2003), reproduced with permission of the Company of Biologists.)

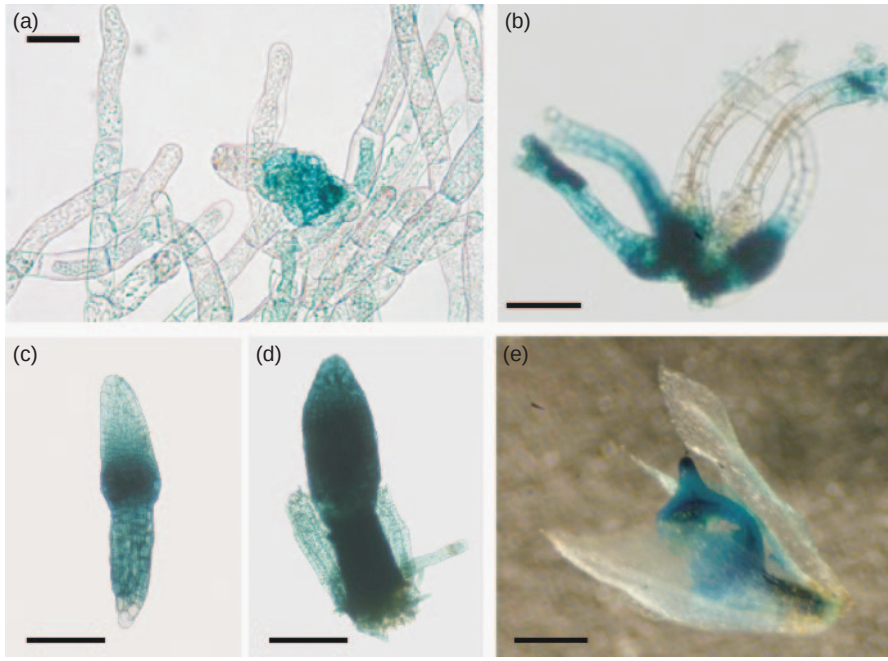


Plate 16 GUS expression in GH3:GUS transgenic *P. patens*. (a) GUS staining in developing bud, tissue was pre-incubated with 10 μM NAA prior to staining (bar represents 50 μm) (*Source*: From Bierfreund et al., (2003), reprinted with kind permission from Springer Science and Business Media). (b) GUS staining in basal part of archegonia (bar represents 50 μm). (c–e) GUS staining during sporophyte development (bar represent 50 μm in (a), (c) and (d), 20 μm in (b), and 125 μm in (e)). Experiments for (b)–(e) were carried out without NAA pre-incubation. (*Source*: Pictures (b)–(e) were obtained by the courtesy of E. Decker and R. Reski (University of Freiburg).)

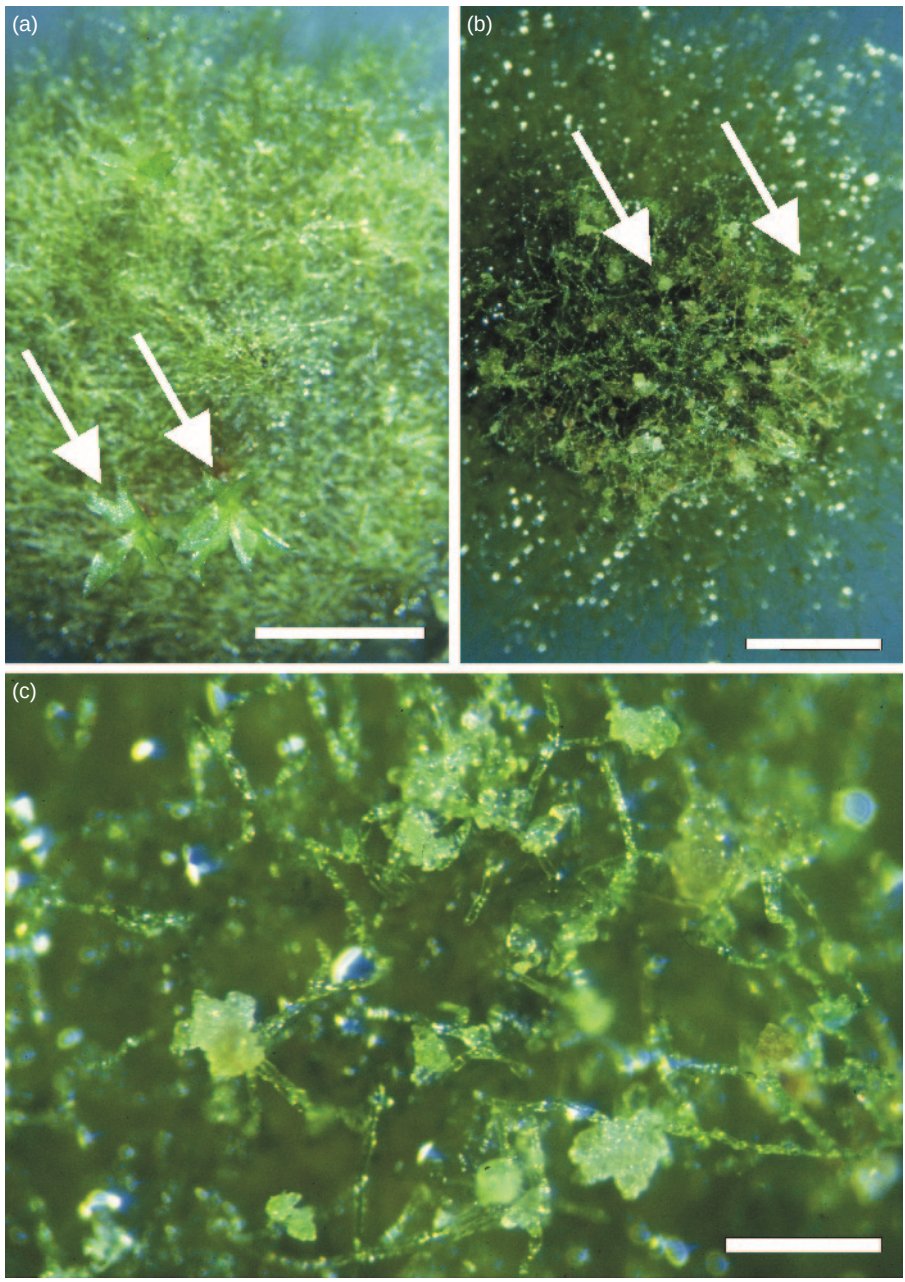


Plate 17 (a) Wild-type culture showing only a few differentiated gametophores (arrows); (b) *ove*B300 mutant exhibiting a high number of buds forming callus-like structures (arrows); (c) same as B at higher magnification. Bar corresponds to 1 mm in (a) and (b) and 0.5 mm in (c).

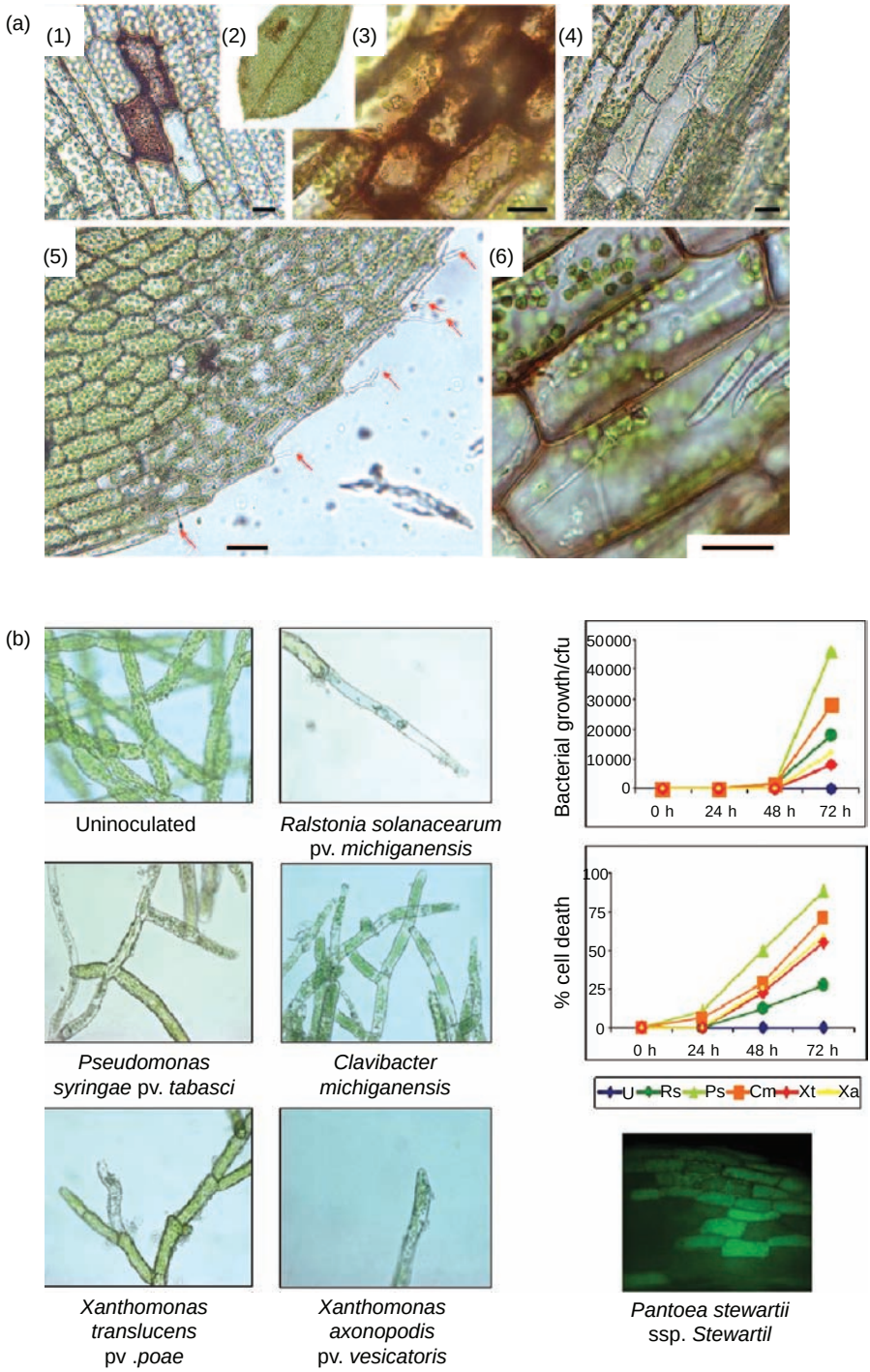


Plate 18 (Continues overleaf)

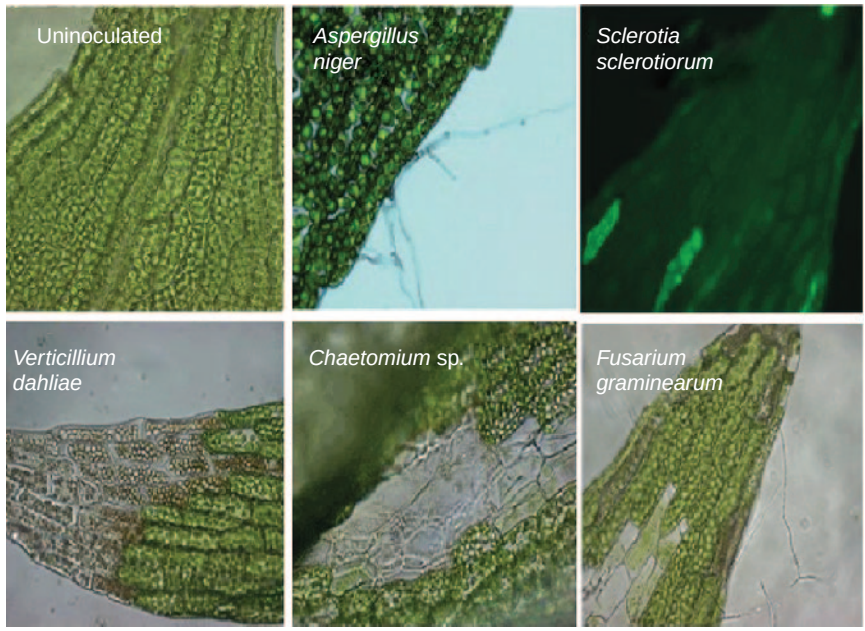


Plate 18 (a) Examples of disease-like symptoms displayed by gametophytic tissues of *P. patens* plants collected directly from the field. (1) A sharp-bordered, pigmented lesion reminiscent of a typical HR. Cytoplasm in the pigmented cells is highly granular and there are no discernable chloroplasts. One cell is completely empty, possibly the result of absorption of cell contents by a pathogen, or the result of cellular autophagy. (2) A diffuse, pigmented lesion on a leaf. The lack of a sharp lesion boundary is consistent with a spreading lesion. (3) Higher magnification of the lesion shown in (2). Cells contain the degraded products of chloroplasts together with crystalline structures. Movement within these cells during a series of time-lapse photographs is consistent with the presence of bacteria. (4) Leaf tissue colonized by an unidentified fungus. Hyphal strands, clearly visible in cells that have lost their entire contents, appear to pass from cell to cell. Cells at various stages of breakdown can be seen in this view. (5) A portion of a leaf showing extensive ramification of fungal hyphae. The progressive wave of cellular breakdown in response to the fungus is apparent. Red arrows indicate individual hyphal tips, which appear to emerge from the ends of each file of plant cells. (6) Reinoculation of an axenically cultured *Fusarium* spp. isolate on *P. patens*. Note the extensive ramification of hyphae within individual cells together with the presence of canoe-shaped, septated conidia. Scale bars represent 20 μm, except for E, which represents 50 μm. (b) Inoculation of *P. patens* with known bacterial pathogens of seed plants. Bacteria were inoculated onto *P. patens* protonemata. The pictures show typical disease symptoms at 24 and 48 h post inoculation. The graphs on the right show the growth of bacteria *in planta*, determined by bacterial plating assays (cfu = colony forming unit) and the extent of *P. patens* cell death measured by Evans blue staining. U: uninoculated; Rs: *Ralstonia solanacearum* pv. *michiganensis*; Ps: *Pseudomonas syringae* pv. *tabasci*; Cm: *Clavibacter michiganensis*; Xt: *Xanthomonas translucens* pv. *poae*; Xa: *Xanthomonas axonopodis* pv. *vesicatoria*. There is an excellent correlation between cell death and bacterial growth, and pathogens that are more effective in killing the host also grow more rapidly. The panel on the lower right-hand side shows the internalization of GFP-labeled *Pantoea stewartii* ssp. *stewartii* in *P. patens* leaves viewed by epifluorescence. (c) Inoculation of *P. patens* with fungal pathogens of seed plants and mosses. *P. patens* gametophore tissues were inoculated with *Aspergillus niger* (which causes black mold on fruits and vegetables), GFP-labeled *Sclerotia sclerotiorum* (which causes leaf drop on lettuce), *Verticillium dahliae* (which causes wilt on a large number of plants), *Fusarium graminearum* (which causes head scab on wheat and barley) and an unknown species of *Chaetomium* cultured from infected moss plants by the authors. Samples were illuminated under bright-field conditions with the exception of GFP-labeled *S. sclerotiorum*, which was viewed by epifluorescence. Note the difference in the morphology of dying cells in *V. dahlaie*-infected plants compared to dying cells in plants infected with *F. graminearum* and *Chaetomium*.

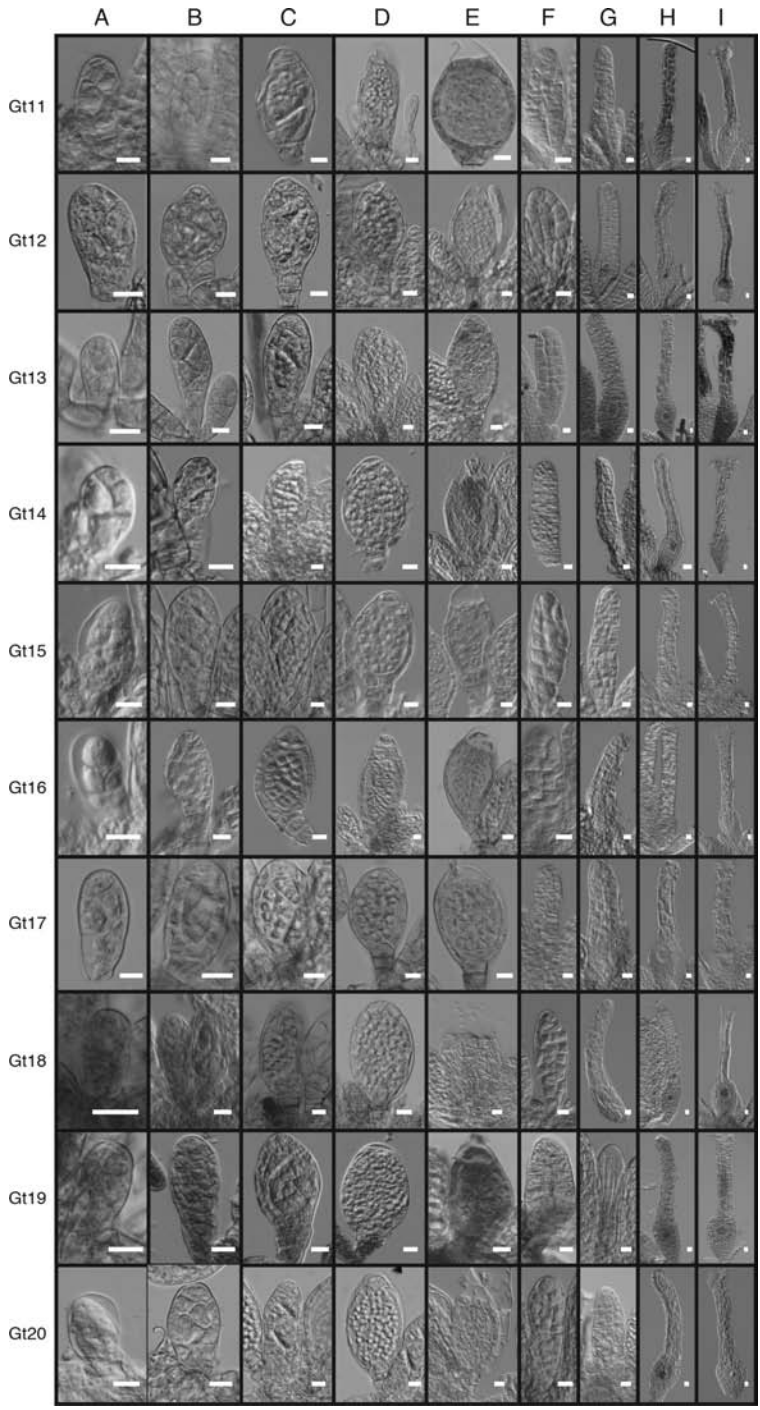


Figure 7.4 Detection of GUS expression in gametangia. Lines Gt11-Gt20. See Figure 7.3 for legend and Plate 8 for color version.

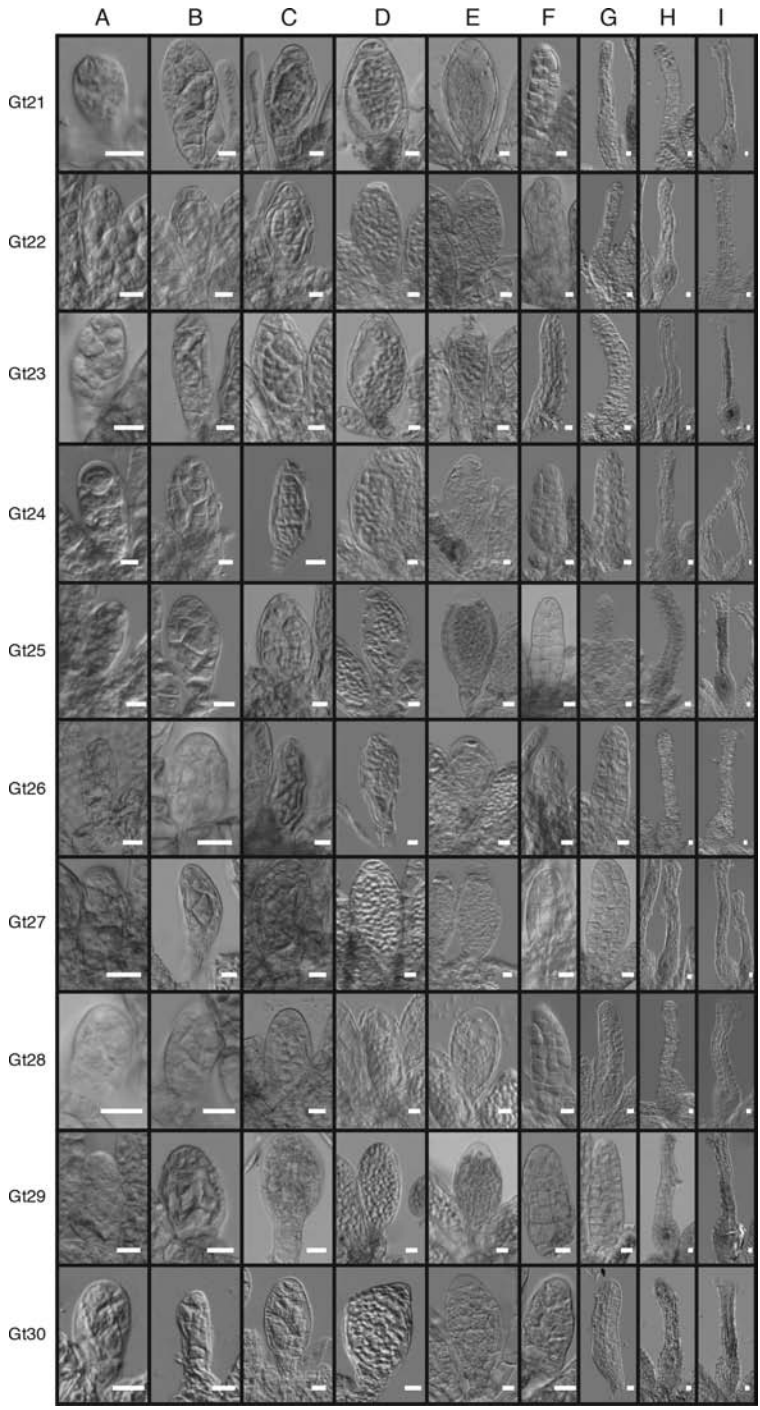


Figure 7.5 Detection of GUS expression in gametangia. Lines Gt21-Gt30. See Figure 7.3 for legend and Plate 9 for color version.

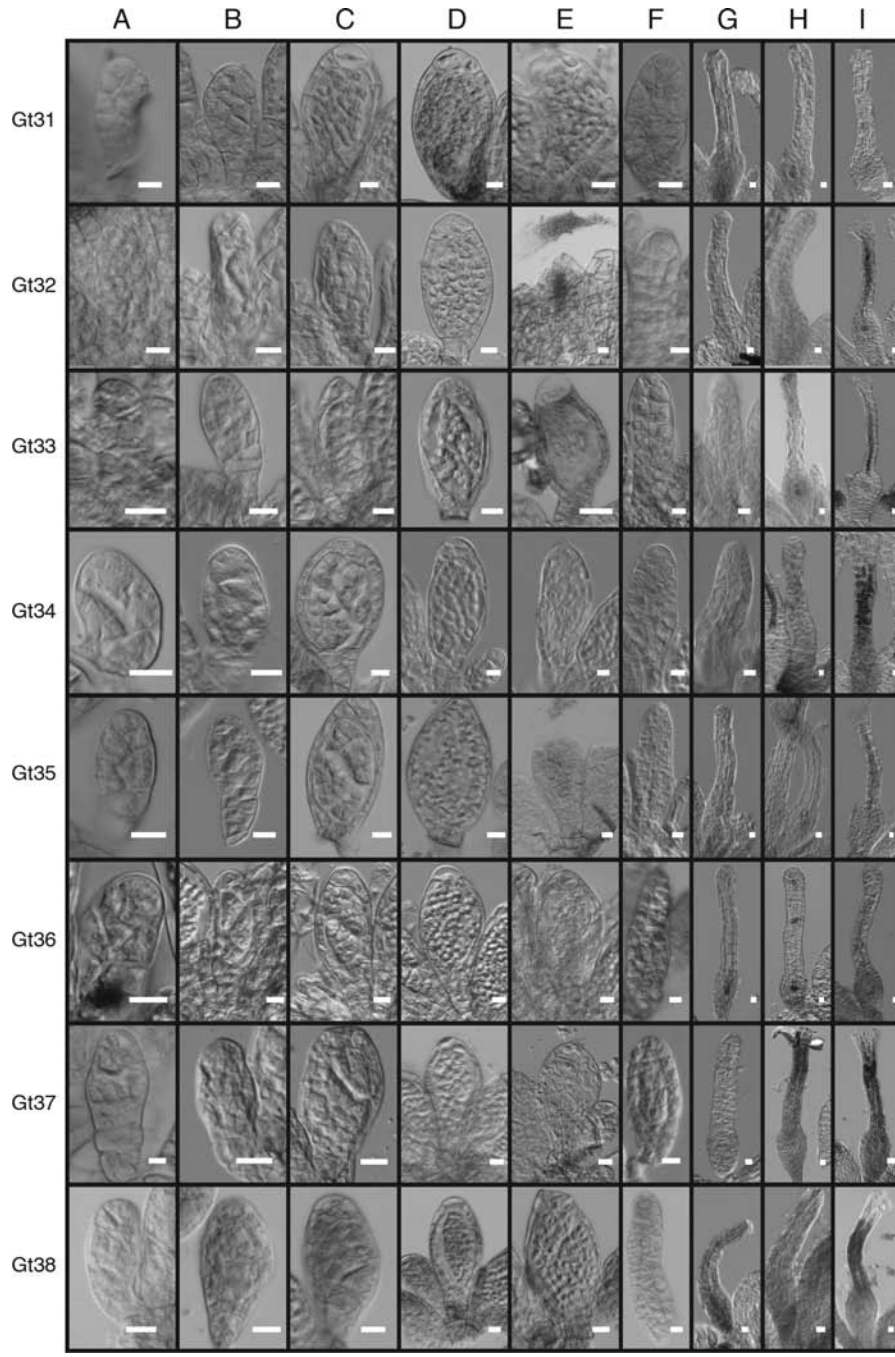


Figure 7.6 Detection of GUS expression in gametangia. Lines Gt31-Gt38. See Figure 7.3 for legend and Plate 10 for color version.

5. Re-introduce the isolated tagged DNA fragment into a wild-type background by gene targeting.
6. Compare the GUS expression patterns of the transformants with those of the original trap line.

For efficient identification of the trapped gene, it is ideal to use RNA extracted from gametangia-rich tissues in the gene-trap line. Several kinds of cDNA are usually found by 5′-RACE because of multiple insertions of the HI-GT construct. The putative coding sequence of the cDNA fused in-frame to one *uid*A should be selected. The release of the genomic sequence of *P. patens* has made this process much easier (Rensing et al., 2008). Information on the genomic sequence surrounding the candidate cDNA is easily obtained using the genome browser at the Joint Genome Institute (JGI *Physcomitrella patens* ssp. *patens* v1.1, `http://genome.jgi-psf.org/Phypa1_1/Phypa1_1.home.html`). The information obtained is then used to assess whether the candidate gene corresponds to the trapped gene by comparing the GUS expression pattern of a knock-in line with that of the original trap line; in the knock-in line, the coding sequence of *uidA* is inserted in-frame just before the stop codon of the candidate gene by gene targeting.

In addition to the isolation of each gene expressed during gametangia development using trap lines, expression profiling will provide a genome-wide view of development, which is indispensable for understanding the genetic network. We have generated expression profiles for 14 842 5′- and 15 076 3′-full-length cDNA ESTs and 211 588 5′-end SAGE (serial analysis of gene expression) (Velculescu et al., 1995) tags using RNA extracted from gametophore tips with gametangia. Other tissues for which EST and SAGE data exist include protonemata, gametophores and sporophytes. Comparative analyses will be useful for selecting additional candidate genes expressed in the gametangia.

The isolation of gametangia-specific RNA from *P. patens* is time consuming because of the small size of the organism, and microarray analyses have not been possible using the standard amount of total RNA (micrograms). It is, however, possible to use amplified mRNA for microarray experiments. Several linear amplification methods for mRNA using in vitro transcription from nanograms of sample have been reported (Wang et al., 2000; Zhao et al., 2002; Schneider et al., 2004). The linear amplification of mRNA maintains the relative amount of existing mRNA in a cell or tissue. Recent advances in the amplification of mRNA from a single cell have also accelerated microarray-based profiling when limited sample is available (Kurimoto et al., 2007). Although tag-based transcriptome analyses, including SAGE, CAGE (Shiraki et al., 2003) and PETs (Ng et al., 2005), have been developed for transcriptome identification, a useful tag number is limited for quantitative profiling (Harbers and Carninci, 2005). The development of high-throughput parallel sequencers will overcome this situation. The ability to sequence more than 3 Gb per run will make it possible to profile quantitatively the number of mRNAs (Ng et al., 2006; Hutchison, 2007). Higher yields of extracted tag

sequences also increase the dynamic range of gene expression. For example, we have developed a protocol for high-throughput mRNA 3′-end sequencing using a 454 sequencing platform (Margulies et al., 2005), and large-scale sequencing has since been conducted using libraries derived from five moss tissue samples, including gametangia. Using this procedure, we obtained a total of 1.9 million tags. In addition, these data may be used to validate the gene models in the genome browser. New transcriptional units are identified with 298 042 tags, indicating that high-throughput sequencing technology can be used to discover novel expressed genes.

The next generation of sequencing technology will provide even more powerful tools for dissecting gene networks. Transcriptional gene networks are used to detect the direct targets of transcription factors. The DNA-binding sites of several transcription factors have been efficiently identified by ChIP-chip (Wu et al., 2006). The genome-tiling array for ChIP-chip is expensive, and large amounts of sample are necessary. Recently, a new sequencing method, called ChIP-seq, was used for the genome-wide identification of transcription factor targets (Johnson et al., 2007). Combined with the conditional activation of a transcription factor (Padidam, 2003; Saidi et al., 2005), ChIP-seq will allow the efficient identification of the immediate targets of transcription factors.

Methods based on gene expression, for example those that rely on gene-trap lines and the next generation of sequencing techniques, are useful for investigating genetic networks. These methods can be used to dissect the process of gametangia development based on gene expression. At the same time, forward genetic approaches can also be used to isolate the genes involved in gametangia development based on mutant phenotypes. However, defects in gametangia development sometimes disrupt the life cycle of the organism. In such cases, it is not possible to use map-based cloning because of the sexual sterility of mutants. Therefore, a system that can isolate a gene directly is needed for the application of a forward genetic approach to the study of gametangia development. Tagged mutants were previously generated by shuttle mutagenesis in *P. patens* (Nishiyama et al., 2000), but most of the mutants contained several tags, which made it difficult to isolate the gene responsible for the mutant phenotype. A method for detecting deletions using a genome-tiling array is better suited to identifying the affected genes in deletion mutants. We created a genome-tiling array for *P. patens* that covers the entire *P. patens* genome (approximately 480 Mb) at a 67-bp resolution, excluding repeated sequences. We are generating deletion mutant lines and identifying the deleted regions by hybridization of the genome-tiling array with genomic DNA. *Agrobacterium tumefaciens*-mediated transformation is a common forward genetic approach in angiosperms; however, this method can also be used to identify a responsible gene using the T-DNA as a tag. This approach is now being established in *P. patens*. Both reverse and forward genetic approaches, including the methods described in this review, will provide a new insight into gametangia development.

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Chapter 8

CHLOROPLASTS

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Abstract: In *Physcomitrella patens*, chloronemal and leaf cells contain numerous large round chloroplasts while caulonemal cells contain fewer and smaller spindle-shaped chloroplasts. Unlike seed plants, plastid differentiation is not usually observed in *P. patens* and the gametophytes always contain chloroplasts that develop even in the dark. The *P. patens* plastid genome (122 890 bp) possesses at least 118 genes. The most interesting feature is that the *rpoA* gene encoding the α subunit of the plastid-encoded plastid RNA polymerase (PEP) is absent from the plastid genome of *P. patens*. Instead, two nuclear counterparts encode the α subunits of PEP enzyme and modulate transcription of the plastid genes. Whether *P. patens* chloroplasts contain a second RNA polymerase, the nuclear-encoded plastid RNA polymerase (NEP), continues to be debated. Here, we provide biochemical evidence supporting the existence of NEP activity in *P. patens*. Plastid transformation, which has been achieved in *P. patens*, allows further investigation of the regulation of plastid gene expression and over-production of foreign polypeptides in the moss chloroplasts. Molecular techniques with the complete genome informatics open a way to study the molecular basis of plastid division and movement, chloroplast import, or circadian rhythm in *P. patens*.

Keywords: chloroplast movement; circadian rhythm; plastid division; plastid DNA; plastid transformation; *rpoA*

Abbreviations:

BLRP	blue-light responsive promoter
GFP	green fluorescent protein
IR	inverted repeat
LSC	large single copy
NEP	nuclear-encoded plastid RNA polymerase
PEP	plastid-encoded plastid RNA polymerase
PPR	pentatricopeptide repeat
RpoT	bacteriophage-type RNA polymerase
SSC	small single copy

8.1 Chloroplasts of *Physcomitrella patens*

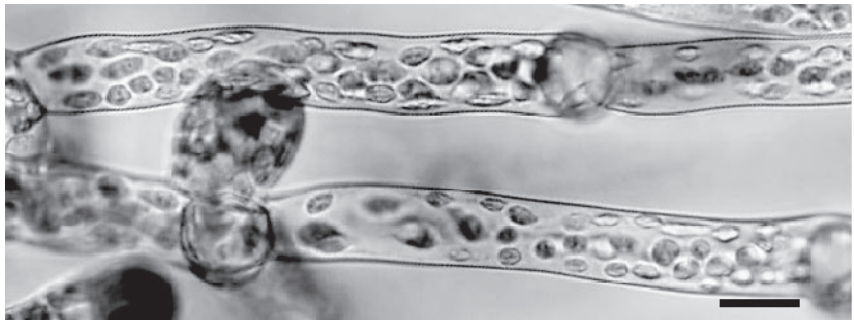
Plastids are DNA-containing organelles found only in algal and plant cells, and are generally accepted to have arisen from a cyanobacteria-like endosymbiont. They are responsible for photosynthesis and for the biosynthesis of key molecules required for plant cellular functions (Hoober, 1984). Plastids vary in size, shape and function. In seed plants, the cells in the young meristematic regions contain proplastids, the precursors of other plastids. In leaves, plastids exist as the chloroplasts that carry out photosynthesis. In non-green cells or tissues, plastids are differentiated to specialized forms, for example leucoplasts (oil-storing plastids), amyloplasts (starch-storing plastids in roots and tubers), chromoplasts (yellow, red or orange plastids in the fruits or flowers) or etioplasts (plastids in the dark-grown seedlings). These plastids are characterized for different tissues and exhibit marked structural and functional differences. Unlike seed plants, plastid differentiation is not usually observed in the moss *Physcomitrella patens*. The moss gametophytes always contain chloroplasts that develop even in the dark and plastid ontogeny of bryophytes is distinctly different from that in vascular plants (Duckett and Renzaglia, 1988).

Mature chloroplasts in seed plants, generally, are regular in shape, occurring as biconcave or lens-shaped structures 1–3 μm wide and 5–7 μm long. Seed plant cells typically contain between 50 and 200 plastids per cell (Hoober, 1984). In *P. patens*, chloronemal and leaf cells contain numerous large round chloroplasts and caulonemal cells contain fewer and smaller spindle-shaped plastids (Reski, 1998; see Figure 8.1, Plate 11). However, the number of chloroplasts is slightly different among the cells of chloronemata, caulonemata and leaves (Table 8.1). The number and size of chloroplasts tend to vary even in the same cell types, due to different growth conditions (Tables 8.1 and 8.2), for example light, temperature or growth medium with or without glucose (Olsson et al., 2003) or phytohormones (Sakakibara et al., 2003). For instance, Nagao et al. (2005) described the presence of nearly round or slightly ovoid-shaped chloroplasts (length 5.9 μm; width 4.4 μm) with large starch grains in chloronemal cells. On the other hand, cells treated with 10 μM abscisic acid (ABA) for 1 day had rather slender, spindle-shaped chloroplasts (length 4.9; width 2.0 μm) with a reduced amount of starch.

Mosses are known to have a low photosynthetic capacity, which represents CO_2 uptake, up to 3 $\mu M/m^2/s$, in comparison with higher CO_2 uptake (10–60 $\mu M/m^2/s$) of seed plant leaves (Valanne, 1984; Larcher, 2003). Chlorophyll *a*/*b* ratios of bryophytes are around 2.7, which is lower than the 3.8 reported in pumpkin (*Cucurbita pepo*) and rye (*Secale cereale*) (Pursiheimo et al., 1998). A recent study showed that the photosynthetic fluorescence activity of the two mosses, *P. patens* and *Ceratodon purpurea*, is more similar to that of vascular plants than cyanobacteria or green algae (Thornton et al., 2005).

In contrast to the numerous studies carried out in seed plants, knowledge of the structure and expression of plastid genes and plastid biogenesis in

(a) Chloronema cells



(b) Caulonema cells

(c) Leafy cells

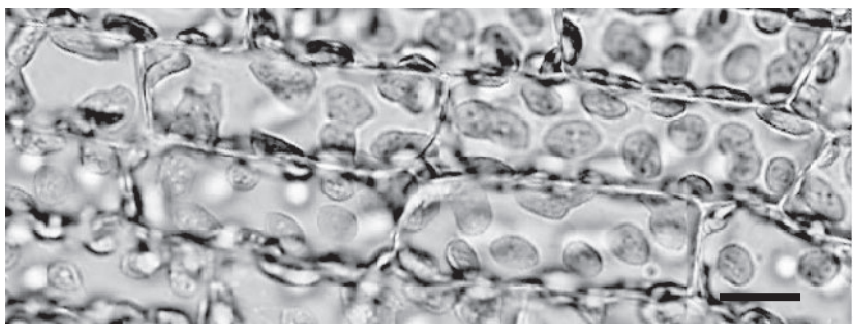


Figure 8.1 Light micrographs of chloroplasts in *P. patens* gametophyte cells. (a) Chloronemal cells. (b) Caulonemal cells. (c) Gametophore leaf cells. Bars = 20 μm (*Source*: Courtesy of Dr Mitsuru Hattori.) (For a color version of this figure, see Plate 11)

P. patens has been limited (Reski, 1994, 1999). During the last decade, however, studies on moss plastids have made rapid progress by recently established technologies that have generated a wealth of information on the genomes of the nucleus and organelles (Cove, 2005; Cove et al., 2006; Quatrano et al., 2007). This chapter summarizes the current state of knowledge of the structure of the plastid genome, transcription machinery in the moss plastids,

Table 8.1 Numbers of chloroplasts per cell in different gametophyte cells of *P. patens*

Chloronema	Caulonema	Leaf	Rhizoid	References
50				Reski, 1998
			30[a]	Sakakibara et al., 2003
41.4 ± 7.0[b]		37.5 ± 6.0[b]	94.2 ± 20.0	Y. Kobayashi, 2004, unpublished observations
47.7 ± 6.0[c]		46.2 ± 4.6[c]		Hayashida et al., 2005
35.1 ± 1.7[d]	37.2 ± 3.2[d]	37.5 ± 1.8[e]		Hattori et al., 2007

[a]Chloroplasts in the second to fourth rhizoid cells from the rhizoid apical cells.
[b]Chloroplasts in chloronemal and caulonemal cells of 5-day-old colonies.
[c]Chloroplasts in the sub-apical chloronemal cells ($n = 100$) and in leaf cells ($n = 100$) from five individual leaves.
[d]Chloroplasts in chloronemal and caulonemal cells ($n = 20$) of the 4-day-old plants.
[e]Chloroplasts in leaf cells ($n = 20$) of 30-day-old gametophores.

genetics and biochemical studies on plastid import, plastid division and movement, and attempts to highlight the differences and similarities between bryophytes, including *P. patens*, and angiosperms.

8.2 Plastid DNA

8.2.1 Overall plastid DNA structure

Plastids are thought to have evolved from an endosymbiotic cyanobacteria-like ancestor, and possess an independent genome distinct from the nuclear genome (Martin and Hermann, 1998; Martin et al., 2002). During evolution,

Table 8.2 Sizes of chloroplasts in different gametophyte cell types of *P. patens*

Cell	Chloroplast length (μm)	Chloroplast width (μm)	References
Chloronema[a]	5.9 ± 1.0	4.4 ± 0.69	Nagao et al., 2005
Chloronema[b]	6.03 ± 0.27	1.51 ± 0.08	Hofmann and Theg, 2005a
Chloronema[c]	6.13 ± 0.69	4.24 ± 0.94	M. Hattori, 2007, unpublished observations
Caulonema[c]	3.22 ± 0.44	1.58 ± 0.26	M. Hattori, 2007, unpublished observations
Leaf[d]	11.4 ± 1.27	8.06 ± 0.72	M. Hattori, 2007, unpublished observations

[a]Protonemata were grown on BCD medium at 23°C under continuous illumination (light intensity 50–80 μE/m^2/s) for 7–10 days.
[b]Protonemata were grown in constant light at 22°C on Knop's medium.
[c]Protonemata from 4-day-old colonies were grown on BCD medium at 25°C under continuous illumination (light intensity 65–80 μE/m^2/s). Sizes of 30 chloroplasts were measured.
[d]Gametophores developed on protonemal colonies 14 days after transfer from BCD medium to BCDAT medium.

most endosymbiotic genes were transferred to the nuclear genome, while a reduced genome was retained in the plastid. Plastid DNAs exist as plastid nuclei (or nucleoids) that are associated with various DNA-binding proteins (Sato, 2001; Kabeya et al., 2004). Plastid DNAs of land plants including bryophytes are relatively uniform in size, from 120 to 160 kb, and their gene content and organization are well conserved. To date, the complete plastid DNA sequences of over 140 species from the major lineages of algae and land plants have been determined (NCBI web site: `http://www.ncbi.nlm.nih.gov/genomes/static/euk_o.html`).

In mosses, Calie and Hughes (1987) constructed the first map of the *P. patens* plastid DNA, using the restriction endonuclease enzymes *Bgl*II, *Cla*I and *Sac*I. Thereafter, the complete plastid DNA sequence (122 890 bp) of *P. patens* was determined and all potential genes were revealed (Figure 8.2, Plate 12; Sugiura et al., 2003). Overall, the GC content of the *P. patens* plastid DNA is 28.5%, which is closer to that of the liverwort *Marchantia polymorpha* (28.8%; Ohyama et al., 1986) and the hornwort *Anthoceros formosae* (= *A. angustus*) (32.9%; Kugita et al., 2003a), than to that of vascular plants (38–39%). Two identical copies of a large sequence (9589 bp) containing the rDNA operon (*rrn16–rrn23–rrn4.5–rrn5*) are present in an inverted orientation (IR_A and IR_B). A large single copy (LSC) region of 85 212 bp and a small single copy (SSC) region of 18 501 bp separate these inverted repeat (IR) sequences from each other.

In contrast to the hepatic bryophytes (*M. polymorpha* and *A. angustus*), an extensive rearrangement involving a 71-kb inversion within the LSC regions is evident in the *P. patens* plastid DNA. The large inversion encompasses a region from *petD* to *rpoB*, its endpoints lying between *rps11* and *rpoB* at one end and *petD* and *trn*C-GCA at the other (Figure 8.2, Plate 12, indicated as *arrows*). However, the other mosses, *C. purpureus*, *Hylocomium splendens*, *Plagiothecium euryphyllum*, *Bartamia pomiformis*, *Takakia lepidozioides* and *Sphagnum girgensohnii*, are not inverted (Sugiura et al., 2003; Sugita et al., 2004). This 71-kb inversion in the plastid genome generally occurs in Funariidae (Bryophyta), including *P. patens* (Goffinet et al., 2007).

8.2.2 Gene content and evolution of plastid DNA

The *P. patens* plastid genome has the potential to code for 83 proteins, 31 tRNAs, 4 rRNA genes and a pseudo tRNA gene (Table 8.3). Four genes encoding proteins (*ccsA*, *cysA*, *cysT* and *rpoA*) that are found in the liverwort *M. polymorpha* and the hornwort *A. angustus* are absent from *P. patens*. The *ccsA* gene (= *ycf5*), which encodes a component involved in *c*-type cytochrome synthesis, is usually found in land plants and algae, except *Euglena gracilis*. Two genes, *cysA* and *cysT*, that encode sulfate transport proteins, are present in the green algae *Chlorella vulgaris* and *Nephroselmis olivacea*, but are absent in charophytes and vascular plants (Martin et al., 2002). The *rpoA* gene encodes the α subunit of the bacterial-type plastid RNA polymerase and is present

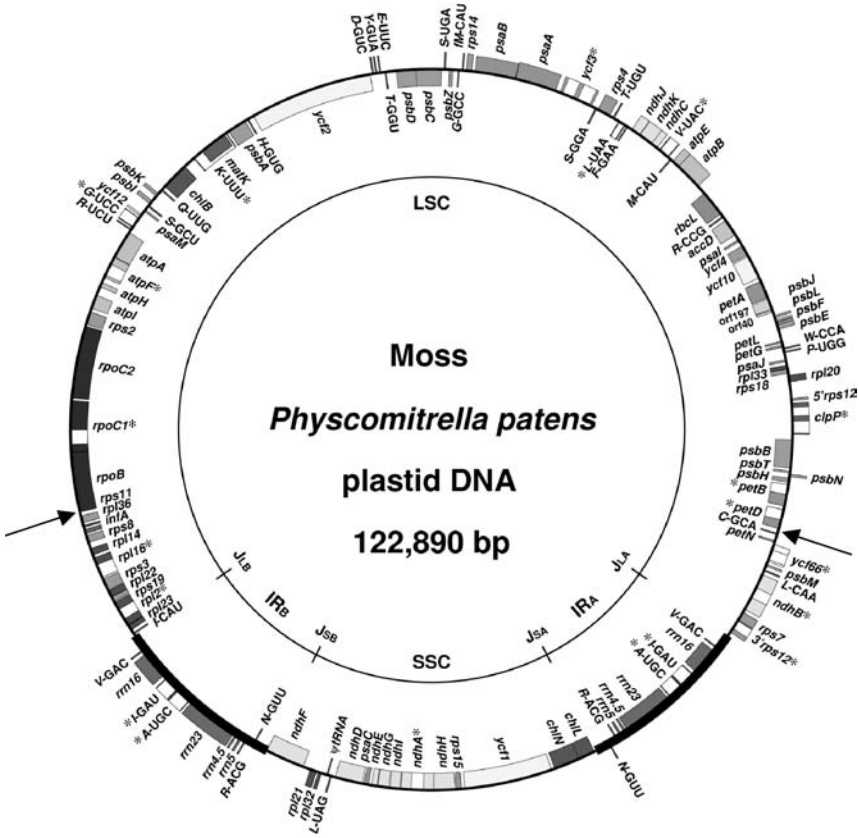


Figure 8.2 Gene map of the moss *P. patens* plastid genome. IR sequences (IR_A and IR_B) are separated by the SSC and LSC regions. Genes on the inside of the map are transcribed clockwise and genes on the outside are transcribed counter-clockwise. Genes with related functions are shown in the same color. Asterisks denote intron-containing genes or split genes. Arrows indicate positions of endpoint of a 71-kb large inversion relative to liverwort and hornwort plastid DNA. (*Source*: Reprinted and modified from Sugiura et al., 2003; copyright 2003 with permission of Oxford University Press.) (For a color version of this figure, see Plate 12)

in all plastid genomes of algae and land plants, but absent in *P. patens* and the malaria parasite *Plasmodium falciparum* (Wilson et al., 1996). Two potential protein-coding sequences (ORF40 and ORF197) unique to *P. patens* are present. *ycf66* is absent in *A. angustus*, but present in *P. patens* and *M. polymorpha*. Three genes, *chlB*, *N* and *L*, encoding subunits of light-independent protochlorophyllide reductase, are present in bryophytes including *P. patens*, algae, ferns and gymnosperms, but absent in angiosperms. Mosses and the gymnosperm pines are known to synthesize and store some chlorophyll components in the dark. A tRNA-like sequence is present between *rpl32* and

Table 8.3 Genes encoded by *P. patens* plastid DNA

Gene products	Genes
Photosystem I	*psaA*, *B*, *C*, *I*, *J*, *M*
Photosystem II	*psbA*, *B*, *C*, *D*, *E*, *F*, *H*, *I*, *J*, *K*, *L*, *M*, *N*, *T*, *Z*
Cytochrome b_6/f	*petA*, *B*[a], *D*[a], *G*, *L*, *N*
ATP synthase	*atpA*, *B*, *E*, *F*[a], *H*, *I*
Chlorophyll biosynthesis	*chlB*, *L*, *N*
Rubisco	*rbcL*
NADH oxidoreductase	*ndhA*[a], *B*[a], *C*, *D*, *E*, *F*, *G*, *H*, *I*, *J*, *K*
Large subunit ribosomal proteins	*rpl2*[a], *14*, *16*[a], *20*, *21*, *22*, *23*, *32*, *33*, *36*
Small subunit ribosomal proteins	*rps2*, *3*, *4*, *7*, *8*, *11*, *12*[a,b], *14*, *15*, *18*, *19*
RNA polymerase	*rpoB*, *C1*[a], *C2*
Translation factor	*infA*
Other proteins	*accD*, *clpP*[c], *matK*
Proteins of unknown function	*ycf1*, *2*, *3*[c], *4*, *10*, *12*, *66*[a]
Ribosomal RNAs	*rrn16*, *23*, *4.5*, *5*
Transfer RNAs	*trnA*(UGC)[a], *C*(GCA), *D*(GUC), *E*(UUC), *F*(GAA), *G*(GCC), *G*(UCC)[a], *H*(GUG), *I*(CAU), *I*(GAU)[a], *K*(UUU)[a], *L*(CAA), *L*(UAA)[a], *L*(UAG), *fM*(CAU), *M*(CAU), *N*(GUU), *P*(UGG), *Q*(UUG), *R*(ACG), *R*(CCG), *R*(UCU), *S*(GCU), *S*(GGA), *S*(UGA), *T*(GGU), *T*(UGU), *V*(GAC), *V*(UAC), *W*(CCA), *Y*(GUA)

[a]Gene containing a single intron.
[b]Gene divided into two independent transcription units.
[c]Gene containing two introns.
Two gene copies due to the inverted repeat are underlined.

trnL-UAG in the SSC region (Figure 8.2, Plate 12) and has been classified as a pseudo gene. Genes encoding stable RNAs other than rRNAs and tRNAs have not been identified in this moss.

Eighteen genes encoding 6 tRNAs and 12 proteins contain introns, as shown in Figure 8.2, Plate 12 and Table 8.3. The gene for ribosomal protein S12 is divided into 5′-*rps12* and 3′-*rps12*, and each gene segment can be transcribed independently and the transcripts are *trans*-spliced. *clpP* and *ycf3* contain two introns in *P. patens*, as in the corresponding tobacco and rice genes, and the other intron-containing genes have a single intron. Hornwort *ycf3* also has two introns, whereas liverwort *ycf3* has only the first of these introns. To investigate transcript profiles of plastid genes, we constructed a *P. patens* plastid DNA microarray containing 102 DNA fragments of all annotated plastid genes (Nakamura et al., 2005). Total RNA was extracted from 5-day-old protonemal colonies of the wild-type mosses and reverse transcribed to generate fluorescent cDNA probes for detection of plastid transcripts. As a result, the transcripts from most plastid genes were detected in the protonemata.

Phylogenetic analysis of the deduced amino acid sequences of 51 plastid-encoded genes from the entire plastid genome sequences of 20 representative

green plant species including *P. patens* provided strong support for the bryophytes as a monophyletic group distinct from extant vascular plants (Nishiyama et al., 2004). On the other hand, recent comprehensive phylogenetic analyses using plastid, mitochondria and nuclear genes do not show a monophyly of bryophytes (Qui et al., 2006). The evolution of bryophytes among lineages of land plants is described and discussed in Chapter 1 of this book.

8.2.3 Loss of *rpoA* gene from the plastid DNA

The most striking feature of the plastid DNA of *P. patens* is the absence of *rpoA*. The *rpoA* gene is also absent in the mosses *H. splendens*, *P. euryphyllum*, *B. pomiformis* and *C. purpureus* (Sugiura et al., 2003). After the divergence of the mosses from the hepatic bryophytes, *rpoA* was lost from the plastid DNA. However, *rpoA* is retained in the plastid DNA of the early diverging mosses *S. girgensohnii* and *T. lepidozioides* (Sugita et al., 2004). Goffinet et al. (2005) surveyed 30 moss species and showed that the loss of *rpoA* from the plastid genome is a general occurrence in the arthrodontous mosses (The Bryopsida constitute the largest class of mosses, having spore capsules with teeth that are arthrodontous). These results suggest that the *rpoA* gene was lost from the plastid genome and transferred to the nucleus during the evolutionary history of the mosses.

8.3 Transcription of plastid genes by two plastid RNA polymerases PEP and NEP

Plastids of seed plants contain two distinct DNA-dependent RNA polymerases: the plastid-encoded plastid RNA polymerase (PEP) and the nuclear-encoded plastid RNA polymerase (NEP) (Maliga, 1998; Hess and Börner, 1999). The PEP enzyme comprises a core complex $\alpha\alpha\beta\beta'\beta''$ encoded by plastid genes *rpoA*, *rpoB*, *rpoC1* and *rpoC2*. The subunit composition of core PEP resembles that of cyanobacterial RNA polymerase, indicating PEP is of cyanobacterial origin (Figure 8.3). Transcription initiation by PEP requires multiple sigma factors, which recognize the bacterial-type promoter sequence of the photosynthesis genes while containing canonical −10 (TATAAT) and −35 (TTGACA) elements. Sigma factors are nuclear encoded and consist of six members Sig1 to Sig6 in *Arabidopsis thaliana* (Shiina et al., 2005); they are also of cyanobacterial origin. PEP activity is inhibited by tagetitoxin, an inhibitor of prokaryote RNA polymerase (Mathews and Durbin, 1990).

NEP preferentially transcribes housekeeping genes such as *accD*, *rpoB* and *clpP*. Transcription of *atpB* and *rrn16* starts from multiple sites, one of which is directed by NEP. NEP recognizes the so-called NEP promoter that is a Y[= T, C]R[= A, G]TA consensus sequence that resembles the plant mitochondrial

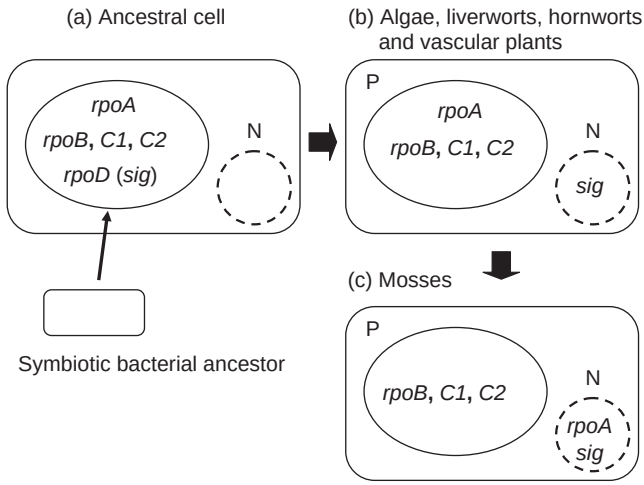


Figure 8.3 Model illustrating the evolutionary history of *rpoA* and *sig* genes transferred from plastids to the nucleus. (a) The ancestral endosymbiont contained all genes, *rpoA, B, C1, C2* and *D* (*sig*) encoding subunits of RNA polymerase holoenzyme. (b) During the establishment of endosymbiosis, the genes encoding subunits of a core PEP retained in the plastid (P) and the *sig* genes were transferred to the nucleus (N) in algae and most lineages of land plants. (c) After divergence of mosses and hepatic bryophytes (liverworts and hornworts), *rpoA* encoding the α subunit of PEP was transferred from plastids (P) to the nucleus (N), and the nuclear *rpoA* genes are distributed among the anthrodontous mosses.

promoter sequence and differs completely from the PEP promoter (Kapoor et al., 1997; Liere and Maliga, 1999). NEP activity is not affected by tagetitoxin. In general, genes for non-photosynthetic components are transcribed by NEP during the early stage of plastid differentiation and chloroplast development (Hajdukiewicz et al., 1997). Subsequent transcription of the genes for photosynthesis-related components (*rbcL*, *psbA* or *psbD*, etc.) is directed by PEP (Hajdukiewicz et al., 1997). Single subunit bacteriophage-type RNA polymerase (RpoT) is encoded in the nucleus of various eukaryotes, and is known to be located in the mitochondria and plastids of vascular plants. Several lines of evidence indicated that a plastid-localized RpoT protein is a NEP (Lerbs-Mache, 1993; Liere et al., 2004; Kühn et al., 2007). No plastid-localized RpoT is found in the green alga *Chlamydomonas reinhardtii* (Kühn et al., 2007). This suggests that the ability to target the RpoT protein to the plastids was acquired after the divergence of algae and land plants.

8.3.1 Existence of multiple PEP enzymes in *P. patens*

The absence of the *rpoA* gene from the plastid genome of *P. patens* raises the question of whether PEP exists in bryophytes. The nuclear genome of *P. patens* encodes two distinct RpoA-like proteins, PpRpoA1 (450 amino

acids) and PpRpoA2 (525 amino acids) (Sugiura et al., 2003; Kabeya et al., 2007). Both proteins were shown to be plastid targeted and have the characteristic arrangement of protein domains identified in the *E. coli* α subunit of RNA polymerase (Kimura and Ishihama, 1995). Two PpRpoA proteins were detected immunologically in the PEP-active fractions separated from the *P. patens* chloroplast lysate by anion exchange column chromatography (Kabeya et al., 2007). This suggests that the two nuclear-encoded PpRpoA proteins constitute core PEP enzyme in the *P. patens* chloroplasts.

The *PpRpoA2* gene was highly expressed in the light but not in the dark, whereas *PpRpoA1* was constitutively expressed (Kabeya et al., 2007). This suggests that the two PpRpoA proteins play different roles in the transcription of plastid genes. To address this possibility, we have generated and characterized the *PpRpoA*-knockout mosses. *PpRpoA1* disruptants were obtained easily. The only *PpRpoA2* disruptants obtained possessed both a wild-type and disrupted *PpRpoA2* gene and were thus likely to be aneuploid. The *PpRpoA1* disruptant displayed a green phenotype like the wild-type but showed slightly retarded growth of the protonemal colonies. Although the *PpRpoA1* gene was disrupted, transcript levels of most plastid genes were not significantly altered. This implies that PpRpoA1 is dispensable for plastid function and that PpRpoA2 plays a central role in plastid transcription. The transcription of most plastid genes may depend on PpRpoA2-PEP activity rather than on that of PpRpoA1-PEP. In contrast, the transcript levels of some plastid genes (*petN*, *psbZ* and *ycf3*) were altered in the *PpRpoA1* gene disruptant, suggesting that these can be categorized as PpRpoA1-PEP-dependent genes. Thus, plastid genes are differentially transcribed by either PpRpoA1 or PpRpoA2 PEP enzymes. Our data indicated that the biosynthesis and assembly of the core subunits of PEP are controlled cooperatively by the plastid and nuclear genomes, and that a relatively more complicated transcription system operates in mosses than in angiosperms.

8.3.2 Promoter sequences of *P. patens* plastid genes

Sigma factors are known to be the most important determinants for the selection and initiation of transcription of plastid genes (Allison, 2000; Shiina et al., 2005) and recognize the promoter which consists of −10 and −35 elements. There are four different plastid sigma factors, PpSig1, PpSig2a, PpSig2b and PpSig5 in *P. patens* (Hara et al., 2001a, 2001b; Ichikawa et al., 2004, 2008). PpRpoA proteins probably interact with one of these sigma factors bound to the promoter. We have determined the positions of the 5′-end of transcripts by primer extension analysis and assigned putative promoter sequences (Kabeya et al., 2007). Like seed plants, the moss photosynthesis genes have canonical −35 and −10-like elements. On the other hand, PpRpoA1-dependent genes (*petN* and *ycf3*) have a −35 element and also a characteristic extended −10 sequence, GAT(G/A)TATATA(T/A)AT (Kabeya et al., 2007). The other putative PpRpoA1-dependent gene, *psbZ*, has a sequence TCGGCCA that is also

found in the upstream region of the *ycf3*−253 position. Such sequences are not found in the plastid genes of *A. thaliana* and tobacco. Therefore, we hypothesize that the strength of transcription activity is modulated by different combinations of the two α subunits (PpRpoA1 and A2) combining with a certain sigma factor. PpRpoA2 possesses a portion of DnaK/HSP70, which may provide an additional function to PpRpoA2 as the α subunit.

8.3.3 Does NEP exist in the chloroplasts of *P. patens*?

Plant RpoT proteins constitute a small family with different targeting properties. For instance, as shown in Figure 8.4, *A. thaliana* RpoT;1 (AtRpoT;1) is targeted to mitochondria, while AtRpoT;3 is targeted to plastids (Hedtke et al., 1997) and AtRpoT;2 is targeted to both organelles (Hedtek et al., 2000). The same observations were reported in *Nicotiana sylvestris* and *N. tabacum* (Kobayashi et al., 2001; Hedtke et al., 2002). Three *RpoT* genes are well conserved in structure and most probably arose by gene duplication from an ancestral gene encoding the mitochondrial RNA polymerase.

In *P. patens*, two *RpoT* genes were identified by two groups (Kabeya et al., 2002; Richter et al., 2002). Kabeya et al. (2002) showed that the two RpoT proteins were targeted only to mitochondria, while Richter et al. (2002) reported that the two proteins were targeted to both mitochondria and chloroplasts. The translated sequence for each moss *RpoT* gene contains two putative translation initiation codons at the N-terminus. *In vitro* translation of the cDNAs revealed initiation of translation at two in-frame AUG codons (Richter et al., 2002). Translation from the first methionine gives rise to a plastid-targeted protein, whereas initiation from the second methionine results in exclusively mitochondrial-targeted protein. Thus, the dual targeting of RpoT protein is caused by, and might be regulated by, multiple translational starts.

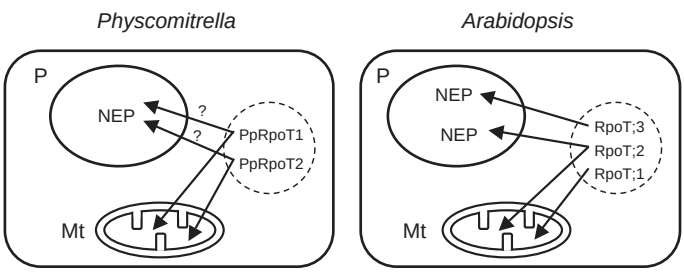


Figure 8.4 Sub-cellular localization of phage-type RNA polymerase RpoTs in the moss *P. patens* and angiosperms. RpoT;1 and RpoT;3 proteins are imported to mitochondria (Mt) and plastids (P), respectively, in *A. thaliana* and *N. tabacum*. RpoT;2 protein is imported to both mitochondria and plastids. The RpoT;3 and the plastid-localized RpoT;2 are considered as NEPs. In contrast, two RpoTs are identified in *P. patens* and their sub-cellular localization is ambiguous. Both are imported to the mitochondria (Kabeya and Sato, 2005) or dual targeted to the mitochondria and plastids (Richter et al., 2002). The existence of NEP in *P. patens* has been debated.

Thereafter, Kabeya and Sato (2005) made several constructs with mutated translation initiation codons, and expressed them transiently or stably in *P. patens*. They reconfirmed their previous conclusion and suggested that both PpRpoT1 and T2 are not translated from the first (upstream) AUG codon in a natural context but are rather translated from the second (downstream) one; furthermore, these proteins are targeted only to mitochondria, although they may be targeted to plastids when translation is forced to start from the first AUG codon, which is fused to the translation leader sequence taken from the *RBC3A* gene of *P. sativum*. Moreover, they carried out a run-on transcription assay using the isolated chloroplasts from the moss protonemata. The transcription activity was completely inhibited by the addition of 10 μM tagetitoxin (an inhibitor of PEP and not NEP enzyme), confirming the absence of NEP in the *P. patens* chloroplasts (Kabeya and Sato, 2005).

We also performed run-on transcription assays (Y. Kobayashi and M. Sugita, unpublished observations). As shown in Figure 8.5, the overall transcriptional activity in the absence of any inhibitors (indicated as control) increased in proportion to the incubation period in wild-type chloroplasts. Actinomycin D, a global inhibitor of transcription, significantly inhibited the incorporation of labeled UTP in both the wild-type and *PpRpoA1*-disruptant mosses. This indicates that the incorporation of [^{32}P]-UTP reflects RNA synthesis. Tagetitoxin strongly inhibited transcriptional activity in the wild-type, indicating that PEP contributes predominantly to the overall transcription

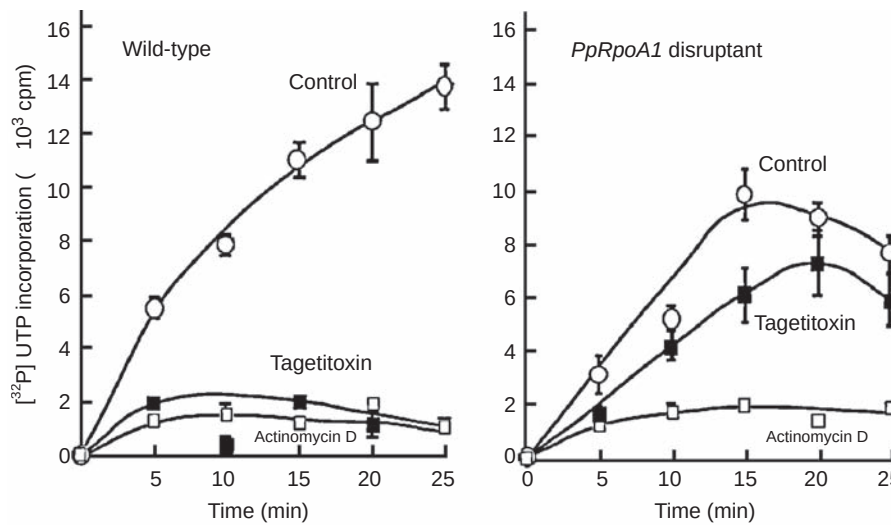


Figure 8.5 RNA synthesis in isolated *P. patens* chloroplasts from 3-day-old protonemata. Intact chloroplasts were broken and incubated in the reaction mixture either with or without 40 μg/mL actinomycin D or 5 μM tagetitoxin. Experiments were repeated three times and standard deviations are shown. Run-on transcription assays were carried out in the absence (control) or presence of inhibitors.

of plastid genes in the protonemata. The result was consistent with that reported by Kabeya and Sato (2005). On the other hand, run-on transcription activity of the PpRpoA1-deficient chloroplasts was only slightly inhibited even by the addition of an effective concentration of tagetitoxin (5 μM). One possibility is that tagetitoxin-resistant plastid RNA polymerase (NEP) exists in *P. patens* chloroplasts and that NEP activity is increased or enhanced in PpRpoA1-deficient moss. These observations imply that plastid genes may be preferentially transcribed by NEP rather than by PEP, in some specific stages in the moss life cycle.

8.4 Rhythmic expression of the plastid *psbD* gene

In plants, as well as in other organisms, circadian clock-controlled gene expression is pervasive in the nuclear genome (Harmer et al., 2000). In *A. thaliana*, myb transcription factors CCA1 and its paralog LHY, which were identified as 'Clock' proteins, regulate the rhythmic transcription of genes by their direct interaction with promoters (Michael and McClung, 2002; Harmer and Kay, 2005). The clock-controlled expression of the nuclear *PpLhcb2* gene, encoding a chlorophyll *a/b*-binding protein, was also reported in *P. patens* (Aoki et al., 2004). However, rhythmic expression in plastid genes has not been extensively studied. The *psbD* gene encoding the D2 protein of photosystem II is one of a few reported examples of plastid genes that are rhythmically expressed. In wheat (Nakahira et al., 1998) and the green alga *C. reinhardtii* (Matsuo et al., 2006), *psbD* showed robust rhythms with a period of 1 day in constant light (LL) or constant dark (DD) conditions as well as in light–dark cycles (LD). Ichikawa et al. (2004) demonstrated that the *psbD* gene also showed rhythmic expression in LD in *P. patens*, although its amplitude was weakened to be nearly arrhythmic in (LL) or (DD). Since the expression of a plastid sigma factor showed a circadian rhythm in wheat, plastid sigma factors were proposed to be a candidate regulator of rhythmic gene expression of plastid genes (Morikawa et al., 1999). In line with this idea, the moss *PpSig5* gene, encoding a plastid sigma factor, showed a very high-amplitude daily rhythm in LD while other sigma genes *PpSig1* and *PpSig2a* were almost arrhythmic (Ichikawa et al., 2004). In addition, *PpSig5* was still rhythmic in DD, though its amplitude dampened within a few days, indicating that *PpSig5* is under the control of the clock. In the *PpSig5*-disrupted strains, the amplitude of *psbD* expression rhythm was lowered (though it was still rhythmic), indicating that *PpSig5* sustains the rhythm of *psbD* (Ichikawa et al., 2008).

The PpSig5 protein is thought to direct, through its rhythmic expression, the PEP holoenzyme to transcribe *psbD* in a rhythmic manner, though we cannot exclude the possibility that direct induction of *PpSig5* by light also contributes to the rhythmic expression of *psbD* in LD. In the double disruptant for *PpCRY1a* and *PpCRY1b* (Imaizumi et al., 2002), encoding the blue-light

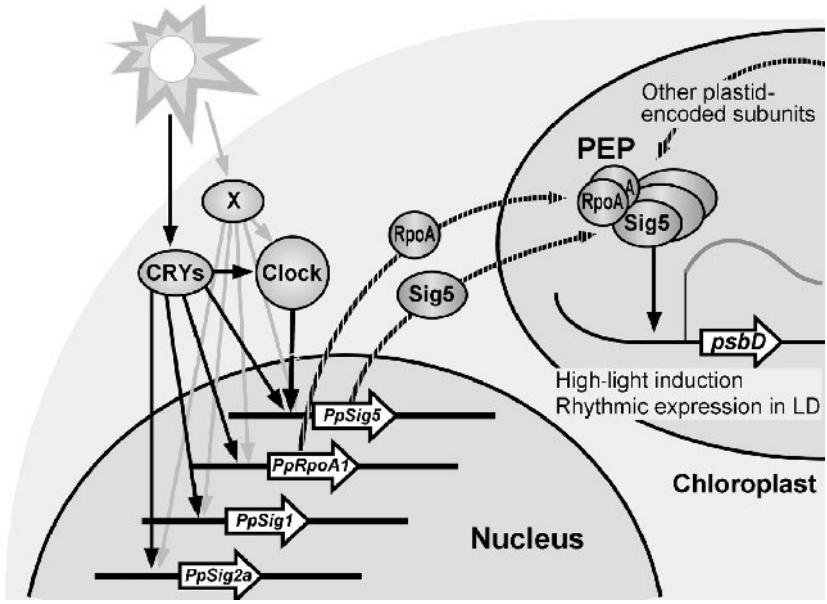


Figure 8.6 The regulation of *psbD* transcription by the plastid sigma factor PpSig5 in *P. patens*. Three sigma genes (*PpSig1*, *PpSig2a* and *PpSig5*) and an *rpoA* gene (*PpRpoA1*) on the nuclear genome and *psbD* on the plastid genome are shown (the other sigma gene [*PpSig2b*] and the other *rpoA* gene [*PpRpoA2*] have recently been identified; Kabeya et al., 2007; Ichikawa et al., 2008). All the four nuclear genes are controlled by blue-light signaling mediated by cryptochromes ('CRYs'), though they are also under the control of other photoreceptors ('X'), possibly phytochromes and/or phototropins. In addition, high-fluence light specifically induces *PpSig5*, though the photoreceptor for this response has not been identified in *P. patens*. The circadian clock ('Clock'), also affected by the blue-light signaling mediated by cryptochromes, specifically controls *PpSig5* expression. In turn, PpSig5 ('Sig5'), the protein product of *PpSig5*, enters into the chloroplast, forms the PEP holoenzyme along with the chloroplast-encoded subunits, and regulates *psbD* transcription. Consequently, the *psbD* gene shows high-fluence light induction as well as diurnal rhythm in LD.

receptor cryptochromes, the waveforms of the rhythmic expression of *PpSig5* and *psbD* changed (Ichikawa et al., 2004). This may be due to some parameters of the clock, that is, its phase or cycle length, being affected by blue light mediated by the cryptochromes. Figure 8.6 diagrammatically represents the regulation of the *psbD* expression by *PpSig5*, which is under the control of the clock and blue-light signaling.

In *P. patens*, the expression of *PpSig5* and *psbD* was induced by high-fluence light (Nagashima et al., 2004; Ichikawa et al., 2008). In the *PpSig5* disruptants, the induction of *psbD* by high-fluence light was totally abolished, indicating that PpSig5 mediates the signaling induced under high-light conditions to direct *psbD* expression in *P. patens*, as in *A. thaliana* (Nagashima et al., 2004). Blue light also induces *psbD* transcription through the blue-light responsive

promoter (BLRP), the sequence of which is widely conserved in seed plants (Shiina et al., 2005). High-fluence light also induces the *psbD* transcription through the BLRP sequence (Nagashima et al., 2004). However, in spite of the functional conservation of Sig5s between *A. thaliana* and mosses, no BLRP-like sequence is found in the upstream sequence of the moss *psbD*. Currently, we do not know the reason for this apparent discrepancy. Future work should attempt to analyze the *cis*-regulatory sequences of *psbD* in *P. patens*.

8.5 Post-transcriptional regulation in plastids

Most plastid genes of land plants are organized in clusters, and are co-transcribed as large polycistronic precursor RNAs that are subsequently processed into shorter RNA species. Precursor (pre-), intermediate and mature RNAs are relatively stable and accumulate at respective steady-state levels. Thus, post-transcriptional RNA processing of pre-RNAs, which includes RNA cleavage and trimming, RNA splicing, RNA editing and RNA stabilization, is an important step in the control of plastid gene expression (Sugita and Sugiura, 1996).

8.5.1 RNA editing in plastids of land plants and bryophytes

RNA editing is a post-transcriptional process that changes individual nucleotides in transcripts, and usually occurs in the plastids of land plants (Bock, 2000; Shikanai, 2006). Plastid RNA editing is generally found as cytidine (C) to uridine (U) conversion in seed plants and results in alteration of amino acid residues and generation of a translation initiation or termination codon. The codon changes resulting from RNA editing restores the identity of conserved amino acids in plant phylogeny.

Approximately 30 RNA editing sites have been identified in the plastid genomes of five seed plant species (Tillich et al., 2006) but there is significant variation among the bryophytes. For instance, RNA editing is absent in the chloroplasts of the liverwort *M. polymorpha* (Freyer et al., 1997), whereas extensive RNA editing, 509 C-to-U and 433 U-to-C conversions, has been found in plastid RNAs of *A. angustus* (Kugita et al., 2003b). Several hundred RNA editing sites have been identified in plant mitochondria of seed plants (Giege and Brennicke, 1999). Editing is apparently absent from the mitochondria of green algae and the liverwort *M. polymorpha* (Gray and Covello, 1993). Recently, we identified more than 300 editing sites in the transcripts of 24 plastid genes of the moss *T. lepidozioides* (Sugita et al., 2006; Yura et al., 2008). Thus, the number of RNA editing sites is significantly divergent among bryophytes compared with the conservation of editing sites in the plastids of vascular plants.

In *P. patens*, we predicted 35 C-to-U editing sites by aligning the DNA and derived protein sequences of interest with the corresponding sequences

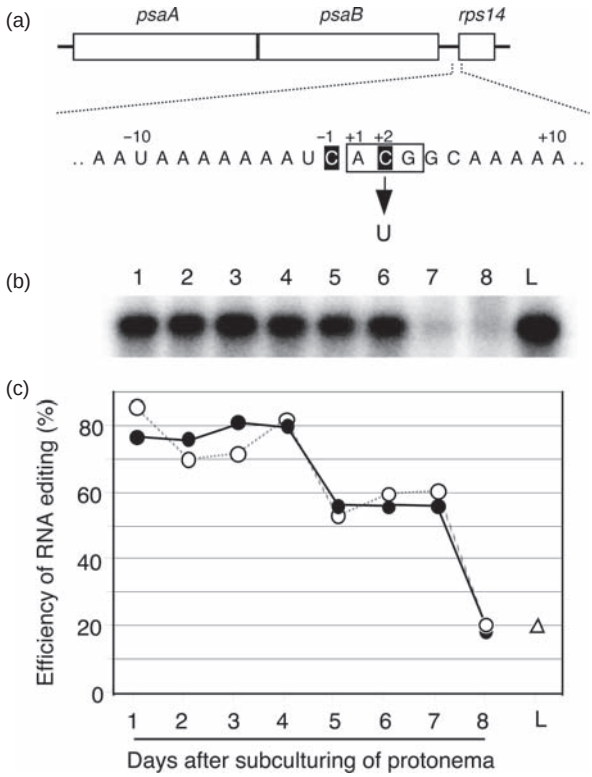


Figure 8.7 RNA editing efficiency of the *rps14* transcript in protonemata and gametophores. (a) *rps14* is transcribed as a 5-kb transcript with *psaA* and *psaB*. The translated region of *rps14* starts at A, position +1. C nucleotide at position +2 is converted to U in the transcript to create an AUG start codon. C nucleotide at position −1 is also edited with a low efficiency. (b) The steady-state level of the transcript containing *rps14* during development of protonemata at 1–8 days after subculturing (lanes 1–8) and in fully developed leafy shoots (gametophores) (L). (c) The editing efficiency at position +2 status of *rps14* cDNAs was determined by sequencing the cDNAs. Two series of experiments were carried out. (*Source*: Reprinted and modified from Miyata and Sugita, 2004.)

from *P. patens* and several plant species. So far, two editing sites have been identified in the transcripts of *P. patens* (Miyata et al., 2002; Miyata and Sugita, 2004). The *P. patens rps14* transcript was edited by a C-to-U transition to create a translation initiation codon, AUG, from the ACG codon. This editing site is unique to the *P. patens rps14* transcript and has not been found in the plastids of other plants. The *P. patens rps14* gene is co-transcribed as a 5-kb pre-mRNA with the *psaA-psaB* genes encoding photosystem I P700 apoproteins A and B. As shown in Figure 8.7, the efficiency of RNA editing was 80% in the young protonemata and decreased to approximately 20% in old protonemata and fully developed leafy shoots (Miyata and Sugita,

2004). This indicates that RNA editing of this site is regulated in a tissue- and stage-specific manner. In addition, the C nucleotide at position −1 relative to the position of A of the ACG codon was also changed to a U with a low efficiency of 5.3% in protonemata. This editing in the 5′ untranslated region may affect the efficiency of translation initiation of *rps14* mRNA. Although transcripts containing the *rps14* sequence accumulate to a substantial level in chloroplasts, the translation efficiency of *rps14* may be low because of the lack of ribosome-binding sequences and incomplete RNA editing of *rps14* transcripts.

Since no other editing sites have been identified, RNA editing may occur only rarely in the chloroplasts of *P. patens*. The *P. patens* mitochondrial genome sequence (105 340 bp) was recently determined and seven possible editing sites are predicted (Terasawa et al., 2007).

8.5.2 RNA processing of *clpP* pre-mRNA in *P. patens*

The *clpP* gene containing two introns is co-transcribed with the 5′-*rps12* and *rpl20* genes, and a primary transcript of 3.2 kb is produced. Then, the primary transcript is cleaved at the intergenic spacer between *clpP* and 5′-*rps12* and spliced to produce a mature *clpP* mRNA of 0.6 kb. This post-transcriptional event proceeds rapidly and therefore the primary transcript accumulates in small amounts. We have recently identified the plastid-localized PPR531–11 protein as a key component involved in this process (Hattori et al., 2007). PPR531–11 is a member of the pentatricopeptide repeat (PPR) proteins. The PPR is a degenerate 35-amino acid repeating motif that is found in animals, fungi and plants (Small and Peeters, 2000). A large gene family encoding PPR proteins exists in plants, from moss (Hattori et al., 2004) to flowering plants (Lurin et al., 2004), but not in fungi and animals. For instance, the *A. thaliana* and *Oryza sativa* genomes have over 450 PPR protein genes (Lurin et al., 2004). The majority of plant PPR proteins are predicted to be localized in the mitochondria or plastids. PPR proteins have been shown to play important roles in a wide range of physiological and developmental functions, that is, cytoplasmic male sterility, photosynthesis and embryogenesis (Saha et al., 2007). Several PPR proteins were shown to be involved in a step of RNA maturation in a gene-specific manner in plastids (Delannoy et al., 2007). We have assigned a total of 103 genes encoding PPR proteins in the *P. patens* genome (O'Toole et al., 2008), of which 32 are predicted to be plastid localized and may have a key role in a certain step in RNA processing or translation of plastid mRNAs.

8.6 Plastid transformation

Targeted disruption of a gene by plastid transformation is the appropriate technique to investigate whether the putative plastid gene is

essential for plastid biogenesis. Plastid transformation was established in the unicellular green alga *C. reinhardtii* (Boynton et al., 1988), and, among the angiosperms, was achieved first in *N. tabacum* (Svab et al., 1990). This technique has been extended to several other plant species, including *A. thaliana*, *O. sativa* and *Lycopersicon esculentum* (Maliga, 2004), and is now available for a variety of species to confer desirable agronomic traits or enable the production of polypeptides (Daniell et al., 2005; Bock, 2007).

Cho et al. (1999) made efforts to perform particle bombardment-mediated plastid transformation in *P. patens* but their results were not convincing. Thereafter, we developed a plastid transformation system for *P. patens* to determine whether *trnR-CCG* is an essential gene for plastid function. There are three distinct arginine tRNAs, *trnR-CCG*, *trnR-ACG* and *trnR-UCU*, present in plastid DNA (Sugiura and Sugita, 2004). Of these three genes, *trnR-CCG* occurs in bryophytes and gymnosperms, but not in angiosperms. For the targeted disruption of *trnR-CCG*, the gene was replaced with a selectable spectinomycin (spec)-resistance (*aadA*) gene on a plastid gene fragment in the transforming plasmid. The plasmid DNA digests were introduced into protoplasts in the presence of polyethylene glycol. Transplastomes were generated by the integration of *aadA* into the plastid genome via the flanking plastid DNA sequences, thus introducing the *trnR-CCG* gene disruption into the transformed lines. This study indicated that *trnR-CCG* is dispensable for *P. patens* plastid biogenesis. Efficiency of plastid transformation is quite reliable and one or two homoplasmic transplastome lines were generated per plastid transformation experiment (Sugiura and Sugita, 2004). Further study will allow investigation of the molecular basis of the regulation of plastid gene expression and the interactions between nuclear and plastid genomes in *P. patens*. Recently, plastid transformation using a biolistic delivery system was achieved for the liverwort *M. polymorpha* suspension-culture cells (Chiyoda et al., 2007).

Little is known about the molecular basis of homologous recombination in plastids. A plastid-localized RecA protein has recently been identified, and could be involved in homologous recombination in plastids (Inouye et al., 2008).

8.7 Chloroplast import

Hofmann and Theg (2003) showed that all of the known Toc/Tic machinery is present in *P. patens*, and so were likely to have arisen before the divergence of non-vascular and vascular plants. Moreover, they showed that isolated moss plastids can be used for *in vitro* protein import assays. With this assay system, they demonstrated that moss plastids import proteins under the same conditions as do *Pisum sativum* plastids, which suggests that the two systems are fundamentally similar. More recently, the same authors generated double disruptants for two genes encoding Toc64-like proteins (Hofmann and Theg,

2005a, b). The disruptants showed slightly altered chloroplast shape in some tissues, although they showed neither an abnormality in growth phenotype nor were defective in *in vitro* protein import ability. These studies illustrate that *P. patens* should be a useful tool, by combining genetic and biochemical approaches, for the study of chloroplast protein targeting.

8.8 Plastid division

It has long been known that chloroplasts multiply by binary fission like their bacterial ancestors. However, the detailed molecular mechanisms have only been elucidated in the past decade (Osteryoung and Nunnari, 2003; Miyagishima, 2005). In *P. patens*, plastid division has been studied for some time; 20 years ago, an interesting mutant, PC22, was isolated by x-ray irradiation of the spores of *P. patens*. The PC22 cells in general contain one single, large, plate-like chloroplast, the 'macrochloroplast' per protonemal cell (Abel et al., 1989). Interestingly, the deficiency in chloroplast division is partly compensated by exogenous cytokinin (Abel et al., 1989), indicating that plastid division is controlled by cytokinin action. During the last decade, several key genes affecting plastid division have been characterized in *P. patens* as well as in *A. thaliana*.

8.8.1 *FtsZ* genes

In bacteria, FtsZ, a self-assembling guanosine triphosphatase (GTPase) that is evolutionarily related to eukaryotic tubulins, plays a critical role in cell division (Miyagishima, 2005). FtsZ assembles into a ring structure at the cell midpoint, and initiates the division process by recruitment of other associated proteins. Genes homologous to the bacterial *ftsZ* genes have been found in the nuclear genome of *P. patens* and in those of angiosperms. This suggests that the *ftsZ* gene of the symbiotic bacterial ancestor of chloroplasts was transferred to the nuclear genome of host cells. Strepp et al. (1998) showed that targeted disruption of *PpftsZ* (later renamed *PpftsZ2–1*), encoding an FtsZ homolog, impeded chloroplast division resulting in a single macrochloroplast per cell in *P. patens*. Osteryoung et al. (1998) made a similar observation in *A. thaliana.* Downregulation of *AtFtsZ1–1* or *AtFtsZ2–1*, encoding FtsZ homologs, by an anti-sense mRNA technique, generated macrochloroplasts. These observations indicated that chloroplasts still use FtsZ proteins in their division like their symbiotic bacterial ancestor. While bacterial genomes encode a single *ftsZ* gene, the *P. patens* genome contains at least four *ftsZ* genes classified into two subfamilies FtsZ1 and FtsZ2 (Rensing et al., 2004). The angiosperm nuclear genomes also encode multiple *ftsZ* genes classified into these two subfamilies, indicating that the two subfamilies generated before the divergence between mosses and other plants. In *A. thaliana*, FtsZ proteins were reported to assemble as a ring structure like FtsZ in bacterial cell

division (Vitha et al., 2001). In contrast, green fluorescent protein (GFP) fusions of FtsZ2–1 and FtsZ2–2 localized to chloroplasts and formed filamentous structures that resemble cytoskeletal networks (Kiessling et al., 2000). This observation suggested a possibility that these FtsZ proteins are not only important for chloroplast division, but could also be involved in the maintenance of plastid shape and flexibility. Moreover, also using GFP fusions, FtsZ1–2 could be localized to both the cytoplasm and the chloroplast depending on the choice of the translation initiation site (Kiessling et al., 2004), which the authors argued could link cell division to chloroplast division (Kiessling et al., 2004).

8.8.2 *MinD* and *MinE* genes

Other than *FtsZ* genes, plant genomes also contain genes encoding factors related to bacterial cell division machinery such as MinD, MinE and Arc6 (Osteryoung and Nunnari, 2003). MinD and MinE are important factors spatially regulating the chloroplast division site. Itoh et al. (2005) investigated two *P. patens* genes, *PpMinD1* and *PpMinD2*, that encode homologs of MinD. While GFP fusions with both gene products are targeted to chloroplasts, disruption of either *PpMinD1* or *PpMinD2* did not show any detectable effects on plastid division. This implies that the two genes either have a redundant function or do not function in chloroplast division. Zhu et al. (2007) isolated a moss gene *PpMinE*, encoding a homolog of MinE. When over-expressed in *E. coli* cells, *PpMinE* caused division site misplacement and mini-cell formation. Moreover, a GFP fusion with the PpMinE protein was localized to chloroplasts in *N. tabacum*. These observations suggest that the PpMinE protein is involved in organelle division. The chloroplast division system uses not only proteins with bacterial origin but also those of host-eukaryotic origin (Osteryoung and Nunnari, 2003; Miyagishima, 2005). However, in *P. patens*, chloroplast division proteins with eukaryotic origin have not yet been investigated.

8.8.3 Peptidoglycan synthesis-related genes

In chloroplast division, peptidoglycans are potentially important because they build the cell wall and are involved in central septum formation in bacterial cell division (Bramhill, 1997). In primary photosynthetic eukaryotes, a cyanobacterial-type peptidoglycan layer (called the cyanelle) has been detected in chloroplasts of glaucophytes, a small group of freshwater algae, but not in other lineages, that is, red and green algae and land plants (Machida et al., 2006). Therefore, chloroplasts of the latter groups apparently seem to have lost peptidoglycan through their evolution from an ancestral endosymbiont. However, treating *P. patens* cells with β-lactam antibiotics such as ampicillin, that inhibit the last step of the peptidoglycan layer synthesis, resulted in only two to three macrochloroplasts per cell (Kasten and Reski, 1997). This

suggests that peptidoglycan synthesis is still involved in chloroplast division in *P. patens*. Fosfomycin and D-cycloserine, inhibiting earlier steps of the peptidoglycan synthesis pathway, similarly caused a decrease in the number of chloroplasts per cell in *P. patens* (Katayama et al., 2003). On the other hand, treating *L. esculentum* cells with β-lactam antibiotics did not affect chloroplast division, indicating that peptidoglycan synthesis is not involved in chloroplast division in angiosperms (Kasten and Reski, 1997). In line with these observations, the nuclear genome of *P. patens* retains at least nine genes potentially involved in the peptidoglycan synthetic pathway, such as *MurA-F*, *Ddl* and *Dac*. The *A. thaliana* genome contains only five genes in the pathway (Machida et al., 2006), suggesting degradation of the peptidoglycan synthesis pathway. Disruption of a gene encoding MurE (*PpMurE*) or PBP (*PpPbp*) caused the formation of macrochloroplasts (Machida et al., 2006), confirming that peptidoglycan synthesis is still involved in chloroplast division in *P. patens*.

It could be due to the thinness of the peptidoglycan layer that no wall-like structure has been detected in *P. patens* chloroplasts. Even under an electron microscope, no wall-like structure is detected near the chloroplast envelopes in moss (Machida et al., 2006). This is also the case, however, in cyanelles. The cyanelle peptidoglycan layer cannot be detected by ordinary electron microscopic techniques, though it can be detected by immunoelectron microscopic observation or a silver methenamine staining method (Steiner and Löffelhardt, 2002; Iino and Hashimoto, 2003). Machida et al. (2006) concluded that plants evolved in three distinct ways: glaucophytes retained peptidoglycans, red algae lost them, and green plants retained some peptidoglycan genes. It seems that green plants have gradually lost peptidoglycan genes throughout their evolution. Ampicillin causes the appearance of macrochloroplasts in the liverwort *M. polymorpha* and the pteridophyte *Selaginella nipponica* (Tounou et al., 2002; Izumi et al., 2003), indicating that these relatively earlier-diverged plants still retain peptidoglycan in chloroplasts. The direct detection of peptidoglycan in chloroplasts from various plants will be of importance in the light of the evolution of chloroplasts.

8.9 Chloroplast movement

Chloroplasts are relocated within the cell depending on ambient light conditions; in general, low-fluence light induces the movement of chloroplasts toward light (accumulation response), whereas high-fluence light causes chloroplasts to move away from light (avoidance response). In *P. patens*, red and blue light induce chloroplast movement in contrast to seed plants, where chloroplast photomovement is predominantly regulated by blue-light signaling mediated by phototropins (Kadota et al., 2000; Sato et al., 2001). The effect of red light is canceled by far-red light, indicating that phytochromes are the photoreceptors (Kadota et al., 2000). Mittmann et al. (2004) isolated four

phytochrome genes, *PHY1–4*, all of which encode canonical plant phytochromes. They generated single disruptants for each single *PHY* gene (*phy1*, *phy2*, *phy3* and *phy4* strains) and analyzed chloroplast movement along with other photomovement responses. Only *phy4* was defective in chloroplast movement (as well as in polarotropism) in response to polarized red light, whereas the other three *phy* disruptants showed normal responses. Therefore, *PHY4* is a major phytochrome for the polarized red-light-induced photorelocation of chloroplasts. On the other hand, in the *phy4* disruptant, chloroplast movement induced by microbeam irradiation of non-polarized red light did not differ from that of the wild-type, indicating functional redundancy of four phytochrome genes in this response. As for the blue-light-induced movement of chloroplasts, phototropin was identified as a photoreceptor in *A. thaliana* (Jarillo et al., 2001; Kagawa et al., 2001). The phototropin protein, named after its identification as a photoreceptor for phototropism, consists of two LOV (light, oxygen and voltage) domains at the N-terminus and a Ser/Thr kinase domain at the C-terminus. *P. patens* has four phototropin genes, *PHOTA1*, *PHOTA2*, *PHOTB1* and *PHOTB2* (Kasahara et al., 2004), structurally classified into two distinct groups, *photA* (with *PHOTA1/2*) and *photB* (with *PHOTB1/2*). Kasahara et al. (2004) generated many disruptants: single disruptants (*photA1*, *photA2*, *photB1* and *photB2*), double disruptants (*photA1photA2* and *photB1photB2*) and a triple disruptant (*photA2photB1photB2*). They examined the chloroplast movement within these disruptants in the basal and tip cells of protonemata. They demonstrated that:

1. photA2 is the predominant photoreceptor, whereas photB1 and photB2 function redundantly, for avoidance movement in basal cells.
2. photA2, photB1 and photB2 (and possibly photA1) mediate the accumulation response redundantly in basal cells.
3. photA1 and photA2 mediate the avoidance response redundantly in the tip cells. Thus, differential regulation can be seen between the two cell types. Moreover, they also demonstrated that the triple disruptant was deficient in both red-light-induced accumulation and avoidance responses, as well as in blue-light-induced responses. Therefore, phototropins may be components of signal transduction pathways for the phytochrome-dependent chloroplast movement in *P. patens*.

Acknowledgments

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Chapter 9

CARBON AND ENERGY METABOLISM

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Abstract: As in all organisms, the primary carbon metabolism in *Physcomitrella patens* provides the cells with free energy and building blocks for the biosynthesis of macromolecules. In particular, carbohydrates play a major role in growth and development in mosses as in other plants, since they are used for storage and allocation of carbon and energy. Reverse genetics in *P. patens* has recently made it possible to address some key questions concerning the carbon and energy metabolism in plants. These questions include: How is the cellular energy status sensed in order to balance anabolism versus catabolism? How does carbon and energy allocation between cells and tissues work? How is whole-plant energy homeostasis maintained? What is the role of sugars in regulating growth and development? And, finally, how similar or different are these processes in bryophytes and vascular plants? The recent release of the complete *P. patens* genome sequence has now made it possible to address the last question using a comparative genomics approach.

Keywords: AMP-activated protein kinase; carbon metabolism; energy homeostasis; hexokinase; SNF1; sugar transport

9.1 Introduction

Primary carbon metabolism has two important functions in all living organisms: to provide free energy in the form of adenosine 5′-triphosphate (ATP), and to provide building blocks for the biosynthesis of macromolecules. One important source of ATP production in all mitochondrial eukaryotes is the Krebs cycle and oxidative phosphorylation, where carbohydrates are fully oxidized to CO_2 and H_2O in the mitochondria, but substrate

level phosphorylation in the glycolytic pathway can also contribute to ATP production. In photosynthetic organisms such as plants, a large amount of ATP is produced in the light reaction. Much of this ATP is used for carbon fixation in the dark reaction, but some of it is used as a source of energy for other metabolic reactions within the photosynthetic cell.

Multicellularity and division of labour between cells and tissues paved the way for a successful colonization of the land by plants (Kenrick and Crane, 1997). A prerequisite for this increase in complexity was the evolution of energy and carbon-allocating systems, in which chemically bound energy and carbon for use in biosynthetic reactions are moved from cells specialized in photosynthesis (source cells) to cells with other primary functions (sink cells). The most important vehicles for transport of carbon and energy between different cells in both animals and plants are sugars. Plants differ from animals in that they mainly use a disaccharide, sucrose, instead of glucose for transport. Mechanisms for the production, transport and metabolism of sucrose are therefore of particular importance in plants.

An equally important aspect of carbon and energy allocation is the ability of different cells and tissues to regulate their carbon metabolism in response to both changes in the carbon and energy supply, and changes in the specific needs of the cell, which in turn may be driven either by changes in the environment or by developmental programs. Such regulation can involve cell–cell signalling, which in plants is mediated by plant hormones such as abscisic acid, auxin, ethylene and cytokinins. There are also several signalling pathways in plants that respond to sugars, and it has therefore been proposed that sugars could play a hormone-like role in plants (Rolland et al., 2006). Studies of these matters have been complicated by the fact that there is extensive cross-talk between different hormone and sugar signalling pathways, which makes a functional dissection of the roles of individual pathways much more difficult.

In addition to cell–cell signalling, there is also internal regulation of the energy and carbon metabolism. Specifically, all eukaryotes possess a strongly conserved homeostatic system for regulating the balance between anabolic and catabolic processes in response to changes in the energy supply. The most important effector in this system is a protein kinase, known as SNF1 in *Saccharomyces cerevisiae*, the AMP-activated protein kinase (AMPK) in animals, and the SNF1-related protein kinase 1 (SnRK1) in plants (Hardie et al., 1998). This kinase is activated under low energy conditions, and it restores the energy level of the cell by inhibiting energy-consuming and stimulating energy-producing reactions. In animals, a low energy status is sensed as a rise in intracellular AMP (adenosine monophosphate), which directly activates the kinase, hence its name. In *S. cerevisiae*, ScSNF1 is inhibited by extracellular glucose (a preferred energy source), through the glucose repression pathway, but the precise mechanism by which this regulation is achieved is poorly understood. Even less is known about regulation of the plant SnRK1 enzyme, but the possibility exists that it could respond to external sugars, like the

S. cerevisiae enzyme, thus integrating cell–cell signalling with homeostatic control of the energy and carbon metabolism (Halford et al., 2003).

The scope of this chapter is carbon and energy metabolism in *Physcomitrella patens* with an emphasis on allocation and regulation. Specifically, we will discuss transport, uptake and metabolism of sucrose, glucose and other simple sugars, sugar sensing and signalling and energy homeostasis. In particular, we will discuss what we can learn about the carbon and energy metabolism in plants through reverse genetics in *P. patens*, and what can be inferred from the presence or absence in the *P. patens* genome (Rensing et al., 2008) of genes known to be involved in these processes in other plants.

9.2 Carbon and energy allocation

Transport of carbon and energy between plant cells is primarily achieved by moving sugars through one of two different routes (Figure 9.1). The first route is symplastic transport between adjacent cells that are connected by plasmodesmata. The second route involves export of sugars from one cell into the apoplastic space followed by uptake into the next cell. This apoplastic route, which enables transport between symplasmically isolated cells, depends on the activity of specific transporters that can facilitate the movement of sugars across cell membranes. Our knowledge about the routes and mechanisms for energy allocation between cells and tissues in *P. patens* is still limited. The topic is therefore discussed below in the light of physiological and ultrastructural studies performed in other mosses. In addition, some deductions can be made by comparing gene families in *P. patens* that are involved in sugar transport and carbohydrate metabolism with their counterparts in other plants. First, though, some key aspects of carbon and energy allocation in vascular plants will be reviewed to serve as a background.

9.2.1 Allocation in vascular plants

The most well-studied system for energy allocation in plants is phloem-mediated transport of sugars in vascular plants. This important transport route is used to transfer energy from source tissues to sink tissues, where it is stored or used to support different functions, such as growth and development. The sugar species used for phloem-mediated transport is normally sucrose, polyols or oligosaccharides that are synthesized in the phloem intermediary cells using sucrose as the substrate (Sauer, 2007). The movement of sucrose to and from the phloem can follow either the symplastic route or the apoplastic route (Figure 9.1; Sauer, 2007).

Uptake of apoplastic sucrose is normally mediated by sucrose transporters but an alternative mechanism also exists which involves hydrolysis of sucrose into glucose and fructose by an invertase located in the cell wall, followed by import of these monosaccharides into the cell by hexose transporters

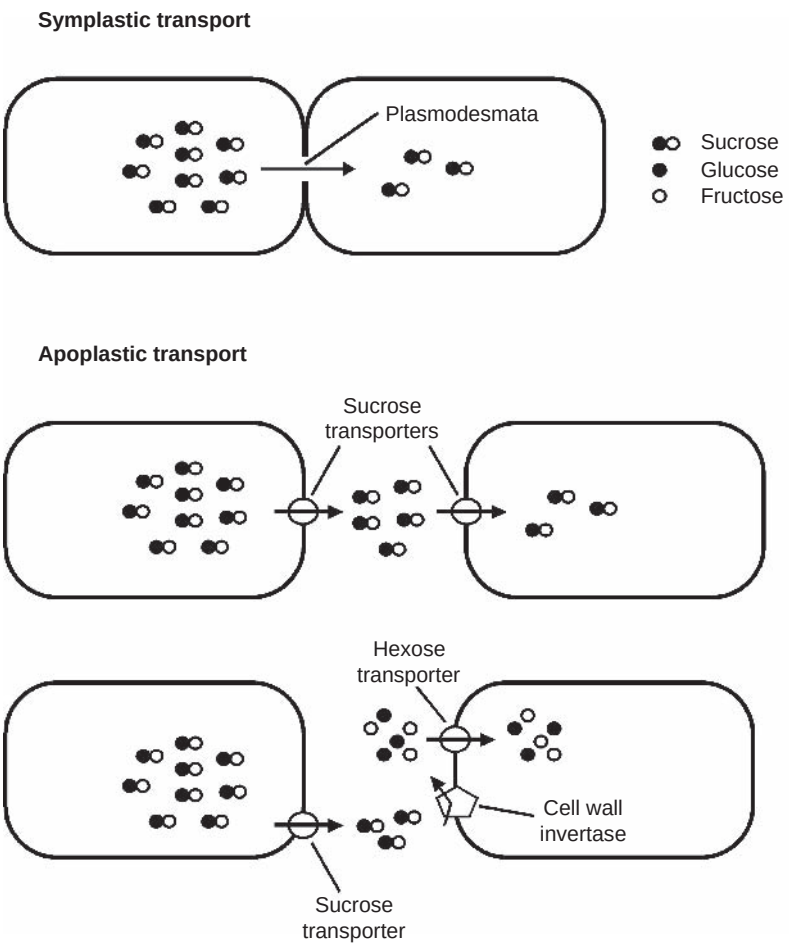

Figure 9.1 Schematic figure showing different routes for sucrose transport between adjacent plant cells. The same basic mechanisms are used also for phloem loading and unloading during long-distance transport in vascular plants (Sauer, 2007). The top panel shows symplastic transport via plasmodesmata whereas the two lower panels show different types of apoplastic transport. In the middle panel, the sucrose is exported from the first cell to the apoplasm from which it is imported into the next cell. Both the export and the import steps are catalysed by sucrose transporters. In the bottom panel, sucrose is first cleaved by a cell wall invertase into glucose and fructose which are then imported using monosaccharide transporters (Büttner, 2007).

(Figure 9.1; Büttner, 2007). This two-step mechanism may at first sight appear redundant, but it differs from direct sucrose uptake in two important ways (Sherson et al., 2003; Roitsch and González, 2004; Büttner, 2007). First, it reduces the local sucrose concentration in sink tissues and thereby contributes to the maintenance of the sink strength. Second, imported sucrose

is not necessarily hydrolysed into glucose and fructose, but can instead be converted into UDP-glucose and fructose in a reaction catalysed by sucrose synthase. Hence, import of glucose and fructose, as compared to direct import of sucrose, can produce different end products, and thus also feed different metabolic reactions and trigger different signalling pathways (Sherson et al., 2003). The importance of a proper balance between the two routes for uptake has been demonstrated in several studies. For example, during *Vicia faba* seed development, glucose stimulates cell division, while sucrose causes a switch to a storage and maturation phase (Weber et al., 1997).

9.2.2 Long-distance transport in mosses

Mosses and other bryophytes are traditionally classified as non-vascular plants. It is true that no moss species has vascular structures with all the diagnostic characters of tracheophyte vasculature (Ligrone et al., 2000). It is far from true though that mosses lack cells specialized in water and nutrient conduction. In fact, most mosses have water-conducting cells, normally referred to as hydroids, and also some kind of nutrient-conducting cells (Ligrone et al., 2000). These cell types are commonly found in the sporophyte seta, and in the stem and the leaf midribs of the gametophores. The picture is, however, complicated by a striking variation in the complexity of these structures between different groups of mosses.

Mosses belonging to the order Polytrichales have highly developed conducting strands (leptoids and hydroids) that share many structural and physiological properties with higher plant phloem and xylem. The nutrient-conducting cells of other mosses are less specialized than the leptoids of Polytrichales while some mosses, finally, appear to lack conducting cells all together (Ligrone et al., 2000). ^{14}C-sucrose feeding experiments in Polytrichales have demonstrated an organized long-distance carbon transport which supports the existence of a source/sink tissue relationship similar to that in seed plants. Thus, directed carbon transport is seen from adult leaves to sites with a high mitotic activity and/or a low photosynthetic capacity, including the growing stem tip, young leaves, developing bud initials and underground axes (Reinhart and Thomas, 1981).

Regarding nutrient and water transporting cell networks in *P. patens*, a conducting strand in the leaf midrib has been described (Sakakibara et al., 2003). It consists of both hydroids and nutrient-conducting cells (deuters or leptoids) and is structurally very similar to the conducting strands found in the leaves of the moss *Funaria hygrometrica* (Wiencke and Schulz, 1983). The leptoids of the latter have been described in detail and represent elongated cells connected by numerous plasmodesmata at their cross walls. They furthermore contain large numbers of longitudinal microtubules hypothesized to be important for the directional transport of organic compounds (Wiencke and Schulz, 1983). The *P. patens* leaf-conducting strands are clearly anchored in the gametophore stem and in transverse sections of gametophores, a bundle

of central stem hydroids is also evident (Sakakibara et al., 2003). Long-distance carbon transport in *P. patens* still remains to be characterized in detail, but based on structural features and extrapolation from other mosses, it appears likely that it is physiologically significant. Future studies in *P. patens* may reveal the extent to which the conducting tissues of mosses represent primitive phloem and xylem precursors.

9.2.3 Allocation in moss protonemata

The *P. patens* gametophyte has two distinct growth phases, the filamentous protonemata and the leafy shoots or gametophores. From its similarity to multicellular structures found in green alga, it seems likely that the protonemal mode of growth is more ancient in its origin. Despite its simplicity, protonemata comprise two distinct cell types, chloronemata and caulonemata. Chloronemal cells are photosynthetically more active, while caulonemata grow much faster, and thus help to expand the surface area of the plant. The caulonemata are also the cells from which gametophores normally develop through the formation of buds. A homeostatic regulation of the balance between chloronemata and caulonemata in response to the energy supply has been proposed to exist (Thelander et al., 2005). The physiological rationale for this hypothesis is the assumption that the formation of adventitious caulonemata should only be allowed if it can be afforded energy wise.

It is conceivable that carbon and energy in the form of sugars could be transported from the photosynthetically active chloronemata to caulonemata, in order to support the rapid growth of the latter. If such allocation exists in protonemata, it is likely to involve symplastic sugar transport. Plasmodesmata are known to connect the cytoplasms of protonemal cells both in *P. patens* (Abel et al., 1989) and in other mosses (Cook et al., 1997; Ligrone et al., 2000). It is, on the other hand, hard to see how apoplastic transport could work in a filamentous tissue type like protonemata.

The fact that plasmodesmata are present both in protonemata and in the nutrient-conducting cells of the gametophore supports the notion that symplastic sugar transport is likely to occur in *P. patens* and related species. A key question is whether apoplastic transport of sugars by transporters exists in mosses, or whether this transport route evolved after tracheophytes diverged from the bryophytes. Loading of extracellularly supplied sucrose into the leptoids of Polytrichales has been suggested to depend on active energy-dependent transport (Reinhart and Thomas, 1981) but this tells us little about the preferred routes for energy and carbon transport *in vivo*.

One way to address the question whether apoplastic sugar transport exists in mosses is to examine whether orthologues of plasma membrane sugar transporters and cell wall-associated invertases from angiosperms are also present in *P. patens*, since there would be little need for these proteins in

an organism, which only has symplastic sugar transport. In the absence of apoplastic transport, one might also expect to find a more limited use of sucrose, and thus a reduced set of genes involved in sucrose metabolism. We will therefore discuss current knowledge of a few relevant genes and gene families below.

9.3 Sucrose metabolism and transport

First, it should be noted that *P. patens* can use sucrose as an energy and carbon source, as shown by its ability to grow in total darkness in the presence of sucrose. Hence, the *P. patens* genome must encode all proteins needed for the metabolism of sucrose. This is not so surprising, since sucrose also has other metabolic functions which appear to be conserved in all plants, and even in cyanobacteria, such as being used as a precursor in polysaccharide and sugar-nucleotide biosynthesis (Salerno and Curatti, 2003). In fact, the two sucrose biosynthetic enzymes sucrose-phosphate synthase (SPS) and sucrose-phosphate phosphatase (SPP), and the sucrose-degrading enzyme sucrose synthase (SUS), which are found in all green plants, are believed to be descended from chloroplast enzymes, since these proteins have clear orthologues in cyanobacteria. With time, the corresponding genes moved to the nucleus, as frequently happens with organelle genes, and the encoded proteins are now expressed in the cytosol (Salerno and Curatti, 2003). The nuclear *P. patens* genome contains SPS, SPP and SUS genes, but as already discussed, this does not prove that sucrose is used for transport in *P. patens*. The situation with respect to the fourth sucrose-metabolizing enzyme, invertase, is more complex, and needs to be discussed in some detail.

9.3.1 Invertases

Invertase catalyses the irreversible hydrolysis of sucrose into glucose and fructose. Plants possess two distinct invertases, which are named after the pH optima of their enzymatic activities: acid invertase and neutral/alkaline invertase (Roitsch and González, 2004). The two invertases differ substantially in both function and sequence, which suggests a dual ancestry of the enzymes (Vargas et al., 2003). Acid invertases related to those in plants, exist in both *S. cerevisiae* and eubacteria, suggesting an ancient evolutionary origin for this family of enzymes (Sturm and Chrispeels, 1990). The genes encoding neutral/alkaline invertases, on the other hand, are likely to have evolved in photosynthetic cyanobacteria and were probably transferred to the plant nucleus after the endosymbiotic event that gave rise to plastids, similar to the SPS, SPP and SUS genes as discussed above (Vargas et al., 2003).

There are nine neutral/alkaline invertases in *Arabidopsis thaliana* which can be divided into two subgroups that differ significantly in sequence (Figure 9.2a). The same two sub-families are present also in *Populus*

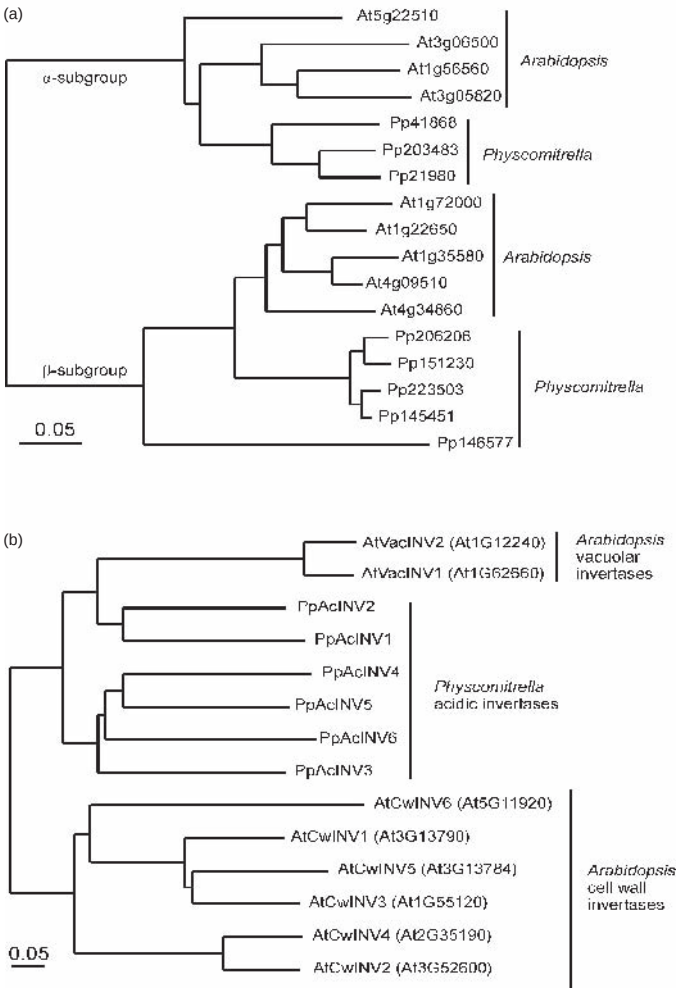

Figure 9.2 Phylogenetic trees based on amino acid sequence alignments of neutral/alkaline and acidic invertases, respectively, in *A. thaliana* (At) and *P. patens* (Pp). (a) Neutral/alkaline invertases. The sequences clearly fall into an α-subgroup and a β-subgroup, both of which have members from both *A. thaliana* and *P. patens*. The α-subgroup has been proposed to comprise organelle-localized enzymes whereas the β-subgroup would comprise cytoplasmic enzymes (Murayama and Handa, 2007). (b) Acidic invertases. The *A. thaliana* sequences form two distinct clades, one comprising the cell wall-localized invertases and the other comprising vacuolar invertases. The six *P. patens* sequences form two subgroups, both of which appear to be more closely related to the vacuolar group of *A. thaliana* invertases. The six *P. patens* sequences used for the alignment were manually deduced from the draft genome sequence (v.1.1) since none of the software generated gene models appeared to be accurate. Protein ID numbers are therefore lacking and instead the resulting sequences, which are available upon request, were named PpAcINV1-6. Except for these six loci, we also found a seventh locus showing clear homology to acidic invertases, but this locus contains two apparently truncated pseudogenes positioned in tandem.

trichocarpa, which suggests that they were created by a relatively ancient duplication event (Bocock et al., 2008). The neutral/alkaline invertases have traditionally been considered to be cytoplasmic enzymes, but this was recently questioned by Murayama and Handa (2007). Based on a combination of software predictions and experimental data, they suggested that while the β-subgroup are indeed cytoplasmic enzymes, the α-subgroup may represent mitochondrial and plastidic enzymes.

A survey of the *P. patens* genome sequence shows that it encodes eight putative neutral/alkaline invertases. From a phylogenetic tree (Figure 9.2a), it is clear that the division into an α-subgroup and a β-subgroup applies also to *P. patens*. Furthermore, the *P. patens* α-subgroup enzymes are more similar to the *A. thaliana* α-subgroup enzymes than to the *A. thaliana* β-subgroup enzymes, and *vice versa*. The gene duplication that generated the two subfamilies of neutral/alkaline invertases therefore clearly occurred before the divergence of mosses from vascular plants. It remains to be shown if the α-subgroup invertases are localized to mitochondria and plastids in mosses. If this is the case, studies of neutral/alkaline invertases in *P. patens* using reverse genetics could shed light on the relation between sub-cellular localization and function of these enzymes also in vascular plants.

The acid invertases of seed plants can also be further divided into two phylogenetically distinct sub-families that differ in their sub-cellular localization: the cell wall invertases and the vacuolar invertases (Figure 9.2b; Roitsch and González, 2004). A survey of the *P. patens* genome shows that it encodes six acid invertases. Interestingly, however, a phylogenetic comparison of the acid invertases in *A. thaliana* and *P. patens* places all six *P. patens* proteins in a position closer to the *A. thaliana* vacuolar invertases, while there are no clear orthologues of the *A. thaliana* cell wall invertases (Figure 9.2b). This might indicate that *P. patens* lacks true cell wall invertases and therefore also lacks the two-step mechanism of sucrose uptake discussed above and outlined in the bottom panel of Figure 9.1. By inference, a lack of cell wall invertases in *P. patens* would suggest that long-distance transport of sucrose via the apoplastic route plays a less prominent role in mosses.

However, it should be noted that the predicted *P. patens* proteins do not form a monophyletic group; two of them are in fact closer to the two vacuolar invertases from *A. thaliana* than to the other four enzymes from *P. patens*. By choosing an asymmetrically located root for the tree, it is therefore possible to group these four enzymes together with the *A. thaliana* cell wall invertases. This interpretation would in turn suggest that the *A. thaliana* cell wall invertases have evolved much faster than other members of this enzyme family, but examples of such accelerated evolution are common, particularly when a protein adapts to a new function or a different environment. It is conceivable that the development of vascular plants puts cell wall enzymes, including cell wall invertases, under additional selection which accelerated their evolution. Clearly, experimental work is needed to answer the question of whether *P. patens* has cell wall invertases or not.

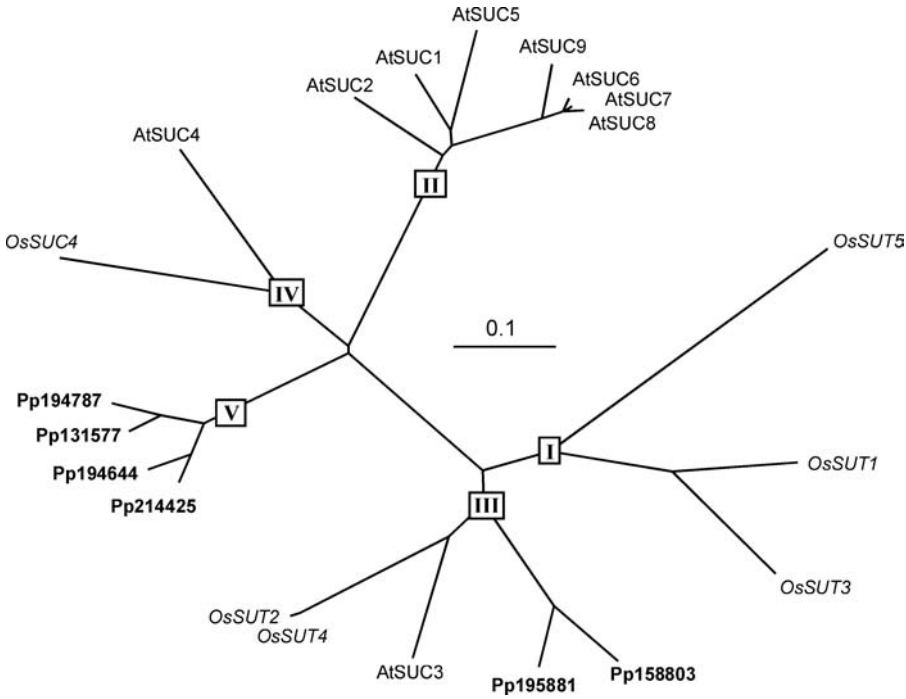

Figure 9.3 Phylogenetic tree based on an amino acid sequence alignment of sucrose transporters in *A. thaliana* (At), *O. sativa* (Os) and *P. patens* (Pp). The sequences fall into five distinct subgroups. Four previously described subgroups contain vascular plant members and have been assigned preliminary functions (Sauer, 2007). Thus, the monocot-specific subgroup I and the dicot-specific subgroup II are plasma membrane-localized transporters needed for phloem loading and sink cell import. Subgroup III, having representatives from all three organisms, is thought to mediate phloem unloading into the apoplasm in vascular plant sink tissues. The fourth subgroup (IV) containing both monocot and dicot sequences probably represents vacuolar transporters. Finally, the last uncharacterized group (V) only contains *P. patens* sequences.

9.3.2 Sucrose transporters

During apoplastic phloem loading and unloading in vascular plants, sucrose is transferred through the plasma membrane by active transport catalysed by a family of sucrose/H^+ symporters. This protein family has several members in both dicotyledonous plants (dicots) and monocotyledonous plants (monocots) which fall into distinct sub-families based on their sequences (Figure 9.3; Sauer, 2007). Furthermore, different sub-families have been suggested to have different functions based on expression patterns and functional characterizations in different vascular plants (reviewed in Sauer, 2007). One sub-family, which contains members from both monocots and dicots, has been proposed to mediate sucrose export from the phloem into the apoplastic space in sink tissues (III in Figure 9.3). In addition, there exist two distinct sub-families which are unique to monocots and dicots, respectively, and which are thought

to function in import of sucrose from the apoplasm into the phloem in source tissues, and into destination cells in sink tissues (I and II in Figure 9.3). Considering their proposed similar functions, these two sub-families are surprisingly divergent in sequence. Finally, there is a fourth sub-family, which has been proposed to encode vacuolar sucrose transporters, with members from both monocots and dicots (IV in Figure 9.3). Sucrose is known to be stored in the vacuoles for shorter or longer periods in several plants.

A survey of the *P. patens* genome sequence reveals six sequences potentially encoding members of the sucrose/H^+ symporter family. Two of the *P. patens* sequences group within the sub-family that contains sucrose transporters from both monocots and dicots, which are believed to be involved in sucrose export from the phloem (III in Figure 9.3). It therefore seems reasonable to assume that these two *P. patens* proteins also function in sucrose export. The other four *P. patens* sequences form a fifth unique group, which is not closely associated with any of the four groups in vascular plants (V in Figure 9.3). Their function and localization will therefore have to await experimental determination. In any case, the presence of several sucrose/H^+ symporters in *P. patens* suggests that sucrose needs to cross the plasma membrane (unless all six *P. patens* transporters are vacuolar proteins, which seems unlikely at least in the case of the two proteins that are related to the sucrose exporting transporters). This in turn suggests that apoplastic sucrose transport may be important in at least some cells and tissues.

9.4 Hexose metabolism and transport

The first step in the metabolism of both glucose and fructose is phosphorylation to glucose-6-phosphate and fructose-6-phosphate, respectively. Fungi and animals both have one major family of hexose-phosphorylating enzymes, the hexokinases (EC 2.7.1.1), and proteins belonging to this protein family are also found in plants. Hexokinases typically have the ability to phosphorylate both glucose and fructose though their specificity for these substrates may vary (Claeyssen and Rivoal, 2007). However, plants also possess a specific fructokinase (EC 2.7.1.4), which is unrelated to the hexokinases (Pego and Smeekens, 2000). The role of different hexose-phosphorylating enzymes in sugar sensing and signalling has been studied extensively in *S. cerevisiae*, where hexokinase plays an important role in glucose-dependent regulation of ScSNF1 activity (Rolland et al., 2002). Much of the work on sugar sensing and signalling in plants has therefore focused on the role of hexokinases, but as discussed below, there are also indications that plant fructokinases might have regulatory functions.

9.4.1 Hexokinases

Beside the fact that hexokinase catalyses the first step in glycolysis, which greatly influences overall carbon metabolism, it has also been proposed to

function as a hexose sensor in plants (reviewed by Rolland et al., 2006). The evidence for this dual role of hexokinases comes mainly from experiments in *A. thaliana*. Therefore, it remains to be proven that plant hexokinases in general have two distinct functions. If this dual function for hexokinase is found also in mosses, which diverged from the ancestors of the flowering plants early in land plant evolution, it would be reasonable to assume that it is conserved in all land plants.

The first evidence that plant hexokinases work as hexose sensors came with the discovery that *AtHXK1* and *AtHXK2* overexpression causes hypersensitivity to glucose. Plants expressing antisense constructs, on the other hand, were hyposensitive to glucose (Jang et al., 1997). In a screen for glucose-insensitive (*gin*) mutants in *A. thaliana*, two mutations in the *AtHXK1* gene were found. The mutant that has been most extensively studied so far is *gin2.* Phenotypic analysis of these mutants indicated that most of the defects could be explained by defects in glucose signalling. Complementation experiments in *A. thaliana* further suggested that the signalling function of AtHXK1 is independent of its enzymatic activity (Moore et al., 2003).

Studies of hexokinase-coding genes in *P. patens* initially focused on *PpHXK1* which was the first hexokinase-coding gene to be cloned. This hexokinase is also the major hexokinase in *P. patens*, accounting for 78% of the glucose phosphorylating activity and 47% of the fructose phosphorylating activity in protonemal tissue (Olsson et al., 2003). Surprisingly, it was found that PpHXK1 differs in localization from previously studied plant hexokinases such as AtHXK1. The latter possess N-terminal membrane anchors which target them to organelle membranes, primarily the outer mitochondrial membrane, though AtHXK1 was recently shown to be present also in the nucleus (Cho et al., 2006b). PpHXK1 has an N-terminal chloroplast transit peptide instead of a membrane anchor, and it is a soluble enzyme which is imported into the chloroplast stroma (Olsson et al., 2003). A survey of available sequences further suggested that most plants possess both chloroplast stromal hexokinases (type A hexokinases) and membrane-localized hexokinases (type B hexokinases) (Olsson et al., 2003). The prediction that the type A hexokinases are localized to the chloroplast stroma also in angiosperms (Olsson et al., 2003) has subsequently been confirmed in several species including *Nicotiana tabacum*, *Oryza sativa and Lycopersicon esculentum* (Giese et al., 2005; Cho et al., 2006a; Kandel-Kfir et al., 2006).

Even though PpHXK1 accounts for most of the glucose phosphorylating activity in moss, the *P. patens* genome potentially encodes no less than 11 hexokinases, localized to different sub-cellular compartments (Figure 9.4, Plate 13). Three of these, PpHXK1, PpHXK5 and PpHXK6, are chloroplast stromal enzymes, seven, including PpHXK2 (Olsson et al., 2003), have hydrophobic membrane anchors that target them to organelle membranes, and one, PpHXK4, is a cytosolic enzyme. A detailed characterization of the *P. patens* hexokinase family will be published elsewhere.

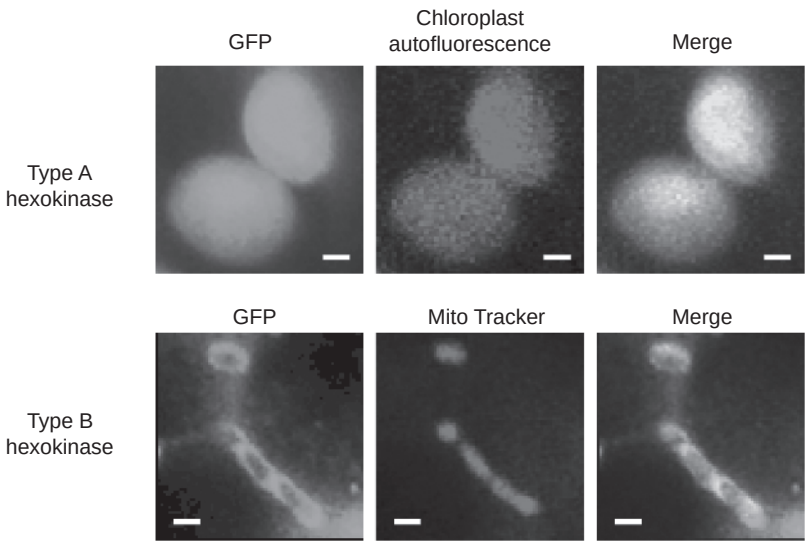

Figure 9.4 The intracellular localization of different hexokinases in *P. patens* visualized by transient expression of fusions of hexokinases to the green fluorescent protein (GFP). Type A hexokinases as shown in the upper panels have N-terminal chloroplast transit peptides, which target them to the chloroplast stroma as is evident from the GFP signal being localized inside the chloroplasts. The red signal shows chloroplast autofluorescence. The merge picture is an overlay of the signal from the hexokinase-GFP fusion protein and the chloroplast autofluorescence channel. Type B hexokinases as shown in the lower panels have N-terminal membrane anchors, which target them to organelle membranes. The GFP signal in this case shows that the hexokinase is localized to mitochondrial membranes. Mitochondria are visualized in red using the mitochondrion-specific dye MitoTracker Orange. The merge picture is an overlay of the signal from the hexokinase-GFP fusion protein and the MitoTracker Orange channel. The bars represent 1 μm. (For a color version of this figures, see Plate 13)

9.4.2 Functional studies of hexokinase

A key question is whether hexokinases in *P. patens* are involved in sugar sensing and signalling, as has been proposed to be the case in *A. thaliana*. In an attempt to study this, wild type and *hxk1* mutant *P. patens* strains were grown in the presence of externally added sugars (Olsson et al., 2003). In the wild type, a low glucose concentration (0.05 M) caused an increase in gametophore formation while an intermediate concentration of glucose (0.15 M) stimulated the formation of caulonemata. Interestingly, the latter effect is reduced several fold in the *hxk1* mutant, suggesting a possible role for PpHXK1 in glucose signalling (Olsson et al., 2003). However, further experiments showed that while caulonemata formation is generally reduced in the *hxk1* mutant, the positive effect of 0.15 M glucose is still of the same magnitude in the wild type and the mutant (Thelander et al., 2005). The response to glucose thus seems to be independent of PpHXK1 function.

The negative effect of the *hxk1* mutant on caulonemata formation is instead probably due to the mutant being in a state of permanent starvation since glucose phosphorylation is severely reduced in the mutant. Consistent with this interpretation, the mutant exhibits reduced growth in the dark in the presence of glucose and also under low-light conditions. As discussed further below (see the SnRK1 kinase), low energy conditions tend to inhibit the formation of adventitious tissues such as caulonemata and stimulate the formation of photosynthetically more active chloronemal cells (Thelander et al., 2005). As also discussed below, this developmental switch is likely to involve the SnRK1 kinase, the loss of which causes a massive overproduction of caulonemata (Thelander et al., 2004).

The absence of an effect of the *hxk1* mutant on glucose induction of caulonemata formation does not rule out the possibility that hexokinase is involved in glucose signalling in *P. patens* also, or that it might function upstream of the SnRK1 kinase, as it does in *S. cerevisiae*. First, the *snf1a snf1b* double mutant (see below) and the *hxk1* mutant have opposite phenotypes, which is consistent with, but does not prove, a model where a hexokinase-dependent signal inhibits the SnRK1 kinase. Second, hexokinases other than PpHXK1 may be involved in glucose signalling. It should be noted that PpHXK1 is located inside the chloroplasts, whereas AtHXK1 is located in the outer mitochondrial membrane (and also, to some extent, in the nucleus). There are in fact type B hexokinases in *P. patens*, such as PpHXK2 (Olsson et al., 2003), that have a membrane anchor which is very similar to that of AtHXK1, and it is not unreasonable to assume that one or several of these enzymes may have a similar regulatory function as AtHXK1 supposedly has in *A. thaliana*. Reverse genetics experiments in *P. patens* may help to answer this question.

9.4.3 Fructokinases

Fructokinase is an enzyme which is unrelated to hexokinase and has a more limited specificity, with fructose as its main substrate. Several fructokinases with different intracellular localizations have been described in angiosperms (Pego and Smeekens, 2000). Some of them are present within the chloroplasts and others in the cytosol (Jiang et al., 2003; Davies et al., 2005; Damari-Weissler et al., 2006). There is also evidence of differential expression of fructokinases in source and sink tissues (Kanayama et al., 1998; Jiang et al., 2003; Davies et al., 2005). The metabolic role of fructokinase is obvious, but as with hexokinase, it has been suggested that it also functions in sugar sensing and signalling. The evidence for this is mainly from genetic studies. Thus, an *A. thaliana* mutant that was isolated in a mannose-insensitive germination screen was identified as an *frk2* null mutation (Pego et al., 2000; Pego and Smeekens, 2000).

The *P. patens* genome contains eight genes potentially encoding fructokinases, while the *A. thaliana* and *O. sativa* genomes have seven and three genes, respectively (Figure 9.5). It should further be noted that the *P. patens* fructokinases form a monophyletic group that can be divided into two distinct subgroups (Figure 9.5). Fructokinases from vascular plants also form

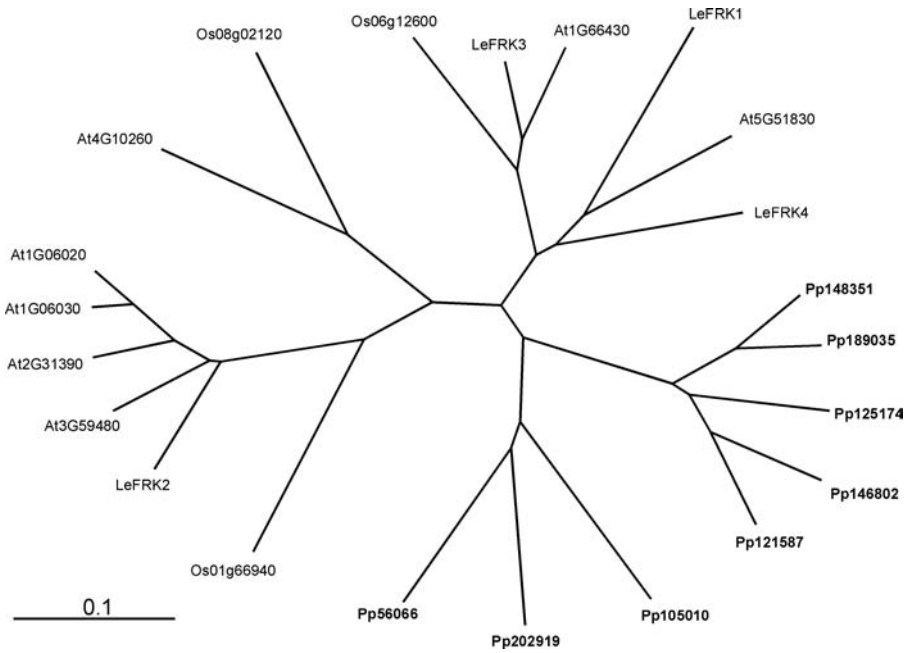

Figure 9.5 Phylogenetic tree of plant fructokinases. This dendrogram shows that there are two subgroups of fructokinases in angiosperms. Two subgroups are also present in *P. patens*, but the *P. patens* fructokinases are all more similar to each other than to either of the two groups in angiosperms. The dendrogram was based on an alignment of protein sequences from all retrieved fructokinase encoding genes in the genomes of *A. thaliana* (At), *O. sativa* (Os) and *P. patens* (Pp). The fructokinase family from tomato (Le) is the most well-characterized fructokinase family, and some tomato fructokinases are therefore also included, even though the complete genome sequence is not yet available in that case (Petreikov et al., 2001; Dai et al., 2002; German et al., 2002, 2004; Damari-Weissler et al., 2006).

two distinct subgroups, with members from both *O. sativa* and *A. thaliana* within each group (Figure 9.5). It has been proposed that these two subgroups of fructokinases function in source and sink tissues, respectively (Pego and Smeekens, 2000). It is conceivable that the two groups of fructokinases in *P. patens* represent the same two ancient sub-families, and that their pattern of expression could tell us something about carbon and energy allocation in moss. However, it would in that case have to be assumed that the moss genes underwent convergent evolution, perhaps mediated by ectopic recombination (i.e. non-allelic gene conversion events), in order to make them all more similar to each other than to their orthologues in higher plants.

9.4.4 Monosaccharide transporters

The *A. thaliana* genome contains no less than 53 genes with the potential to encode monosaccharide transporter-like (MST) proteins belonging to

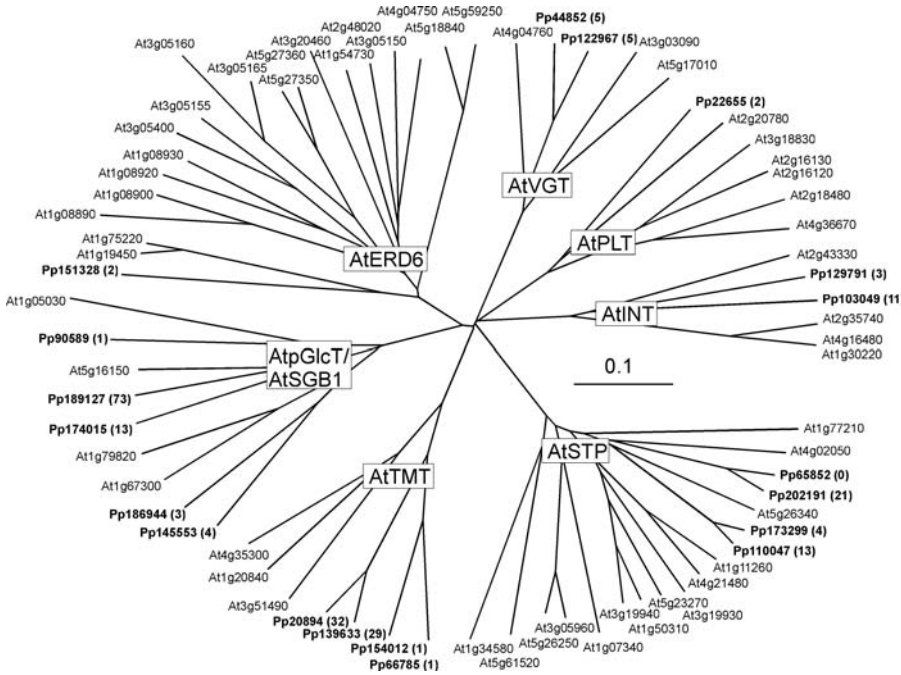

Figure 9.6 Phylogenetic tree based on an amino acid sequence alignment of monosaccharide transporters from *A. thaliana* (At) and *P. patens* (Pp). The sequences fall into seven previously described subgroups named after prominent members of this protein family in *A. thaliana* (Büttner, 2007). Each subgroup includes members from both *A. thaliana* and *P. patens*, which suggests that they are of ancient origin. Numbers within parentheses show the number of individual EST sequences that have been associated with each identified locus in the *P. patens* genome database (v.1.1).

seven clearly distinct sub-families, each with three or more members (Figure 9.6; Büttner, 2007). The majority of these potential proteins are likely to be monosaccharide/H^+ symporters that catalyse the transport of hexoses, but also in some cases polyols, across membranes. Surprisingly, little is known about the functions of most of these proteins but it is clear that some localize to the plasma membrane and are likely to mediate import of hexoses from the apoplastic space (Büttner, 2007). Other proteins localize to the membranes of different intracellular organelles such as vacuoles and chloroplasts, and are believed to be responsible for transport of monosaccharides within the cell (Büttner, 2007).

The *P. patens* genome has at least 19 genes which potentially encode proteins related to the *A. thaliana* MST super-family. Significantly, a phylogenetic analysis reveals that members of all seven *A. thaliana* sub-families exist also in *P. patens* (Figure 9.6). Johnson et al. (2006) has previously reported the presence of six of the seven MST sub-families in *P. patens*, but since their survey was based on expressed sequence tag (EST) data, they may have missed the single gene belonging to the missing class (Polyol Transporters; PLT) due to low

expression. It can be concluded that the organization of the plant MST genes into seven sub-families is ancient in origin and must have existed already in the last common ancestor of mosses and flowering plants. It is interesting to note that gene copy numbers within the MST super-family are generally lower in *P. patens* than in *A. thaliana* (Figure 9.6). The most prominent example is the poorly characterized ERD6 sub-family, which has 19 members in *A. thaliana* but is represented by a single gene in *P. patens* (Figure 9.6). Similarly, the PLT sub-family has six members in *A. thaliana*, but only a single member in *P. patens*. The much lower number of MST genes in *P. patens* makes functional analysis of this gene family by reverse genetics in *P. patens* a particularly interesting option.

The best-characterized MST sub-family in flowering plants is the AtSTP (*Sugar Transport Protein*) group of plasma membrane-localized monosaccharide/H^+ symporters (Figure 9.6; reviewed in Büttner, 2007). This subclass has 14 members in *A. thaliana*, most of which are expressed in different sink tissues. These proteins mediate uptake of hexoses, including but not limited to glucose, from the apoplastic space into sink tissue cells. In *P. patens*, there are four genes belonging to the AtSTP subclass of the MSTs. The existence of multiple EST sequences for three of the genes suggests that they are indeed expressed. This fact clearly indicates that *P. patens* is able to take up hexoses from the apoplastic space and/or its surroundings. The latter is no surprise, given the fact that *P. patens* can grow in darkness if provided with glucose as an energy and carbon source.

The physiological function of these plasma-membrane-localized hexose transporters in *P. patens* is not known but their presence suggests that apoplastic transport of hexoses between different cells may be important in mosses. It should be pointed out that hexose transport could be more important at certain developmental stages or during particular phases in the life cycle. It could, for example, be crucial for feeding of the zygote and the developing sporophyte by surrounding gametophytic tissue through so-called matrotrophy (Graham and Wilcox, 2000). It should also be pointed out that plasma membrane-localized hexose transporters have been suggested to function in retrieval of sugar molecules that are leaked out of cells or released as a result of cell wall degradation during growth and development (Sherson et al., 2000). In addition, it cannot be ruled out that *P. patens* uses these transporters to scavenge its surroundings for nutrients, in analogy to how plasma membrane sugar transporters function in *S. cerevisiae* and other microorganisms. Given the nutrient poor status of its natural biotope (fresh clay surfaces; Hallingbäck et al., 2006), such a function does, however, seem unlikely.

9.5 Energy homeostasis and Snf1-related kinases

The Snf1-related kinases (SnRKs) play a key role in regulating the energy status of the eukaryotic cell. Protein kinases that belong to this family exist in all eukaryotes, and have been suggested to function as cellular energy gauges

(Hardie et al., 1998). Thus, they are activated in response to a low energy status, and once activated, they act to restore the energy supply by stimulating energy-producing and inhibiting energy-consuming processes through phosphorylation of multiple substrates including both metabolic enzymes and transcription factors. The function of this kinase was first studied in *S. cerevisiae*, where it is known as the sucrose non-fermenting (ScSNF1) kinase, and in animals, where it is known as the AMPK. More recently, data has accumulated that support a role in energy homeostasis for members of this protein kinase family also in plants (Halford et al., 2003; Thelander et al., 2004; Baena-González et al., 2007). In animals and fungi, the kinase forms a heterotrimeric complex made up of a catalytic α subunit and regulatory β and γ subunits. Homologues of all three subunits exist also in plants, but the situation is more complicated due to the presence of larger and more diverse gene families, as further discussed below.

9.5.1 SNF1-related kinases in plants

Interestingly, the gene family encoding the catalytic α subunits of the SNF1/AMPK-related kinases is much more extensive and diverse in plants than in animals and fungi. Thus, in addition to the true orthologues of *S. cerevisiae* SNF1 and mammalian AMPK, the SnRK1 sub-family, plants also possess two divergent sub-families of SnRKs which are named the SnRK2 and SnRK3 kinases (Figure 9.7; Halford et al., 2003). These two plant-specific kinase sub-families differ significantly in sequence from the SnRK1 sub-family, and as discussed below, they are thought to mediate intracellular signalling triggered by abiotic stress rather than by cellular energy deprivation. Still, they are more closely related to the SnRK1/AMPK/SNF1 sub-family than to any other family of protein kinases (Hrabak et al., 2003). It is thus clear that all SnRKs share a monophyletic origin (Hrabak et al., 2003).

Furthermore, it is clear that the gene duplications which generated the SnRK2 and SnRK3 sub-families are much older than the separation of the crown eukaryotes (plants, animals and fungi) since the SnRK1 enzymes are more similar to their orthologues in animals and fungi than to the SnRK2 and SnRK3 enzymes (Figure 9.7). Accordingly, it seems likely that the SnRK2 and SnRK3 sub-families were present in a last common ancestor of the crown eukaryotes, but subsequently were lost in animals and fungi. Furthermore, we note that the *Chlamydomonas reinhardtii* genome contains SnRK1 and SnRK2 genes, but no SnRK3 gene (Figure 9.7), so it appears that the SnRK3 enzyme was lost in algae after their divergence from land plants.

9.5.2 The SnRK1 catalytic subunit

The *P. patens* genome has 16 genes with the potential to encode proteins that are related to the catalytic α subunits of the SnRKs. As shown by the dendrogram in Figure 9.7, it is clear that the three distinct sub-families of

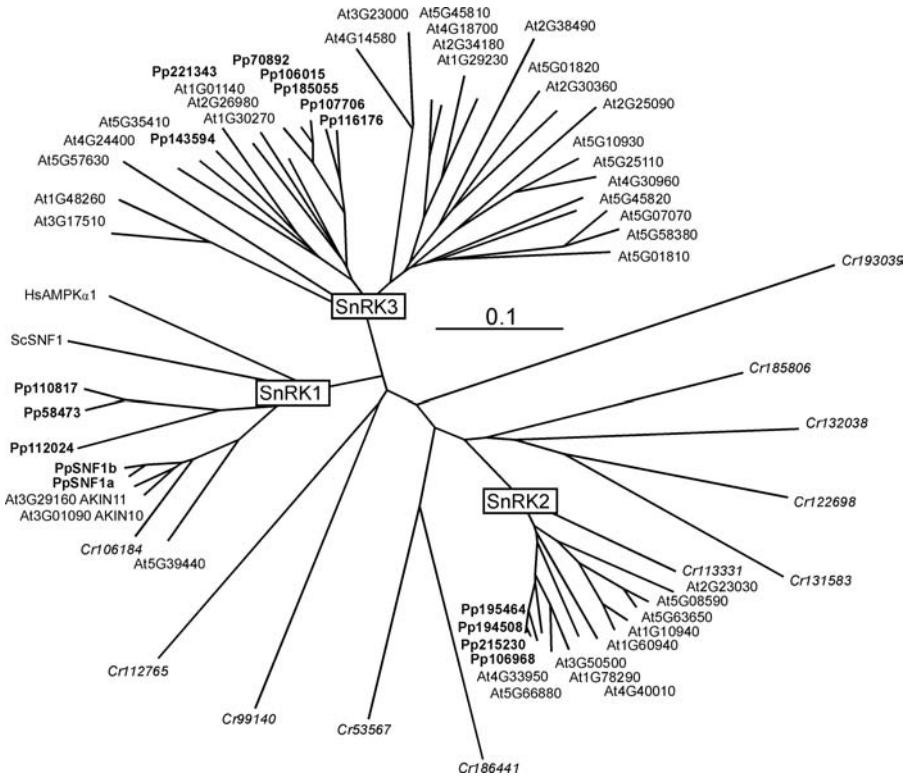

Figure 9.7 Phylogenetic tree of the catalytic α subunits of SNF1-related protein kinases. All *A. thaliana* and *P. patens* sequences clearly fall into one of the previously described sub-families: SnRK1, SnRK2 and SnRK3 (Halford et al., 2003; Hrabak et al., 2003). *C. reinhardtii* appears to have a single SnRK1 kinase and a single SnRK2 kinase but no SnRK3 kinase. However, there are also nine more divergent *C. reinhardtii* sequences which could not reliably be classified into one of the three sub-families. Most of these divergent sequences appear to be somewhat more similar to the SnRK2 group. The dendrogram was based on protein sequence alignments of the full complements of SnRK kinase α subunits in *A. thaliana* (At), *P. patens* (Pp) and *C. reinhardtii* (Cr). *Homo sapiens* (Hs) AMPKα1 and *S. cerevisiae* (Sc) SNF1 were included for reference. The alignment used to calculate the dendrogram was limited to the N-terminal kinase domains which, unlike the C-terminal regulatory domains, are clearly homologous across the whole super-family of SNF1-related kinases.

SnRKs that are found in flowering plants also are present in *P. patens*. In *A. thaliana*, there are 3 SnRK1 genes, 10 SnRK2 genes and 25 SnRK3 genes (Figure 9.7; Halford et al., 2003; Hrabak et al., 2003). In contrast, *P. patens* has as many as five genes belonging to the SnRK1 sub-family, while the other two sub-families have fewer members than in *A. thaliana*, with only four SnRK2 genes and seven SnRK3 genes. In *C. reinhardtii*, finally, there is one gene each clearly belonging to the SnRK1 and SnRK2 groups, but there are

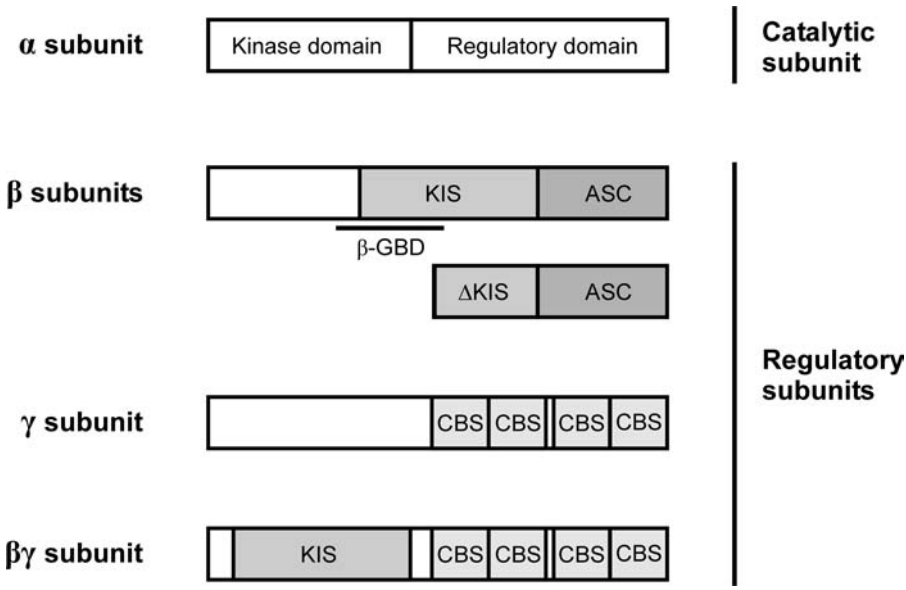

Figure 9.8 Schematic illustration of the domain structures of different SnRK1 subunits. The various domains within the different subunits are not drawn to scale. SnRK1 is the catalytic subunit in the SnRK1 complex which is also referred to as the α subunit. The catalytic subunit is composed of a kinase domain and a regulatory domain that interacts with the regulatory β and γ subunits. Two different types of regulatory β subunits have been identified in plants. The *Association with SNF1 Complex* (ASC) domain is conserved in both types of β subunits while the *kinase interacting sequence* (KIS) domain is partially truncated in the AKINβ3-like subunits. This truncated variant is not found in *P. patens*, and has so far only been found in vascular plants. In those β subunits that possess a full-length KIS domain, an overlapping glycogen-binding domain (β-GBD) has been identified in animals. There are at least two different types of γ subunits in plants which share the conserved domain structure with four Cystathionine-β-Synthase (CBS) motifs found in γ subunits from animals and fungi. The plant-specific βγ subunit also contains a KIS domain, which is usually found in β subunits, in addition to the conserved CBS domain.

no clear SnRK3 homologues. There are, however, nine additional genes in *C. reinhardtii* which cannot clearly be associated with any of the three families (Figure 9.7).

Two SnRK1 genes have previously been cloned and characterized in *P. patens* (Thelander et al., 2004). These genes, named *PpSNF1a* and *PpSNF1b*, encode highly similar proteins with the typical SNF1/AMPK architecture, composed of a conserved N-terminal kinase domain followed by a C-terminal regulatory domain (Figure 9.8). Both single and double knockout mutants were generated and a lack of apparent phenotypes in the former combined with a severe pleiotropic phenotype in the latter suggest that the encoded proteins have largely redundant functions *in vivo*. Assays of SnRK1-related kinase activity in the mutants showed that the *snf1a snf1b* double mutant was devoid of detectable SnRK1 activity.

As shown in Figure 9.7, PpSNF1a and PpSNF1b are very closely related to the two characterized SnRK1 kinases of *A. thaliana* (AtAKIN10 and AtAKIN11) as well as one SnRK1 kinase encoded by the *C. reinhardtii* genome (Cr106184). We conclude that this clade within the SnRK1 sub-tree, which is represented in all three species, most likely contains the true orthologues of the ScSNF1 kinase and the mammalian AMPK. In contrast, the other three SnRK1 sequences in *P. patens* and the third *A. thaliana* sequence are all located on divergent branches within the SnRK1 sub-tree, outside the clade containing AtAKIN10/11, PpSNF1a/b and Cr106184. This could mean that these sequences either are pseudogenes that have diverged more rapidly over time, or else encode proteins that have diverged due to selective adaptation to new functions. Our finding that the three extra SnRK1 genes do not contribute significantly to the total SNF1-like kinase activity in protonemal extracts (Thelander et al., 2004) is consistent with both these explanations. Preliminary studies further suggest that the additional three SnRK1-like genes are silent under normal growth conditions (Thelander, unpublished data). Still, it is possible that they are expressed only in specific tissue types or under specific conditions, and that, in those cases, they provide important functions. The same might be true for the divergent *A. thaliana* gene. Finally, this reasoning may also be valid for two of the nine divergent *C. reinhardtii* sequences discussed above, which appear loosely associated with the SnRK1 branch of the tree (Figure 9.7; Cr112765 and Cr99140).

9.5.3 The SnRK1 regulatory subunits

SnRKs in animals and fungi form a heterotrimeric protein complex made up of a catalytic α subunit and regulatory β and γ subunits (reviewed by Hardie, 2007). There are genes potentially encoding true homologues of both the β and γ subunits in *P. patens*, as in other plants. However, as discussed below, plants also possess unique regulatory subunits which are not found in animals or fungi. The functions of these extra regulatory subunits in plants are still poorly understood.

The role of the β subunit in the plant SnRK1 complex, as in animals and fungi, is not only to function as a platform which enables the α and γ subunits to align closely to each other, but also to recognize and bind to various SnRK1 targets (Lumbreras et al., 2001). It should be noted that there is a more diverse set of β subunits in plants than in *S. cerevisiae*, which has three β subunits (ScSIP1, ScSIP2 and ScGAL83), and in mammals, which usually possess only one or two β subunits. All β subunits share a common structure with two conserved domains: the internal kinase interacting sequence (KIS) domain and the association with SNF1 complex (ASC) domain (Figure 9.8). The work that revealed the functions of these two conserved domains was done in *S. cerevisiae* (Jiang and Carlsson, 1997), but it is believed that they have analogous functions also in β subunits from other organisms. Analysis of the AMPK β subunits in animals has further revealed a glycogen-binding

domain (β-GBD) which overlaps with the KIS domain (Hudson et al., 2003; Polekhina et al., 2003). This newly defined sub-domain is also found in two of the three *S. cerevisiae* β subunits (Wiatrowski et al., 2004).

The β subunit is also involved in sub-cellular localization of the kinase complex in both *S. cerevisiae* and animals (Vincent et al., 2001; Warden et al., 2001). Studies of plant β subunits have so far been performed mainly in *A. thaliana* (Bouly et al., 1999; Gissot et al., 2004). In one study, the two β subunits AtAKINβ1 and AtAKINβ2 were described in detail. They share the classical features of a β subunit, with a KIS and an ASC domain. An atypical β subunit, AtAKINβ3, has also been discovered in *A. thaliana*, which lacks the N-terminal part including most of the KIS domain (Gissot et al., 2004). This new β subunit type has been shown to interact with the other subunits of the SnRK1 complex.

An evolutionary tree with β subunits from different plants is shown in Figure 9.9. The *P. patens* genome encodes two β subunits of the classical type, which are related to AtAKINβ1 and AtAKINβ2 and possess both KIS and ASC domains. Interestingly, however, there are no atypical β subunits like AtAKINβ3 encoded in the *P. patens* genome. This group of proteins therefore seems to be present only in seed plants. It is conceivable that AtAKINβ3 has a more specialized function in certain cells or tissues that are absent in mosses. Consistent with the notion of a more specialized function, it has been reported that AtAKINβ3 is highly expressed in stems and pollen grains (Gissot et al., 2004).

The situation is even more complex for the regulatory γ subunits, since there are at least two distinct types of γ subunits in plants (Figure 9.8). *S. cerevisiae* and animal γ subunits share a conserved domain composed of four CBS (Cystathionine-β-Synthase) motifs. A γ subunit with this overall structure is present also in *A. thaliana* (Bouly et al., 1999). The sequence of this protein, named AtAKINγ, is, however, rather distantly related to those of the *S. cerevisiae* and mammalian γ subunits. Sequences that are more closely related to the latter are instead found among the so-called βγ subunits. These are hybrid proteins which possess both a domain homologous to the *S. cerevisiae* and animal γ subunits and an N-terminal part highly similar to the KIS and GBD domains found in β subunits (see above). Since plants store energy as starch, it has been proposed that the GBD domain is a starch-binding domain in plants. This atypical group of βγ subunits has so far been found only in plants (Lumbreras et al., 2001; Gissot et al., 2006; Hardie, 2007).

A survey of EST and genome sequences reveals that genes encoding both the γ and βγ subunits are present in *P. patens* (Figure 9.10). It therefore seems likely that all land plants possess these two distinct types of γ subunits. The reasons for this extra complexity in plants remain to be elucidated. Possibly, it is related to other functions of the SnRK1 kinases outside energy homeostasis. In this context, it is interesting to note that the KIS/GBD domain found in the βγ subunits has also been shown to interact with proteins suggested to be involved in plant pathogen resistance (Gissot et al., 2006).

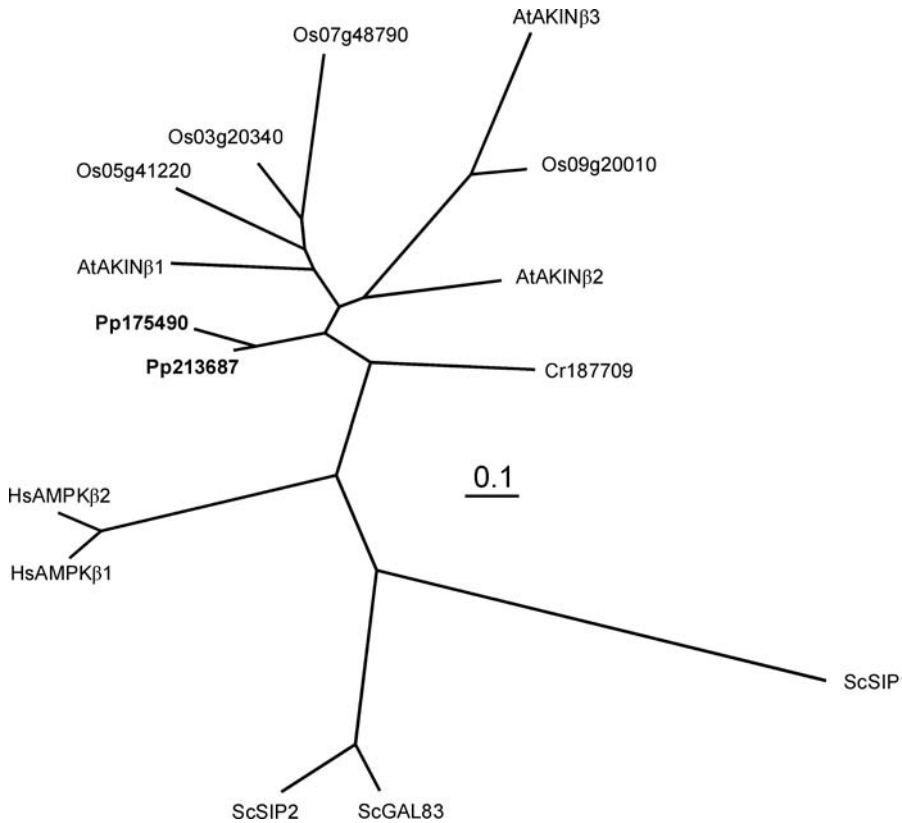

Figure 9.9 Phylogenetic tree of the regulatory SnRK1/SNF1/AMPK β subunits. All the plant β subunits group together, and the branching order fits with the evolutionary hypothesis that green algae is the most basal among the green plants. There are true β subunits present in all plants investigated but the atypical β3 subunits (Figure 9.8) are only found in the vascular plants (AtAKINβ3 and Os09g20010). The dendrogram was based on a protein sequence alignment derived from all retrieved β subunit encoding genes in *A. thaliana* (At), *O. sativa* (Os), *P. patens* (Pp) and *C. reinhardtii* (Cr). The β subunits from *H. sapiens* (Hs) and *S. cerevisiae* (Sc) were included for reference. The alignment used to calculate the dendrogram was limited to the conserved KIS and ASC domains (Figure 9.8).

9.5.4 Functional studies of SnRK1 kinases

The *snf1a snf1b* double mutant exhibits an array of complex phenotypes affecting both growth and development (Thelander et al., 2004). First and foremost, it requires a constant supply of energy in the form of either relatively high-intensity light or externally added carbohydrates in order to grow. Significantly, it is unable to grow in low-intensity light, but also in a normal day–night light cycle, where high-intensity light is present during 16 h each day. Thus, even if there is an abundant energy supply, the *snf1a snf1b* double

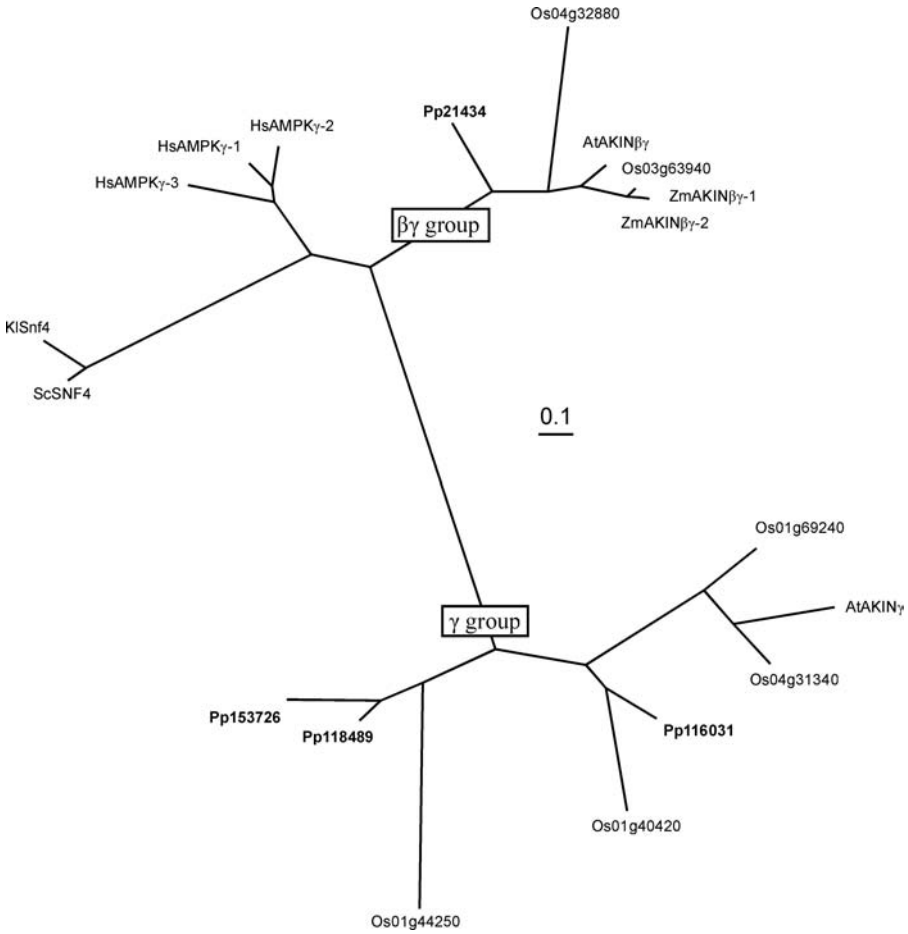

Figure 9.10 Phylogenetic tree of the regulatory SnRK1/SNF1/AMPK γ subunits. In plants, there are several different types of γ subunits, of which AKINγ and AKINβγ share the conserved structure with four CBS domains found in γ subunits from animals and fungi (Gissot et al., 2006). The tree shows that the γ and βγ subunits are very distantly related to each other, and furthermore, that the plant-specific βγ group is also present in *P. patens*. The dendrogram was based on a protein sequence alignment derived from all retrieved γ and βγ subunit encoding genes in the genomes of *A. thaliana* (At), *O. sativa* (Os) and *P. patens* (Pp). The initial discovery of the plant-specific βγ subunit was made in maize, and as a reference the ZmAKINβγ-1 and ZmAKINβγ-2 proteins were therefore also included (Lumbreras et al., 2001). For comparison, the regulatory γ subunits of the AMPK and SNF1 complex from Human (Hs) and the two yeast species *Saccharomyces cerevisiae* (Sc) and *Kluyveromyces lactis* (Kl) were also included. The alignment used to calculate the dendrogram was limited to the conserved region comprising the four CBS domains.

mutant is unable to handle rapid variations in this supply. This fits well with the proposed role for the SnRK1 kinases as cellular energy gauges (Hardie et al., 1998; Halford et al., 2003; Thelander et al., 2004).

However, the severe and pleiotropic phenotype of the *snf1a snf1b* double mutant suggests a more complex role, which also affects differentiation and development. Thus, the double mutant has an excess of tissues with low photosynthetic capacity (caulonemata, rhizoids) at the expense of photosynthetically active tissues (chloronemata, shoot part of gametophore). This suggests that plant SnRK1 kinases are needed not only for short-term regulation of metabolism in response to a reduced energy supply, but also for long-term adaptation which affects plant development. Our interpretation is that the SnRK1 enzyme suppresses the formation of adventitious tissue types with a low photosynthetic capacity under low-energy conditions when an increase in photosynthetic activity is needed (Thelander et al., 2004).

The importance of the SnRK1 kinase for changes in development in response to the metabolic status was recently confirmed in *A. thaliana*, where an inducible silencing system was used to circumvent the difficulty of generating an AtAKIN10 AtAKIN11 double mutant (Baena-González et al., 2007). This study supported the notion obtained from work in *P. patens* that plant SnRK1 is involved not only in metabolic control but also in growth and development (Thelander et al., 2004). The report also identified several hundred genes that are likely to be regulated by AtAKIN10 and AtAKIN11. Essentially, genes promoting catabolism and suppressing anabolism were found to be co-activated by AtAKIN10 and a limited energy supply. Finally, it was suggested that AtKIN10 and AtKIN11 might regulate target promoters via G-box binding bZIP transcription factors (Baena-González et al., 2007).

The potential role of the SnRK1 kinase in senescence is particularly intriguing. We found that a *P. patens snf1a snf1b* double mutant has an accelerated senescence phenotype, indicating a possible role for SnRK1 in the control of senescence in plants (Thelander et al., 2004). Similar observations have subsequently been made in *Medicago truncatula* (Rosnoblet et al., 2007) and *A. thaliana* (Baena-González et al., 2007). Previous work has also implicated the SNF1/AMPK kinase in the control of aging both in *S. cerevisiae* (Ashrafi et al., 2000; Lin et al., 2003; Harkness et al., 2004) and human fibroblasts (Wang et al., 2003). The precise nature of this connection remains to be elucidated, but the fact that SNF1/AMPK is activated under low-energy conditions suggests a possible role of regulating the metabolic rate, which is known to control aging in diverse organisms from *S. cerevisiae* to mammals. Indeed, resveratrol, which prolongs the lifespan by activating the ScSIR2 deacetylase, causes an increase in AMPK activity, and it has been proposed that the effect of caloric restriction on the lifespan in part is mediated by this mechanism (Baur et al., 2006). It remains to be seen to what extent senescence in plants is linked to energy homeostasis and the metabolic rate, but the fact that SnRK1 is involved in both (Thelander et al., 2004) suggests that such a link may exist.

9.5.5 Sugar sensing and regulation of SnRK1 activity

A major unresolved issue is the mechanism by which SnRK1 activity is regulated in response to changes in the cellular energy status. The key question is at what level is a reduced cellular energy status sensed and how? In *S. cerevisiae*, ScSNF1 is inhibited in the presence of glucose, a regulatory step which requires the major hexokinase ScHXK2 (Rolland et al., 2002). Hexokinase has been proposed to be a sugar sensor also in *A. thaliana* (Jang et al., 1997; Moore et al., 2003; Cho et al., 2006b), but recent evidence suggests that SnRK1 activity in *A. thaliana* is regulated in a hexokinase-independent way (Baena-González et al., 2007). It remains to be seen if hexokinase-dependent glucose signalling exists in *P. patens*, and if SnRK1 activity in *P. patens* is regulated by glucose or hexokinase. Studies of *snf1a snf1b hxk1* triple mutants may help to answer these questions.

Two novel putative SnRK1 regulators have recently been identified in *P. patens* (Thelander et al., 2007). The two proteins were isolated as SnRK1 interactors in a yeast two hybrid screen and were therefore named SnRK Interactor 1 and 2 (PpSKI1 and PpSKI2). Heterologous co-expression in *S. cerevisiae* showed that the novel proteins can inhibit the ability of PpSNF1a and PpSNF1b to complement a *S. cerevisiae snf1* mutation. This suggests that the novel proteins may inhibit SnRK1 activity *in vivo*. The phenotypes of *ski1* and *ski2* knockout mutants further suggest that the two proteins may function in regulation of development in response to a limited energy supply (Thelander et al., 2007). Thus, the *ski1* knockout exhibits an overproduction of leafy shoots during conditions of low energy supply, where leafy shoot formation is normally suppressed. Given this phenotype, and the ability of PpSKI1 and PpSKI2 to interact with PpSNF1a and PpSNF1b and inhibit their functions in *S. cerevisiae*, it is possible that PpSKI1 and PpSKI2 are involved in SnRK1-dependent control of differentiation in response to limitations in the energy supply. The *PpSKI* genes belong to a plant specific and previously uncharacterized gene family with representatives in all land plants, and their homologues may therefore have analogous functions in tracheophytes (Thelander et al., 2007).

It is well known that moss development is regulated by many of the same plant hormones that regulate seed plant development, including auxin and cytokinin (see Chapter 10). An obvious question is therefore if SnRK1-dependent regulation of plant development works independently of plant hormones, or if these signalling pathways act together. The extensive cross-talk between hormone response and sugar signalling pathways in plants suggests the latter to be likely. Consistent with this notion, we found that the *snf1a snf1b* double mutant is hypersensitive to the inhibitory effects of auxins on growth (Thelander et al., 2004). One possible explanation would be if reduced SnRK1 activity makes the cells more sensitive to auxin signalling, in which case the role of SnRK1 in development could be to reduce auxin sensitivity in certain cells and tissues when the energy supply is limited. An

alternative mechanism would be if SnRK1 activity inhibits auxin biosynthesis, in which case the increased auxin sensitivity of the double mutant could be due to an already elevated endogenous auxin level.

9.5.6 The SnRK2 kinases

As stated above, the SnRK2 kinases in seed plants are believed to be involved in signalling cascades that are triggered by osmotic stress and/or the stress hormone abscisic acid (ABA; Hrabak et al., 2003). Mutant analysis in *A. thaliana* has shown that SnRK2 kinases are required for a variety of classical ABA-regulated processes including seed dormancy (Fujii et al., 2007), stomatal aperture (Mustilli et al., 2002), root growth (Umezawa et al., 2004) and gene expression (Umezawa et al., 2004; Fujii et al., 2007). The involvement of an SnRK2 kinase therefore seems to be a hallmark character of most, if not all, ABA signalling regardless of the tissue-specific response which is triggered. The ten SnRK2 kinases encoded by the *A. thaliana* genome can be further subdivided into two classes, the first class being post-translationally activated exclusively by osmotic stress, while the second class is activated by both osmotic stress and the stress hormone ABA through apparently distinct mechanisms (Boudsocq et al., 2007).

There are four SnRK2 kinases in *P. patens*, which form a separate and internal clade within the *A. thaliana* SnRK2 sub-part of the SnRK tree (Figure 9.7). Their closest *A. thaliana* homologues are the well characterized and ABA-activated second class of SnRK2 kinases including At4G33950, which regulates stomatal aperture (Mustilli et al., 2002), and the closely related At5G66880 and At3G50500, which regulate seed germination and seedling growth (Fujii et al., 2007). This suggests that the SnRK2 kinases are likely to be involved in ABA-activated signalling also in mosses. This would further emphasize the ancient origin and conservation of the ABA signalling pathway, as already shown by the finding that the ABA-responsive *Em* promoter from wheat remains responsive to both ABA and osmotic stress when transferred to *P. patens* (Knight et al., 1995). A function for the *P. patens* SnRK2 kinases in ABA-mediated stress signalling is also in line with the recent finding that at least one of these genes is upregulated in response to drought (Cuming et al., 2007).

It is also interesting to note that the remaining seven *A. thaliana* SnRK2 kinases have no orthologues in *P. patens*. The single *C. reinhardtii* SnRK2-like sequence occupies a basal position in the SnRK2 sub-tree. Taken together, these findings suggest that the SnRK2 gene family has undergone a significant expansion in land plants. It is conceivable that this reflects adaptation to new functions and in particular to new forms of abiotic stresses such as drought in the new non-aquatic environment.

The role of the SnRK2 kinases in ABA signalling thus seems to be an ancient function which was already present in early plants. It is possible that the evolutionary forces that converted a kinase that regulates the energy

homeostasis and carbon metabolism into an effector in the stress response included the use of carbohydrates as osmo-protectants by eukaryotic cells. It should further be noted that energy limitation (starvation) is also a form of stress. It is possible that the ancestor of the SnRK2 kinases was a duplicated copy of the SNF1/AMPK kinase in an early eukaryote, which adopted a different mode of regulation, responding to other types of stress such as osmotic stress, while at least initially retaining its ability to regulate the carbohydrate metabolism.

9.5.7 The SnRK3 kinases

The SnRK3 group is the largest SnRK sub-family in vascular plants and is represented by 25 genes in *A. thaliana* (Figure 9.7; Halford et al., 2003; Hrabak et al., 2003; Kolukisaoglu et al., 2004). Functional characterization of vascular plant SnRK3 genes has suggested functions in diverse signalling pathways that are triggered either by different kinds of abiotic stress (Kim et al., 2003; Zhu, 2003) or by nutrient availability (Cheong et al., 2007; Pandey et al., 2007). This involvement in seemingly unrelated physiological responses may be due to their common function as sensors of intracellular Ca^{2+} signals.

Transient and highly specific changes in intracellular Ca^{2+} concentrations are triggered by a large number of environmental and cell-generated stimuli (Sanders et al., 2002). A specific Ca^{2+} signature, triggered by a given stimulus, must therefore somehow be recognized, that is, distinguished from other Ca^{2+} dependent signals, and converted into a proper stimulus-specific response. The SnRK3 sub-family represents one of several protein kinase families that serve as sensors of such unique Ca^{2+} signatures (Kolukisaoglu et al., 2004). Regulation of SnRK3 activity in response to Ca^{2+} is mediated by a calcineurin B-like (CBL) protein and a more common name for SnRK3 kinases is therefore CBL-interacting protein kinases (CIPK). To our knowledge, no systematic inventory for CBL proteins has been made in *P. patens* but at least four such genes are known to exist (Kolukisaoglu et al., 2004).

The 25 SnRK3/CIPK encoding genes in *A. thaliana* can be further subdivided into two groups based on protein sequence similarities (Figure 9.7; Kolukisaoglu et al., 2004). The significance of this grouping is further substantiated by the fact that genes encoding proteins in the smaller subgroup show conservation in their exon/intron organization while the members of the larger subgroup essentially lack introns (Kolukisaoglu et al., 2004). The same grouping and correlation with exon/intron organization is seen also among *O. sativa* SnRK3 encoding genes. There are seven putative genes in *P. patens* which clearly belong to the SnRK3 encoding genes and all group together with the smaller *A. thaliana* subgroup (Fig. 9.7). In analogy to their closest *A. thaliana* homologues, the moss genes all possess multiple introns. In contrast, there are no *P. patens* sequences grouping in the larger, intronless subgroup. This subgroup is therefore likely to have originated after the

divergence of mosses from tracheophytes, but before the separation between monocots and eudicots.

The seven *P. patens* sequences are found on a branch of the tree which is shared with five *A. thaliana* genes, out of which four have been assigned specific functions. Thus, *At5G35410* (*AtSOS2*, *AtCIPK24*) is needed for salt stress signalling (reviewed in Zhu, 2003), *At1G01140* (*AtCIPK9*) and *At1G30270* (*AtCIPK23*) are involved in signalling related to potassium deprivation (Pandey et al., 2007; Cheong et al., 2007) and *At2G26980* (*AtCIPK3*) is involved in cold and ABA signalling (Kim et al., 2003). Future studies will reveal whether these functions are shared also by the *P. patens* SnRK3 kinases. Finally, it should be emphasized again that we were unable to find any sequences in the *C. reinhardtii* genome that clearly belong to the SnRK3 subgroup (Figure 9.7).

9.6 Conclusions

One important question is whether *P. patens* (and other mosses) have mechanisms for energy and carbon allocation that involve apoplastic transport of sugars, similar to carbon allocation in seed plants. A definite answer to this question will have to await further experimental studies, but a wealth of indirect evidence suggests that this is the case. *P. patens* has no less than six sucrose transporters and numerous MSTs, at least some of which are likely to function in facilitated uptake of sugars across the plasma membrane. The fact that *P. patens* can use both sucrose and glucose as carbon and energy sources when grown in the dark supports the notion that it is capable of such transport. It is hard to see what purpose this transport could serve other than moving sugars from one cell to another within the plant. A scavenging function like that in microorganisms seems unlikely, and a transient role in sugar recovery during reassembly of the cell wall (Sherson et al., 2000), while possibly of some selective value, can hardly explain the evolution of a complex multigene system for sugar import.

Further support for the notion that transport of sugars may occur in *P. patens* comes from the studies of Sakakibara et al. (2003) who found water-conducting hydroids and nutrient-conducting cells both in the leaves and in the gametophore stem. It is harder to see how apoplastic transport could occur in the filamentous growth phase, but sugars could still move from cell to cell in that case through symplastic transport. It remains to be seen if the analogy to seed plants can be taken one step further, equating photosynthetically active chloronemal cells with source cells and adventitious cells like caulonemata and rhizoids with sink cells (Thelander et al., 2005). Another question which remains to be answered is whether sugars can play a hormone-like role in cell–cell signalling in moss, as has been suggested to be the case in angiosperms.

There is now considerable evidence that the SnRK1 kinase in plants has a function in energy homeostasis which is analogous to that of its orthologues

in animals and fungi, the AMPK and SNF1 kinases (Hardie, 2007). Our findings that SnRK1 in *P. patens* controls both energy metabolism during the day–night cycle as well as differentiation and senescence (Thelander et al., 2004) have recently been confirmed in *A. thaliana* (Baena-González et al., 2007) and are most likely true also in other plants. Still a number of important questions remain to be answered. How is SnRK1 regulated *in vivo*, and what are its most important downstream targets? In particular, the question whether SnRK1, like *S. cerevisiae* SNF1, is regulated by hexokinase-dependent glucose signalling remains to be determined. There is evidence from *A. thaliana* that SnRK1 is inhibited by glucose, but independently of hexokinase (Baena-González et al., 2007). Epistasis analysis with multiple knockout mutants in *P. patens* may help to provide a more definite answer to this question.

Another interesting question is to what extent SnRK1 functions in the control of senescence in plants. There is evidence that SNF1/AMPK affects ageing in both *S. cerevisiae* and mammalian cells (Ashrafi et al., 2000; Lin et al., 2003; Wang et al., 2003; Harkness et al., 2004), but the effect is still poorly understood. Possibly, it is connected to the well-known effect of caloric restriction, which delays ageing in many different organisms (Rine, 2005; Baur et al., 2006). A cell with excess SNF1/AMPK activity is in a state of artificial starvation, which to some extent resembles caloric restriction, while a cell that lacks this kinase is unable to sense and respond to starvation. We found that the *snf1a snf1b* double knockout in *P. patens* exhibits accelerated senescence (Thelander et al., 2004), which is consistent with an inhibitory effect of SnRK1 on cellular ageing in plants.

The functions of the two plant-specific families of SnRKs, the SnRK2s and SnRK3s, in *P. patens* remain to be determined. While there is substantial evidence from other plants that several of the SnRK2s are involved in ABA-dependent stress signalling, and that the SnRK3 kinases are involved in Ca^{2+}-dependent stress signalling, their precise roles are still poorly understood. Reverse genetics in *P. patens* may help to shed light on this, particularly since there are only four closely related SnRK2s and seven SnRK3s in *P. patens*, something which will facilitate the genetic analysis.

9.7 Technical note on database searches and tree construction

The moss and algal sequences used for tree construction were obtained by screening the draft sequences of *P. patens ssp. patens (v.1.1)* and *C. reinhardtii (v3.0)* derived at the Joint Genomics Institute. Prototype members of different protein families from *A. thaliana* or other seed plants were used as query sequences in homology searches using the TBLASTN algorithm with the default settings. For some proteins, their published names are used in the

figures. In most cases though, locus names are used for *A. thaliana* sequences, while the protein ID from the best existing gene model for a certain locus is used for most *P. patens* and *C. reinhardtii* sequences. Details about sequences from other organisms are given in the corresponding figure legends. All phylogenetic trees shown in this chapter were based on amino acid sequence alignments made using the ClustalX 2.0 software (Thompson et al., 1997) with default settings. Trees were computed from clearly homologous parts of the aligned sequences using the neighbour-joining method with corrections for multiple substitutions and exclusion of gaps in the alignments. The bars in the phylogenetic trees represent PAM values (percent accepted point mutations) as indicated in each figure.

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Chapter 10

HORMONAL REGULATION OF DEVELOPMENT BY AUXIN AND CYTOKININ IN MOSS

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Abstract: Since the early days of plant hormone research, bryophytes have attracted studies on developmental and reproductive processes. It is a striking characteristic of bryophytes that they respond to several plant hormones, and model plants such as the moss *Physcomitrella patens* therefore offer insights into the hormonal signalling processes that were established in a land plant lineage which has diverged from seed plants ca. 430 million years ago. This review aims to summarise knowledge on auxin and cytokinin in mosses, thereby outlining hormonal action on a morphogenetic and molecular level as well as biosynthesis and metabolism of these hormones. In this respect, the recent assembly of the *P. patens* genome sequence provides extremely valuable data enabling comparative approaches to decipher the evolution of hormone-mediated regulatory networks in plants.

Keywords: auxin; bryophytes; cytokinin; moss; phytohormones; plant hormones

Abbreviations:

Auxins

2,4-D	2,4-dichlorophenoxy acetic acid
IAA	indole-3-acetic acid
NAA	naphthylacetic acid
PCIB	parachlorophenoxyisobutyric acid (anti-auxin)

Cytokinins

BA	N^6-benzyladenine
BAR	N^6-benzyladenosine
cZ	*cis*-zeatin
cZR	*cis*-zeatinriboside
cZRMP	*cis*-zeatin-riboside-5′-monophosphate
cZOG	*cis*-zeatin-*O*-glucoside

cZROG	*cis*-zeatin-riboside-*O*-glucoside
DHZ	dihydrozeatin
DHZR	dihydrozeatinriboside
DHZROG	dihydrozeatinriboside-*O*-glucoside
iP	N^6-(Δ^2-isopentenyl)adenine
iPR	N^6-(Δ^2-isopentenyl)adenosine
iPRMP	N^6-(Δ^2-isopentenyl)adenosine-5′-monophosphate
mT	*meta*-topolin
oT	*ortho*-topolin
tZ	*trans*-zeatin
tZR	*trans*-zeatinriboside
tZRMP	*trans*-zeatin-riboside-5′-monophosphate
tZOG	*trans*-zeatin-*O*-glucoside
tZROG	*trans*-zeatinriboside-*O*-lucoside

10.1 Major plant hormone routes are established in bryophytes

Mosses are especially interesting organisms for plant hormone research, as they have a simple organ structure on the one hand but on the other hand respond to many plant hormones. The development of model plants such as *P. patens* comprises only a few cell types and differentiation processes, the generation of which is controlled and influenced by plant hormones. It is a striking beauty of this system that many regulatory mechanisms known from seed plants are already apparent in this early land plant. Deciphering hormonal processes in mosses and algae can provide essential information on the evolutionary steps involved in establishing the hormonal network that controls the morphogenetic systems of highly developed seed plants. The assembly of the *P. patens* genomic sequence (Rensing et al., 2008) together with tools available in the field of comparative genomics will in future allow deep insights into the organisation and evolution of genes involved in plant hormone homeostasis and signal transduction chains.

In Figure 10.1, Plate 14, the developmental steps and the morphogenetic effects of the plant hormones auxin, ABA and cytokinin are schematically summarised for moss. This review will mainly focus on auxin and cytokinin. The action of abscisic acid in moss, which is also well documented in the literature, will be treated in the context of stress tolerance in Chapter 11.

Setting the focus on auxin and cytokinin does not imply that other plant growth regulators are regarded as less important. Results on other plant growth regulators and other moss species will be mentioned if they complete the general picture, but cannot be reviewed in detail due to space limitations. Summaries of older work will be referred to by citing the corresponding review articles.

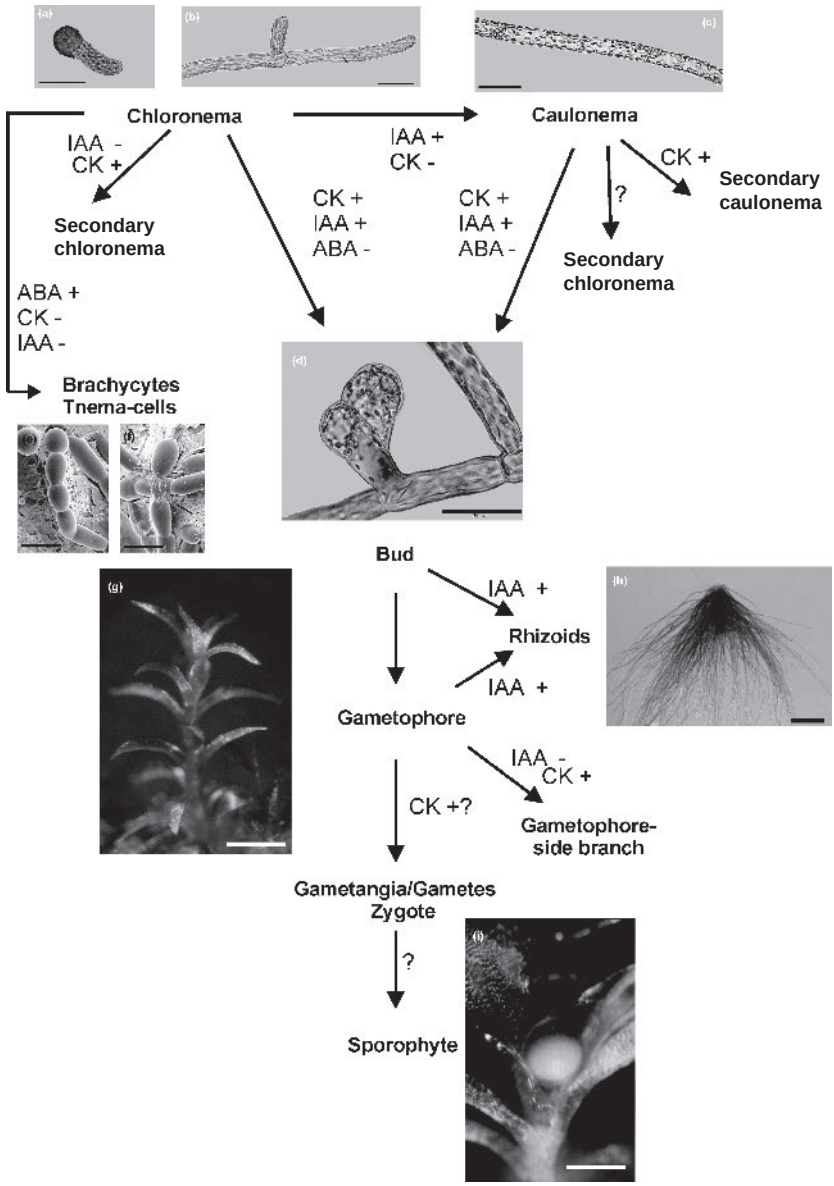

Figure 10.1 Simplified scheme summarising known morphogenetic actions of the plant hormones ABA, auxin (IAA) and cytokinins (CK) in mosses. '+' promoting effect, '−' inhibiting effect. For references, see Tables 10.1 and 10.2, and for ABA, see Table 11.1. Brachycytes (e) and tnema-cell (f) were visualised by scanning electron microscopy (*Source:* From Decker et al. (2006), printed with kind permission of University Freiburg). Rhizoids on gametophore (h) were induced by growth on 1 μM NAA during 6 weeks, picture was obtained by courtesy of K. Sakakibara (Monash Univ. Australia) and M. Hasebe (NIBB, Okazaki, Japan). Pictures (a)–(d), (g) and (i) were taken by S. Bringe, N. Wegner and K.v.S. (University Hamburg). Scale bars correspond to 50 μm in (a)–(f), to 350 μm in (g), (h) and to 150 μm in (i). (For a color version of this figure, see Plate 14)

10.2 Auxin

10.2.1 Auxin responses in moss

The effects of exogenously applied auxin on mosses were identified many years ago (Fries, 1943; Bopp, 1953; Gorton and Eakin, 1957). However, clear evidence that auxin acts as a plant hormone by inducing a specific morphogenetic response was not provided until the auxin-induced chloronema to caulonema transition in *Funaria hygrometrica* was discovered (Johri and Desai, 1973; Johri, 1975). Strong support for auxin being a morphogenetic substance came later from Sood and Hackenberg (1979), who were able to revert the differentiation of chloronema to caulonema by adding the anti-auxin PCIB (parachlorophenoxyisobutyric acid). The same authors also showed that cytokinin-induced bud formation was inhibited by the anti-auxin, thus demonstrating the dependency on auxin for both morphogenetic responses.

Genetic evidence for the implication of auxin in the development of mosses was obtained from complementation studies in which mutants of both *F. hygrometrica* and *P. patens*, that were impaired in certain developmental processes, were restored to the wild-type phenotype by addition of exogenous auxin (Hatanaka-Ernst, 1966; Ashton et al., 1979a; for reviews, see Ashton and Cove, 1990; Bopp and Atzorn, 1992a, 1992b). Examples for auxin-mediated growth responses in mosses are listed in Table 10.1.

Interestingly, the analogue 2,4-dichlorophenoxy acetic acid (2,4-D), which is a very potent auxin in seed plants, induces auxin responses in moss, such as stimulation of budding, only at far higher concentrations compared to naphthylacetic acid (NAA) or indole-3-acetic acid (IAA) (Bopp and Atzorn, 1992b).

Table 10.1 Examples of auxin effects on moss growth and development

Effect	Species	References
Influence on protonemal growth (cell division)	*Funaria hygrometrica*	Fries, 1943; Bopp, 1953
Induction of caulonema formation	*F. hygrometrica* *Physcomitrella patens*	Johri and Desai, 1973; Johri, 1975 Ashton and Cove, 1990
Repression of secondary chloronemal formation	*P. patens*	Ashton and Cove, 1990
Suppression of tmema cell formation	*F. hygrometrica*	Bopp and Atzorn, 1992b
Bud formation in synergy with cytokinins	*F. hygrometrica P. patens*	Gorton and Eakin, 1957; Bopp, 1990; Cove and Ashton, 1984
Suppression of lateral buds on gametophores	*Splachnum ampullaceum* *Plagiomnium cuspidatum*	Maltzahn, 1959; Nyman and Cutter, 1981
Induction of rhizoids on gametophores	*P. patens*	Sakakibara et al., 2003; see Figure 10.2

Based on phenotypic observations, the interaction of auxin and cytokinin was demonstrated by Cove applying a system of continuous medium replacement (drip-feed cultures). It became obvious that the simultaneous presence of both auxin and cytokinin is necessary to control the morphogenesis of moss with auxin being mainly responsible for caulonemal induction and cytokinin being mainly responsible for bud induction. These results, which included the analysis of auxin and cytokinin-resistant *P. patens* mutants, have been reviewed in detail by Cove and Ashton (1984), Ashton and Cove (1990) and Cove et al. (2006).

Cyclic AMP (cAMP) has been described as an antagonist for the auxin-induced chloronema to caulonema transition (Handa and Johri, 1976; Bhatla and Chopra, 1983). Although considerable information on the distribution and metabolisation of cAMP by cyclic nucleotide diesterases has been collected for *F. hygrometrica*, the exact role of cAMP in the hormonal network of mosses has not yet been elucidated. Here, it is referred to in the reviews of Bopp (1990) and Bhatla and Dhingra-Babbar (1990).

Another strong morphogenetic auxin response is the induction of additional rhizoids on gametophores (see Figures 10.1 and 10.2, Plates 14 and 15). The additional rhizoids originate mostly from stem epidermal cells in the cell files of the midrib cells, as is the case with non-treated gametophores (Sakakibara et al., 2003).

Auxin action in corn coleoptiles and parsley hypocotyls has been reported to result in a rapid increase of intracellular Ca^{2+} (Gehring et al., 1990) and a similar effect was also described for *F. hygrometrica* by Bhatla et al. (1996). Using a pharmacological approach, the distribution of L-type Ca^{2+}-channels was visualised in *F. hygrometrica* by *in vivo* staining with a fluorochrome-labelled phenylalkamine (Bhatla et al., 2002) and an activation of Ca^{2+}-channels in the apical region of polarly-dividing protoplasts was reported. Obviously, this is important for the establishment of an intracellular Ca^{2+}-gradient which is formed within minutes after IAA treatment and could be visualised indirectly by labelling Ca^{2+}-calmodulin complexes. A tip-to-base gradient of Ca^{2+} was demonstrated for chloronemal apical cells and related to the action of IAA (Bhatla et al., 2003). Interestingly, a rapid Ca^{2+}-influx has also been reported for the action of cytokinin in *F. hygrometrica* (Hahm and Saunders, 1991; Schumaker and Gisinski, 1993; Schumaker and Dietrich, 1998) and for abscisic acid in seed plants (Gehring et al., 1990). So far, the exact positions of the intracellular Ca^{2+}-increase in the hormonal signal transduction chains are unclear and need further elucidation regarding the specificity of the responses in the different target cells.

The interaction of hormone action and light requirement in the context of developmental processes has been subject to intensive investigations in the group of Cove (Cove and Ashton, 1984; Ashton and Cove, 1990; Cove et al., 2006). In work published by Imaizumi et al. (2002), the interaction between blue light and auxin responses in *P. patens* was corroborated at a molecular level by creating knockout mutants for the blue-light receptors cryptochrome

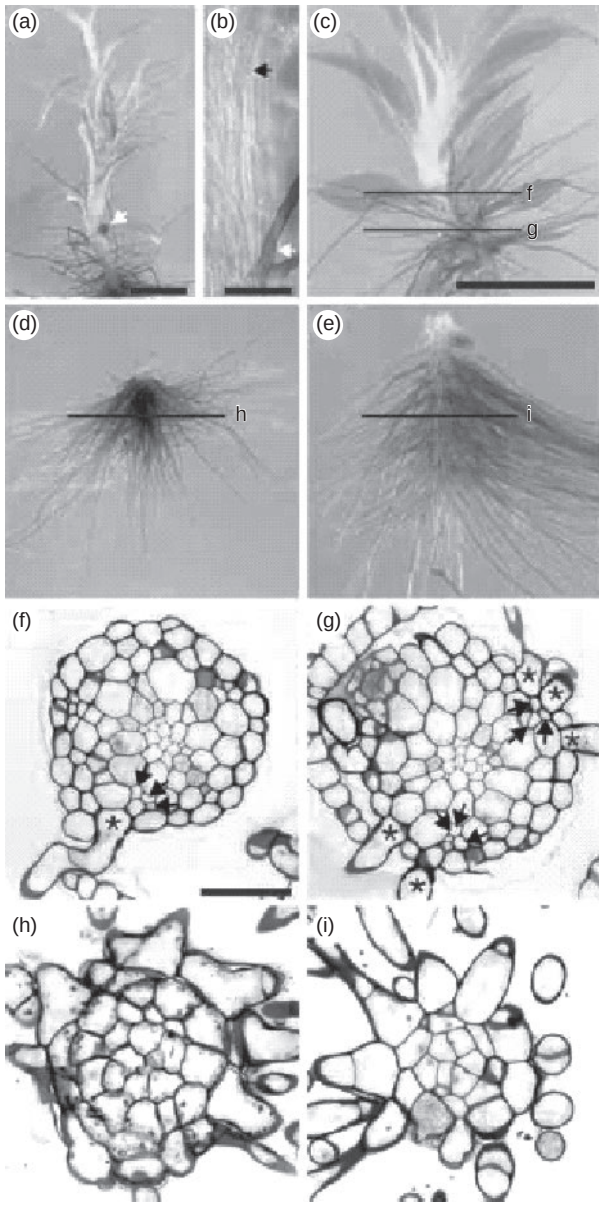

Figure 10.2 The effects of exogenous auxin on wild-type *P. patens*. (a) Gametophore cultured in 1 μM NAA for a week with adventitious gametophore (white arrow). (b) The uppermost mid-stem rhizoid of the gametophore (white arrow) in (a). The black arrow indicates a midrib. (c–e) Gametophores grown with 0.1 (c), 1.0 (d), and 10 (e) μM NAA for 6 weeks. The positions of the transverse sections in (f–i) are indicated by the lines. The asterisks and arrows in (f) and (g) indicate rhizoids and cells of the leaf traces, respectively. Scale bars: (a) 1 mm; (b) 100 μm; (c–e) 1 mm; (f–i) 100 μm. (*Source*: From Sakakibara et al. (2003), reproduced with permission of the Company of Biologists.) (For a color version of this figure, see Plate 15)

PpCRY1a and PpCRY1b. Using blue light, Imaizumi and co-workers showed that in *CRY* single or double knockout plants, the chloronema to caulonema transition, as one of the typical auxin responses, is enhanced compared with the wild type, thus revealing that cryptochrome specifically inhibits the auxin response. GFP-based localisation studies of the PpCRY gene products led to the model that this inhibition takes place in the nucleus. The CRY-mediated suppression of the auxin response was further supported at the level of gene expression for the auxin responsive elements *PpGH3L1* and *PpIAA1*. A strong increase in the activity of both homologous and heterologous *GH3*-promotors was demonstrated in the *cry1a cry1b* double knockout in the presence of NAA. In addition, higher levels of the *PpIAA1* transcript were found when compared against the wild type. *Aux/IAA* are well characterised in seed plants and represent mostly large gene families encoding for early auxin responsive elements which function as transcriptional repressors (Bishopp et al., 2006); for *P. patens*, two *AUX/IAA* orthologues have been listed (Rensing et al., 2008).

Imaizumi et al. (2002) also described a significant increase in the number of gametophores in the *cry1a and cry1b* knockout plants. Although the authors do not discuss this phenomenon in the context of auxin–light interaction, it seems possible that bud induction and proliferation, known to be promoted by auxin (Cove, 2005), was de-repressed in the CRY-deficient plants.

Further examples of auxin acting on gene expression in moss are the induction of *PpExpB2*, an expansin isoform in *P. patens* (Schipper et al., 2002) and the homeodomain-leucine zipper I gene *Pphb7*, which is expressed in the course of auxin-induced rhizoid formation (Sakakibara et al., 2003).

The fact that ten gene families involved in auxin homeostasis and signalling (including homologues for *SAUR, AUX1/LAX* and *PIN* genes) are present in *P. patens*, but are absent in the green algae *Chlamydomonas rheinhardtii* or *Ostreococcus tauri*, underlines the point that auxin signalling was established already in the bryophyte lineage. Auxin perception might involve the TIR1/AFB-like auxin receptors for which four orthologues are present in the *P. patens* genome (Rensing et al., 2008).

Although considerable progress has been made concerning the elucidation of auxin action, the picture of molecular events occurring during the perception of the hormone signal, and involving the transcriptional regulation of genes leading finally to the establishment of a morphogenetic response, remains incomplete.

10.2.2 Auxin metabolism, biosynthesis and transport

A long time after the demonstration of auxin action on moss (Bopp, 1953), the first unequivocal determination that IAA was an endogenous auxin of moss was achieved using gas chromatography mass spectrometry (Ashton et al., 1985). Quantities of 2.1 ng IAA g^{-1} dry weight were measured for *P. patens*. IAA was also shown to be an endogenous compound in *F. hygrometrica*

and the liverworts *Pellia epiphylla* and *Plagiochila arctica* (Thomas et al., 1983; Jayaswal and Johri, 1985; Law et al., 1985), and IAA is therefore assumed to be a ubiquitous auxin in bryophytes. To date, there is no detailed knowledge about the occurrence of other auxins known from seed plants such as indolebutyric acid or phenyl acetic acid (Ludwig-Müller, 2000; Ludwig-Müller and Cohen, 2002).

With respect to auxin biosynthesis in plants, there is broad evidence for several pathways contributing to the generation of IAA (Pollmann et al., 2006). While there is evidence that IAA biosynthesis in *F. hygrometrica* is tryptophan dependent (Jayaswal and Johri, 1985), Sztein et al. (2000) collected data for *Polytrichum ohioense* indicating that IAA formation is tryptophan independent.

Based on feeding experiments using tritiated tryptophan, a precursor of IAA in *F. hygrometrica*, it was concluded that, at least in protonemata, IAA biosynthesis takes the indole-pyruvate pathway (Jayaswal and Johri, 1985). This pathway includes a transamination reaction to give indole-pyruvyl acetic acid and its subsequent decarboxylation in order to form IAA. It is still not clear the extent to which the indole-pyruvate pathway is used for IAA formation in bryophytes. The analysis of the *P. patens* genome revealed six genes similar to YUCCA/FLOOZY flavin mono-oxygenases (Rensing et al., 2008), which catalyse the *N*-oxygenation of tryptamine to hydroxytryptamine being finally converted to indole-3-acetaldehyde and IAA (Zhao et al., 2001; Pollmann et al., 2006). The characterisation of further enzymes such as nitrilases, amidases and IAA synthases putatively involved in IAA formation should provide answers concerning the alternative pathways of auxin biogenesis.

For *F. hygrometrica*, there is evidence that moss-associated methylobacteria, classified as phytosymbionts, are a source of auxin providing sufficiently high quantities to influence growth (Hornschuh et al., 2002, 2006). The presence of methylobacteria in moss cultures was shown to enhance cell length and number. The inhibitory effect of the anti-auxin PCIB on protonemal growth could be reversed by adding methylobacteria to the cultures.

When measuring IAA in the *F. hygrometrica* mutant 87.13, which is unable to form caulonema without an exogenous supply of auxin, a reduced level of IAA compared with the wild type was clearly measured by an enzyme immunoassay (Atzorn et al., 1989; Bopp and Atzorn, 1992b). It was unclear whether the reduced IAA level was due to impaired biosynthesis or was caused by enhanced degradation. This question was answered by adding the IAA precursor tryptophan to both the mutant 87.13 and the wild type. Tryptophan application led to an increase of IAA in both genotypes indicating that the biosynthesis rate was not affected; however, the degradation was much faster in the mutant 87.13 compared with the wild type (Atzorn et al., 1989). By radiolabelling experiments using tritiated IAA, it was shown that IAA was first inactivated by the formation of IAA-aspartate, which was further oxidised to dioxyindole-aspartate. It is likely that GH3 gene products

which are known to catalyse the formation of IAA-conjugates in seed plants (Staswick et al., 2005) are involved in this conjugation reaction. Rensing et al. (2008) comment that *P. patens* genes with the potential to code for proteins similar to class II GH3 amidosynthetases (and IRL1/LL IAA amidohydrolase) do not group directly with the corresponding flowering plant genes.

In the *P. patens* genome, two GH3 encoding genes, *PpGH3-1* and *-2*, have been described and *GH3* single knockout lines did not exhibit a distinct phenotype (Bierfreund et al., 2004). Recently, functional studies on recombinant proteins revealed that PpGH3-1 and -2 are able to form IAA-conjugates, PpGH3-2 taking a large spectrum of amino acids as substrates (Ludwig-Müller et al., 2009). Mosses obviously employ a conjugation–hydrolysis strategy to control their levels of active auxin (Sztein et al., 1999, 2000; Cooke et al., 2002), in contrast to liverworts which use a biosynthesis–degradation strategy. As double knockout mutants for *PpGH3-1* and *PpGH3-2* have almost no IAA amide conjugates, there is now convincing evidence that *P. patens* GH3 proteins play an important role in auxin homeostasis (Ludwig-Müller et al., 2009).

Information on spatial auxin distribution and its changes during development was obtained by the generation of stable transformants carrying either the synthetic auxin-responsive element DR5 or a heterologous GH3 promoter transcriptionally fused to the β-glucuronidase gene (*GUS*) (Bierfreund et al. 2003). The analysis of the GUS staining of IAA treated and untreated DR5:GUS and GH3:GUS plants revealed that all cell types are able to respond to IAA, but the two promoters showed a different behaviour especially in the time needed to cause detectable GUS activity. Strong GUS staining was observed in buds of *P. patens* transformed with GH3:GUS but also archegonia and developing sporophytes indicated auxin responses (see Figure 10.3, Plate 16). Previous results showing IAA accumulation in protonemal tip cells (Bopp and Atzorn, 1992a) were confirmed and extended to the gametophore, where the highest auxin responses were found in the apex (Bierfreund et al., 2003). Recently, Fujita et al. (2008) confirmed the activation of the GH3-promotor in the apex of *P. patens* gametophores.

This latter finding seems important as it can be related to early reports on apical dominance in gametophores, which was shown to be mediated by the interaction between auxin and cytokinin (Maltzahn, 1959; Nyman and Cutter, 1981). The results of Bierfreund et al. (2003) and Fujita et al. (2008) demonstrate that the gametophore apex indeed represents a site of high IAA response, thus suggesting that a gradient of endogenous IAA might cause the suppression of lateral branching as demonstrated by Maltzahn as well as by Nymann and Cutter for exogenously applied IAA. As outlined below, Fujita et al. (2008) collected strong evidence that the auxin gradient in the gametophores is likely to be a consequence of basipetal diffusion from the site of biosynthesis in the apex.

As for seed plants, it can be assumed that auxin transport plays a crucial role for the coordination of hormonal responses. Evidence that auxin efflux is essential for cell division of isolated protoplasts of *P. patens* was given by

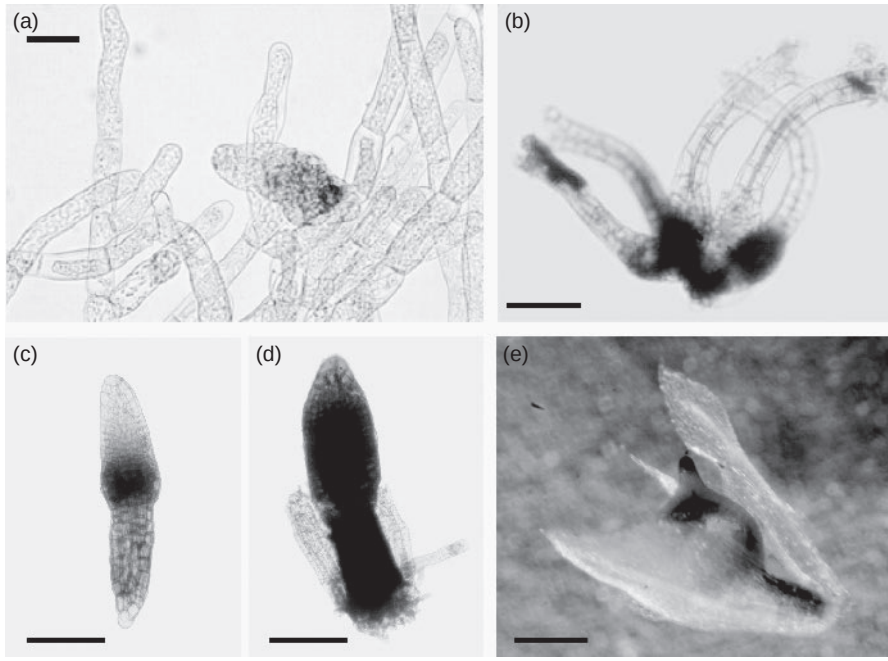

Figure 10.3 GUS expression in GH3:GUS transgenic *P. patens*. (a) GUS staining in developing bud, tissue was pre-incubated with 10 μM NAA prior to staining (bar represents 50 μm) (*Source*: From Bierfreund et al. (2003), reprinted with kind permission from Springer Science and Business Media). (b) GUS staining in basal part of archegonia (bar represents 50 μm). (c–e) GUS staining during sporophyte development (bar represent 50 μm in (a), (c) and (d), 20 μm in (b), and 125 μm in (e)). Experiments for (b)–(e) were carried out without NAA pre-incubation. (*Source*: Pictures (b)–(e) were obtained by the courtesy of E. Decker and R. Reski (University of Freiburg).) (For a color version of this figure, see Plate 16)

Bhatla et al. (2002). These authors showed that addition of the IAA efflux inhibitor naphthyl acetic acid (NPA, 20 μM) to the culture medium blocked cell division without visibly affecting protoplast viability.

PIN auxin efflux facilitators are known to be essential elements for the establishment of auxin gradients and polarity. For *P. patens*, PIN-like genes have not been functionally characterised so far but sequence comparisons place them close to the PIN5 group from seed plants (Paponov et al., 2005).

New insights on polar auxin transport in various moss species was published by Fujita et al. (2008). These authors carried out analyses of the distribution of radiolabelled auxins applied to gametophores and sporophytes and compared the results with those obtained for shoots of *Arabidopsis thaliana*. In vascular plants where, in contrast to mosses, shoots form in the diploid generation, it is well known that polar auxin transport is a prerequisite for morphogenetic pattern formation of shoots (Benková et al., 2003; Friml et al.,

2003). In gametophores, representing shoot-like structures in mosses, surprisingly Fujita and co-workers revealed no evidence for polar auxin transport. For cuttings of gametophores, it was shown that, in contrast to *A. thaliana* shoots, radiolabelled IAA moved from the basal end at similar rates as from the apex. The authors conclude that an auxin gradient forming in basipetal orientation from the apex is more likely to be a result of diffusion than of polar auxin transport. However, strong evidence for polar auxin transport was obtained in sporophytes of mosses, which do not form shoot-like structures. This was shown by monitoring the distribution of ^{14}C-IAA in the seta of sporophytes of several mosses and further supported for sporophytes of *P. patens*, where the activity pattern of the auxin-inducible GH3-promotor changed dynamically during development (Fujita et al., 2008). In younger stages of development, the GUS-signal was stronger in the apical part and in later stages stronger in the basal part. The lack of polar auxin transport in gametophytic haploid moss shoots suggests that the developmental mechanisms differ significantly from those in the sporophytic diploid flowering plants shoots (Fujita et al., 2008).

10.3 Cytokinin

10.3.1 Cytokinin responses in moss

Among the effects of cytokinins described for mosses (Table 10.2, see also Figure 10.1, Plate 14) are those which can be classified as non-specific growth responses. For example, growth of chloronema and caulonema was shown to be stimulated in *Ceratodon purpureus* as well as in *F. hygrometrica* (Szweykowska

Table 10.2 Examples of cytokinin effects on moss growth and development

Effect	Species	References
Promotion of chloronema and caulonema growth[a]	*Funaria hygrometrica, Ceratodon purpureus*	Szweykowska et al., 1971, 1972
Changes of length and width of protonemal cells	*Physcomitrella patens*	Schwartzenberg et al., 2007
Inhibition of chloronema to caulonema transition	*P. patens*	Thelander et al., 2005
Induction of branching of unbranched caulonema	*F. hygrometrica*	Bopp and Jacob, 1986
Induction of buds on protonema	Twenty-three species *Trematodon brevicalyx* *Hyophila involuta*	Listed in Cove and Ashton, 1984; Chopra and Dhingra-Babbar, 1984; Rahbar and Chopra, 1982
Reactivation of lateral buds on gametophores	*Splachnum ampullaceum*	Maltzahn, 1959

[a]Not a specific morphogenetic response.

et al., 1971, 1972). This growth response is not regarded as a morphogenetic hormonal cytokinin response as it can also be caused by addition of non-hormones such as adenine or adenosine (although at much higher concentrations). This non-specific effect might therefore be comparable to that of various species of algae reacting with enhanced growth rates when treated with cytokinins (see Mooney and van Staden, 1986).

It was observed that cytokinins have an impact on the differentiation of protonema. Thelander et al. (2005) demonstrated for *P. patens* that the application of cytokinins to chloronema blocks the formation of caulonemal cells, thus influencing the spreading of moss colonies on the substrate. In *F. hygrometrica*, it was shown that branching of caulonemata can be stimulated at very low concentrations (fmolar) of cytokinins (Bopp and Jacob, 1986).

The major point attracting many researchers to work with cytokinins and moss was the most obvious morphogenetic effect that cytokinins can induce budding on protonemata. Shortly after the discovery of kinetin (N^6-furfurylaminopurine) and its growth-stimulating and morphogenetic action on seed plant tissue (Miller et al., 1955, 1956), this cytokinin was also reported to influence the development of the moss *Tortella caespitosa* (Gorton and Eakin, 1957), where the addition of kinetin to protonemata resulted in a strong induction of buds. It was concluded that kinetin-like substances (together with auxin) play a crucial role for the onset of gametophores.

As a variety of cytokinin molecules including the isoprenoid forms (*trans*-zeatin, *trans*-zeatin riboside, isopentenyladenine) and aromatic forms, for example benzyladenine, have been shown to act on 23 different moss species, bud induction is generally accepted as being a major effect of cytokinin on moss protonemata (for a review, see Cove and Ashton, 1984). Christianson and Hornbuckle (1999) found that phenylurea-type cytokinins (thidiazuron and CPPU) also cause bud formation on *F. hygrometrica*.

Bopp (1979) established the concept that auxin and cytokinin act sequentially, based on developmental observations with the moss *F. hygrometrica*: auxin causing the transition from chloronema to caulonema and cytokinin inducing the formation of buds on caulonema (see Figure 10.1, Plate 14). Thus, the auxin action creates the target cells for the following cytokinin action. It should be noted, however, that for *P. patens*, in contrast to *F. hygrometrica*, cytokinins induce buds not only on caulonema cells but also on chloronema (Reski and Abel, 1985). At least for protonema of *P. patens*, and possibly other mosses, it is not possible to distinguish between cytokinin target cells (i.e. those that form buds after treatment with cytokinin) and non-target cells (Bopp, 1990).

The finding that there is a dose–response relationship for the concentration of cytokinins applied to the moss (Bopp and Diekmann, 1967) led to a bioassay for the estimation of cytokinin activity in biological samples (Hahn and Bopp, 1968). As no bud-inducing activity could be obtained with other plant hormones, this assay is considered as cytokinin specific and highly sensitive because concentrations as low as 15 nM (iP) can be detected.

Schumaker and Gisinski (1993) demonstrated that cytokinin-induced budding is mediated by the activation of Ca^{2+}-influx carriers. Apparently, active cytokinins acting on dihydropyridine-sensitive Ca^{2+}-channels cause a strong voltage-driven Ca^{2+}-influx. Convincing evidence that the stimulation of Ca^{2+}-influx is part of the cytokinin signal transduction chain was also provided (Saunders and Hepler, 1982, 1983; Conrad and Hepler, 1988; Hahm and Saunders, 1991). In this work, it was demonstrated that Ca^{2+}-agonists can cause bud induction in *F. hygrometrica* in the absence of cytokinins and it was accordingly shown that Ca^{2+}-antagonists blocked the cytokinin-induced budding.

Using *F. hygrometrica* as a model, the cytokinin-induced budding response was further dissected by Christianson (1998), who described two phases in the perception of the cytokinin signal. The transfer of protonemata between media with different cytokinin concentrations revealed that the number of buds formed is not determined in the early phase of hormonal perception. It was however shown that for the dose-dependent response, a later phase of cytokinin recognition is important, in which target cells become stably committed to buds.

Schwartzenberg et al. (2007) analysed the bud-inducing activity of 14 different cytokinins of isoprenoid and aromatic type in a *P. patens* bioassay. The nucleotides iPRMP, tZRMP, BARMP and the *cis*-zeatin forms cZ and cZR had no detectable effects, while the activities displayed by other selected cytokinins were in the following order: iP>tZ>BA>BAR>iPR>tZR>mT>DHZ>oT. These data generally confirm data previously obtained for *F. hygrometrica* by Whitaker and Kende (1974), who showed that cytokinin bases have considerably higher hormonal activity than the corresponding N^9-riboside forms. Also for *F. hygrometrica*, a low activity of cZ and cZR had previously been reported by Spiess (1975), thus confirming results obtained for seed plants. Astonishingly, cZ-type cytokinins showed no bud-inducing activity at all in *P. patens*. For seed plants, the low activity of N^9-ribosides (Skoog et al., 1967) as well as of cZ cytokinins has been reported (Schmitz et al., 1972; Mok et al., 1978). These similarities in the pharmacology of the cytokinin response point out that the basic mechanisms in the perception of different cytokinin forms are conserved between mosses and seed plants.

Interesting conclusions can also be drawn from experiments taking a complementary approach in which moss cultures are deprived of hormones. A loss of endogenous cytokinins in moss can be achieved by overexpressing cytokinin catabolic genes such as cytokinin oxidase/dehydrogenase (CKX). CKX irreversibly inactivates cytokinins by cleaving off the N^6-side chain from the adenine residue (Werner et al., 2006) (Figure 10.4a). The constitutive overexpression of the *A. thaliana AtCKX2* gene in *P. patens* mainly resulted in a reduction of extracellular iP and iPR (Schwartzenberg et al., 2007). Phenotypic changes related to the loss of cytokinins occurred in protonemata as well as in gametophores. Chloronemal filaments showed irregular growth and cells tended to be shorter exhibiting at the same time an increased diameter. The onset of budding was reduced and retarded due to the increase in cytokinin

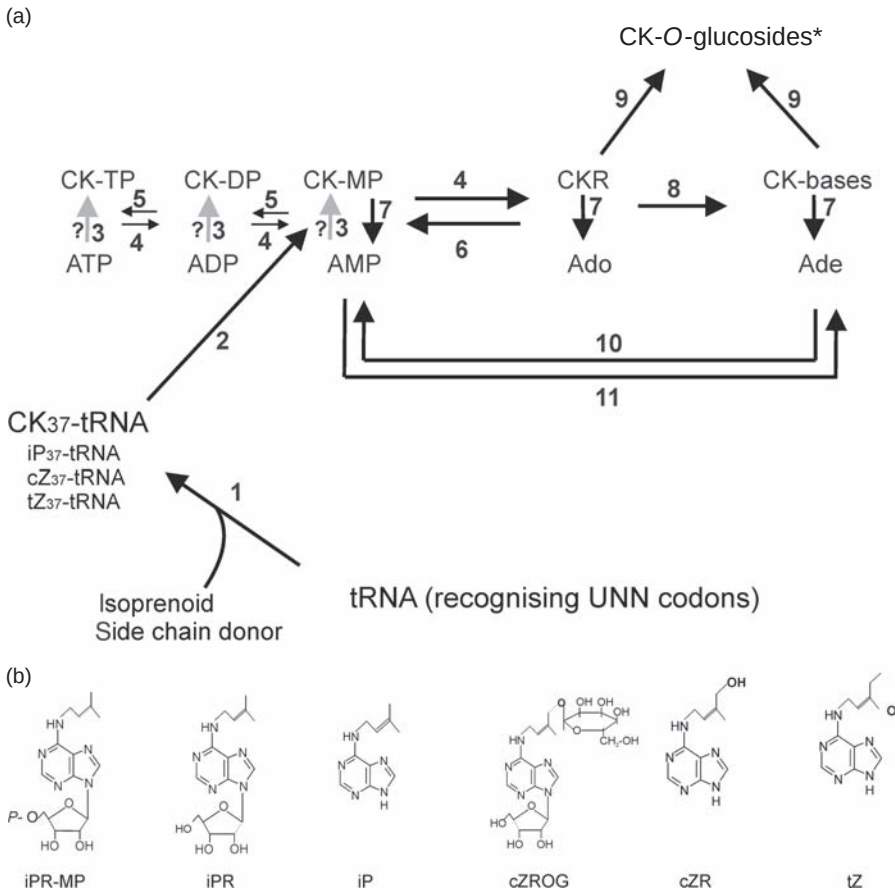

Figure 10.4 (a) A simplified scheme summarising isoprenoid cytokinin biosynthesis, interconversion and metabolism as proposed for *P. patens*. Ade – adenine, Ado – adenosine, AMP, ADP, ATP – adenosine-5′-mono, di-, tri-phosphate (respectively), CK – cytokinins, CKR – cytokinin ribosides, CK-MP, CK-DP, CK-TP – cytokinin mono-, di-, tri-phosphates (respectively). * – only zeatin forms (1) tRNA-isopentenyltransferase (tRNA-IPT); (2) tRNA degradation; (3) adenylate isopentenyltransferase (not identified in *P. patens* genome); (4) nucleotidase/phosphatase; (5) adenylate kinase; (6) adenosine kinase (ADK); (7) cytokinin oxidase/dehydrogenase (CKX); (8) adenosine nucleosidase; (9) zeatin-*O*-glycosyltransferase; (10) adenine phosphoribosyl transferase; (11) cytokinin phosphoribohydrolase. (b) Major cytokinins detected in *P. patens* (see Schwartzenberg et al., 2007). iPR-MP – isopentenyladenosine-monophosphate, iPR – isopentenyladenosine, iP – isopentenyladenine, cZROG – *cis*-zeatin-riboside-*O*-glucoside, cZ – *cis*-zeatin, tZ – *trans*-zeatin.

degradation, as expected. This developmental effect of CKX-overexpression could be partially reverted by adding iP to the culture medium.

The gametophores forming on cytokinin-deficient plants showed a reduction in size and cell number in leaves in comparison with the wild type. On agar plates, gametophores from cytokinin-deficient plants turned out to

be sterile, and under intensities of white light above 100 $\mu E/m^2/s$, CKX-transformants tended to turn pale and white after 4–6 months whereas wild-type cultures stayed green (Fernandez Nunez, Turčinov, Schwartzenberg, unpublished).

Thus, it was concluded that endogenously produced cytokinins are involved in (a) the regulation of protonemal development, (b) the regulation of budding initiation and (c) gametophore development and generative multiplication. In summary, analysis of cytokinin-deficient *P. patens* plants to some extent confirms previous studies and also supplements and extends results obtained from the application of external hormones. The altered protonemal morphology caused by the CKX-overexpression underlines the significance of cytokinins for protonemal growth, which was also outlined for exogenously supplied cytokinins promoting protonemal cell division in *F. hygrometrica* and *C. purpureus* (Szweykowska et al., 1971, 1972). However, other cytokinin roles, such as the influence on gametangia formation as well as on gametophore viability, have not yet been described and need further detailed analyses.

Many experiments have attempted to identify the causal chain from cytokinin perception to morphogenetic change. Cytokinins have been reported to cause various molecular and biochemical changes, which might be involved as intermediate steps of morphogenic responses or which could also be physiological responses independent from developmental processes (Cove et al., 2006).

The *P. patens* mutant PC22, which is blocked in gametophore development as well as in chloroplast division (Abel et al., 1989), was analysed intensively for molecular changes after the addition of cytokinins. This mutant could be rescued by external application of cytokinins as well as by overexpression of the *IPT* (isopentenyltransferase) gene from *Agrobacterium tumefaciens*; a high number of malformed buds occurred in the *IPT* transgenics due to cytokinin overproduction (Reutter et al., 1998; Schulz et al., 2000). The ability to complement the mutation in PC22 led to numerous approaches in the laboratory of R. Reski to describe cytokinin action at a molecular level. In PC22 plants grown under white-light conditions, cytokinins were shown to induce a transient increase of *rbcL* transcripts (Reski et al., 1991). Under blue-light conditions, however, this effect was not observed, thus suggesting a phytochrome-mediated interaction of light and cytokinin signalling.

In chloroplasts, it was shown that the mRNA stability of a certain plastidic transcription unit, which is increased in the PC22 mutant, was reduced to wild-type levels by the addition of cytokinin (Kruse et al., 1995). It was also demonstrated that cytokinins modulate transcription of the plastome-encoded *zfpA* gene (Kasten et al., 1992).

At the protein level, Neuenschwander et al. (1994) reported the specific induction of a 14 kDa extracellular protein by cytokinin and Kasten et al. (1997) showed that several nuclear and plastome-encoded proteins accumulate in chloroplasts of cytokinin-treated plants. As it has been demonstrated in seed plants that the cytokinin signal transduction chain involves phosphorylation

relays, a study on the phosphoproteome was carried out by Heintz et al. (2006). Among 172 phosphorylated proteins, 13 were identified for which the abundance was specifically altered by cytokinin action within minutes of hormone application. None of these 13 phosphoproteins had previously been shown to be involved in cytokinin signalling, thus promising new future insights into cytokinin signalling components of moss.

As the cytokinin-induced budding response is a clear example of hormonal action on a single cell, it served as a model for the description of hormone-mediated growth control on biochemical, molecular, anatomical and ultra-structural levels, and a high number of publications emerged on this topic (see reviews by Bopp (1979, 1990), Bopp and Atzorn (1992a, b), Cove (1992, 2005), Cove and Ashton (1984), Cove et al. (2006), Schumaker and Dietrich (1997, 1998), Reski (1998), Decker et al. (2006) and Schwartzenberg (2006)).

Brandes and Kende (1968) showed a strong accumulation of radiolabelled ^{14}C-benzyladenine in bud-forming protonemata of *F. hygrometrica*, whereas non-target cells only contained a small amount of the radiolabel. Similar results were described by Erichsen et al. (1978) using ^{14}C-Kinetin. The reason for the strong binding of radiolabelled cytokinins in caulonemal cells was seen in the presence of caulonema-specific proteins (CSFs), which were regarded to be related to the competence of these cells to respond to cytokinins (Erichsen et al., 1977; Bopp et al., 1978). Although *in vitro* binding studies provided evidence that there are three receptor-like proteins responsible for cytokinin binding (Gardner et al., 1978), a detailed report on cytokinin receptors has not yet been published for any moss. However, *in silico* searches in the *P. patens* genome reveal the presence of three genes encoding histidine-kinases (Rensing et al., 2008; see also Troullier et al., 2004) similar to the two component cytokinin receptors CRE1, AHK2 and AHK3 known from *A. thaliana* (Kakimoto, 1996; Inoue et al., 2001; Suzuki et al., 2001; Schmülling, 2004). These putative cytokinin receptors contain the CHASE domain, which is responsible for cytokinin binding (Heyl et al., 2007). Reverse genetic studies on all three receptors will show how far each of these is responsible for the budding response in moss. The expression of GFP-fusion proteins will show whether the cytokinin receptors accumulate in target cells. Beyond receptor proteins, *P. patens* contains genes for all members of the cytokinin signal transduction pathway known so far in plants (Rensing et al., 2008).

10.3.2 Cytokinin metabolism, distribution and biosynthesis

The first report on cytokinin extracted from moss was given by Bauer (1966), who isolated a kinetin-like compound from a hybrid callus derived from *F. hygrometrica* (L.) Sibth.x *Physcomitrium pyriforme* Brid. The compound was found in micromolar concentrations in tissue as well as in culture medium and was named 'bryokinin' until Beutelmann (1973) and Beutelmann and

Bauer (1977) determined its chemical structure to be identical with isopentenyladenine (iP).

10.3.2.1 Cytokinin-overproducing mutants and transformants

Overproduction of buds was a strong phenotype observed in the *ove* mutants of *P. patens*, which were first isolated by Ashton et al. (1979b). The mutant phenotype resembled wild-type cultures treated with high concentrations of cytokinins. Compared with wild type, buds of the *ove* mutants occur in high numbers and functional gametophores are not formed as buds develop callus-like structures (see Figure 10.5, Plate 17). Work published by Wang et al. (1980) revealed that the *ove* phenotype correlated with a strong overproduction of the cytokinin iP, and that higher amounts of *trans*-zeatin (tZ) were also found (Wang et al., 1981a, b). Genetic analyses carried out by somatic hybridisation revealed that the recessive *ove* mutations can be assigned to three different complementation groups (Featherstone et al., 1990). Temperature-sensitive cytokinin mutants were also described. *Ove*A409 and *ove*ST25 show wild-type-like growth at a temperature of 15°C but exhibit an *ove* phenotype when grown at 25°C. This phenotypic change was shown to be correlated with a strong overproduction of cytokinins, which are released into the culture medium (Futers et al., 1986; Schulz et al., 2001; Schwartzenberg, 2006). Although labelling experiments with radioactive adenine showed increased amounts of radioactivity in the cytokinin fraction of *ove* mutants (Wang et al., 1981a), it was not clear whether the reason for cytokinin overproduction was increased biosynthesis or impaired cytokinin degradation. Schulz et al. (2001) showed by enzyme assays for cytokinin oxidase/dehydrogenase that the strains *ove*A78, *ove*A201 and *ove*B300 had degradation rates similar to wild type. In addition, it was found that *ove* mutants had a higher rate for the conversion of the cytokinin riboside isopentenyladenosine (iPR) to its base iP (see Figure 10.4a). Taken together, the biochemical and genetic results indicated that cytokinin production in *ove* mutants is increased due to a de-regulation of cytokinin biosynthetic steps. It is likely that the loci affected in the recessive *ove* mutants are involved in the negative regulation of cytokinin biosynthesis (Schwartzenberg, 2006). Although *ove* mutants are unique in the plant kingdom due to their drastic cytokinin overproduction, the corresponding loci have not been identified yet. Their future identification could contribute considerably to our understanding of the regulation of cytokinin biosynthesis.

Cytokinin-overproducing plants of the *P. patens* mutant PC22 (Abel et al., 1989) have been generated by the expression of an *IPT* gene from *A. tumefaciens* and the plants were analysed for phenotypical changes as well as for cytokinin status (Reutter et al., 1998; Schulz et al., 2000). The analysis of cytokinins in these plants corroborated that, when excess cytokinins are formed, these are released into the culture medium. This phenomenon, which had previously been described for cytokinin-overproducing *ove* mutants (see above), underlines the importance of the extracellular space in moss. It seems that the

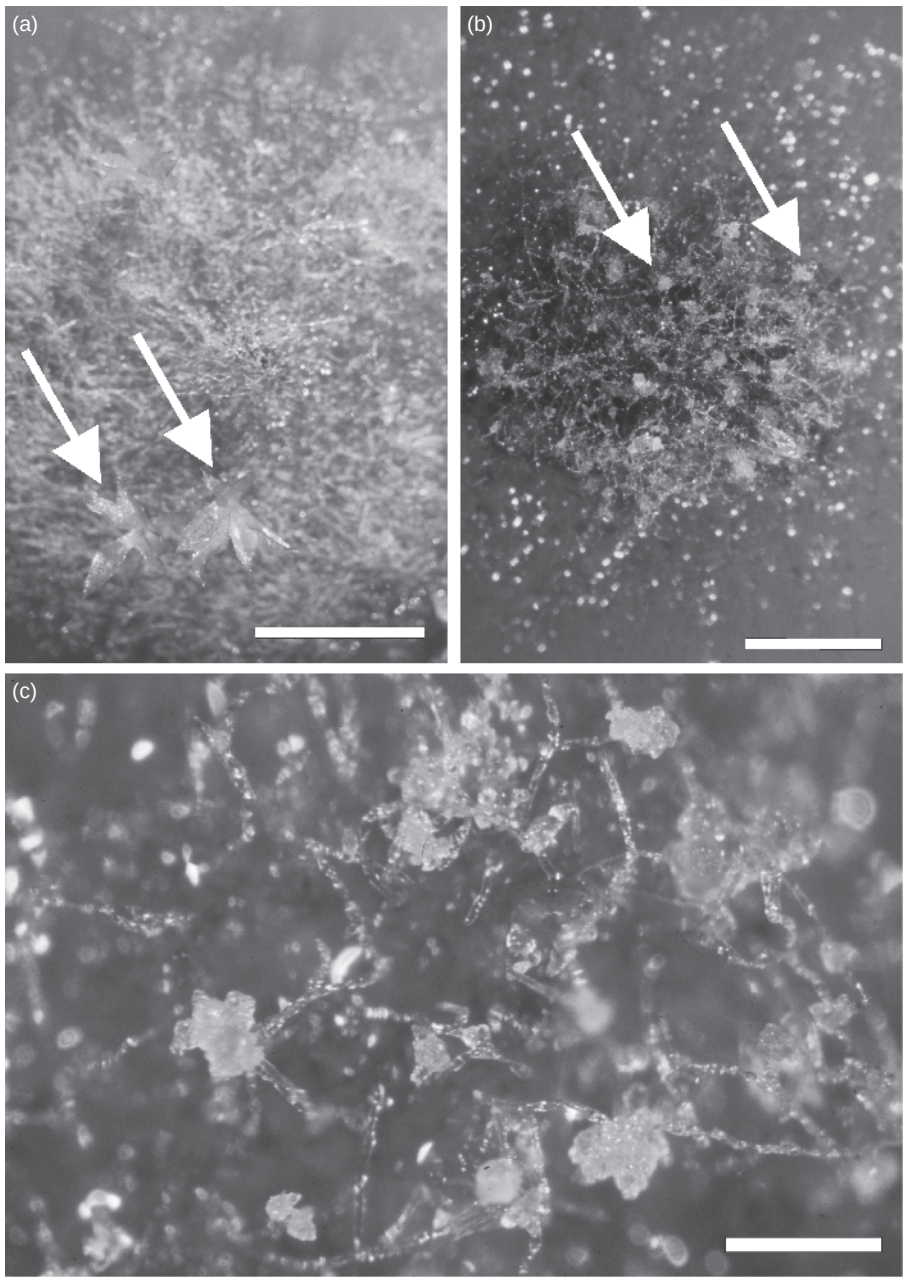

Figure 10.5 (a) Wild-type culture showing only a few differentiated gametophores (arrows); (b) *ove*B300 mutant exhibiting a high number of buds forming callus-like structures (arrows); (c) same as B at higher magnification. Bar corresponds to 1 mm in (a) and (b) and 0.5 mm in (c). (For a color version of this figure, see Plate 17)

release of excess hormones into the culture medium prevents cytokinin concentration within the cells accumulating to cytotoxic levels. Also by *AtCKX2* overexpression in *P. patens* wild type, a reduction of cytokinins in the culture medium could be correlated to phenotypic changes (Schwartzenberg et al., 2007). Obviously, as non-vascular plants, mosses use the extracellular space for cytokinin transport and hormonal homeostasis.

10.3.2.2 Cytokinin interconversion

As cytokinins are interconvertable between the forms of bases, ribosides and nucleotides (see Figure 10.4), a considerable amount of radioactivity is usually found in the fraction of nucleotides when carrying out *in vivo* labelling experiments with radioactive cytokinins.

The reactions leading to nucleotide formation are usually catalysed by adenine phosphoribosyltransferase (APT) and adenosine kinase (ADK) enzymes, which also have adenylic compounds as substrates (Chen, 1997). With respect to this cytokinin interconversion in *P. patens*, Schwartzenberg et al. (1998, 2003) revealed that, unlike seed plants, ADK rather than APT is responsible for the formation of iP-type nucleotides in moss (compare Figure 10.4a). Under conditions favouring overproduction, the cytokinin base iP, a major cytokinin in *P. patens*, is found in high concentrations in the culture medium. The enzyme responsible for the release of iP is thought to be a purine nucleosidase, for which the coding genes are currently being characterised (Turčinov and Schwartzenberg, unpublished). This enzyme can be regarded as catalysing the last step in the biosynthetic chain of cytokinin bases. It is so far unclear whether cytokinin-specific phosphoribohydrolases, which were recently identified in rice and *A. thaliana* (Kurakawa et al., 2007), also play a role in cytokinin activation in *P. patens*.

In contrast to cytokinin metabolism in seed plants, no cytokinin *N*-glucosides have been found in *P. patens* (Schwartzenberg et al., 2003, 2007).

10.3.2.3 Cytokinin catabolism

Gerhäuser and Bopp (1990) first demonstrated in *F. hygrometrica* that the radiolabelled cytokinins kinetin and benzyladenine (BA) are degraded *in vivo* to adenylic compounds. This cleavage of the N^6-substitution results in an irreversible loss of hormonal activity and is catalysed by cytokinin oxidase/dehydrogenase. Interestingly, the degradation of radiolabelled cytokinins was higher when cultures were pre-treated with high concentrations of kinetin prior to the application of the radiolabelled substrates. This homeostatic regulation demonstrates that cytokinins can induce their own breakdown in mosses, as has also been shown for seed plants (Laloue and Pethe, 1982; Armstrong, 1994; Motyka et al., 1996; Galuszka et al., 1999).

For cytokinin oxidase/dehydrogenase from *A. thaliana* and other seed plants, it is known that certain isoforms are targeted towards the secretory pathway (Werner et al., 2006). Although no detailed functional data on cytokinin oxidase/dehydrogenase have been published yet for *P. patens*, a secretion of cytokinin oxidase/dehydrogenase by wild type was described

by Schwartzenberg et al. (2007). When the heterologous gene *AtCKX2* was expressed in *P. patens*, the specific activity in the culture medium compared with wild type increased by up to 157-fold versus only 26-fold in the tissue extract.

Preliminary studies on substrate specificity of native CKX enzymes in a crude extract of wild-type *P. patens* revealed that cZ is degraded at a higher rate compared to iP and tZ (Gajdošová, Motyka, Schwartzenberg, unpublished). The preference of cZ versus other isoprenoid cytokinins can be understood as an adaptation of the cytokinin degradation machinery to the actual cytokinin profile in *P. patens*, where cZ-forms prevail over other cytokinin forms (Schwartzenberg et al., 2007).

10.3.2.4 Cytokinin profiles of moss: comparisons to algae and seed plants

Recently, a systematic profiling based on ultra-performance liquid chromatography coupled to mass spectrometry (UPLC-MS/MS) was performed by Schwartzenberg et al. (2007). From 40 cytokinins tested, 20 different cytokinin species were detected in *P. patens*.

The most abundant cytokinins determined in tissue were *cis*-zeatin-riboside-*O*-glucoside (cZROG), followed by N^6-(Δ^2-isopentenyl)adenosine-5′-monophosphate (iPRMP), *trans*-zeatinriboside-*O*-glucoside (tZROG), *cis*-zeatin-riboside-5′-monophosphate (cZRMP), *trans*-zeatin-riboside-5′-monophosphate (tZRMP), *cis*-zeatin-*O*-glucoside (cZOG) and N^6-(Δ^2-isopentenyl)adenosine (iPR), for which the maximum concentrations were 646, 332, 170, 72, 68, 52 and 36 pmol/g dry weight (DW), respectively. In addition, the aromatic cytokinins N^6-benzyladenosine (BAR), N^6-benzyladenine (BA), *meta*- and *ortho*-topolin (mT, oT) were detected. The most abundant extracellular cytokinin was the nucleotide iPRMP. Major cytokinins found in *P. patens* are given in Figure 10.4b.

With respect to the evolution of cytokinin signalling, it is helpful to compare the studies on endogenous cytokinins to those for other evolutionary distant organisms such as algae and seed plants. Ördög et al. (2004) presented studies on cytokinins in three genera of unicellular algae (Chlorophyta). Interestingly, the cytokinin profiles found for *Protococcus viridis*, *Chlorella minutissima* and *Scenedesmus sp*. showed certain similarities to the one presented for *P. patens*. The dominant forms found in the algae were also zeatin-*O*-glucoside compounds. However, in contrast to *P. patens*, the *trans*-zeatin-*O*-glucosides tZOG and tZROG were more abundant (e.g. 19 and 1 pmol/g DW, respectively, in *P. viridis*) than the corresponding *cis* isomer forms cZOG and cZROG. A considerable amount of aromatic cytokinin forms (BA, mT, oT, pT) were found in the unicellular algae (up to 27 pmol BA/g DW in *P. viridis*). As in *P. patens*, dehydrozeatin-cytokinins occurred only in traces in the algae and also no *N*-glucosides were found. For *C. reinhardtii*, Ivanova et al. (1992) reported the presence of high amounts of iP representing ca. 90% of the total cytokinins. Quantifications in this study were based on immunoenzymatic assays in which tZ- and DHZ-forms were also found at low concentrations (cZ forms were not analysed).

In the multicellular green algae *Cladophora capensis* and *Ulva sp*., both iP- and cZ-type cytokinins were found, and again *N*-glucosides were absent (Stirk et al., 2003). Thus, the moss *P. patens* shares certain features in its cytokinin profile with other non-vascular plants. The prevailing amounts of cZ-type cytokinins in *P. patens* and multicellular green algae present a major difference in comparison to dicotyledonous seed plants such as *A. thaliana* and *Nicotiana tabacum*, where tZ-type cytokinins are usually dominant (Werner et al., 2001; 2003a, 2003b). It seems likely that the differences found in the cytokinin profile of *P. patens* and seed plants reflect differences in the major cytokinin-forming pathways.

10.3.2.5 Cytokinin biosynthesis – importance of the tRNA-mediated pathway in *P. patens*

Cytokinin nucleotides are important constituents of tRNAs that recognise UNN codons and occur ubiquitously in all organisms except archeae (Edmonds et al., 1991). The main function of cytokinins as hypermodified adenosine in position 37 (3′- adjacent to the anticodon) of tRNAs is the stabilisation of the codon–anticodon binding, thus contributing to the maintenance of the correct reading frame and increasing the precision of protein biosynthesis (Konevega et al., 2006). tRNA is known to be the origin of free cytokinins produced by many prokaryotes (Koenig et al., 2002) and its turnover can also contribute to cytokinin biosynthesis in plants. However, for seed plants, tRNA is generally regarded as not sufficient as the only source of cytokinins (Miyawaki et al., 2006).

As *cis*-zeatin and iP-type forms have been described to be major constituents of plant tRNA (Taller, 1994), the prevalence of these forms in the soluble fraction of *P. patens* as presented by Schwartzenberg et al. (2007) suggests that the tRNA-dependent biosynthetic pathway might be of greater importance in *P. patens* than in seed plants, where cytokinins are generated to a large extent independently from tRNAs by the catalytic action of adenylate IPTs (Kakimoto, 2001; Takei et al., 2001; Miyawaki et al., 2006).

A systematic search in the moss genome revealed no homologues for adenylate *IPT* genes (Yevdakova and Schwartzenberg, 2007). Two orthologues *PpIPT1* and *PpIPT2*, for which expression evidence exists, were shown to be very similar to known plant *tRNA-IPT* genes (see Figure 10.6). However, *PpIPT2* seemed to be only very weakly expressed in wild-type gametophytes. Yevdakova and Schwartzenberg confirmed that the PpIPT1 gene product carries out the isopentenylation of *tRNA*, using a yeast complementation system based on the tRNA-IPT deficient mutant MT-8 (Dihanich et al., 1987). Preparations of tRNA-hydrolysates derived from the yeast mutant demonstrated that hormonally active iPR was formed by the action of the PpIPT1 gene product. Additional evidence that PpIPT1 is involved in cytokinin biosynthesis in *P. patens* was obtained from semi-quantitative RT-PCR studies, which revealed a drastically stronger transcription of

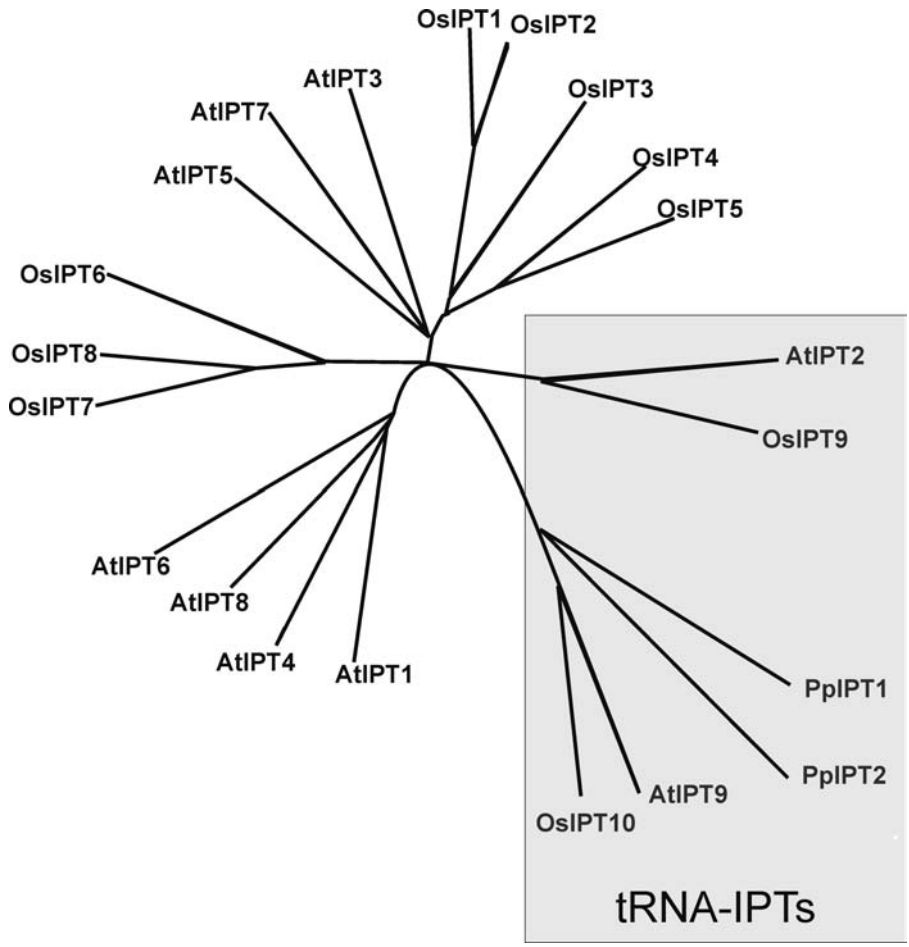

Figure 10.6 Neighbor-joining tree reflecting homology relationship between isopentenyltrasferases (IPTs) from *Arabidopsis thaliana* (*AtIPT1–9*), *Oryza sativa* (*OsIPT1–10*) and *P. patens* (*PpIPT1* and *PpIPT2* on basis of deduced amino acids sequences). tRNA-IPTs are boxed (Yevdakova and Schwartzenberg, unpublished).

PpIPT1 in the cytokinin-overproducing mutant *ove*ST25 compared with wild type (Yevdakova and Schwartzenberg, 2007).

The absence of adenylate *IPT* genes in the moss genome and the high transcriptional expression of the tRNA-IPT gene *PpIPT1* in *ove*ST25 suggest that in moss, in contrast to seed plants, cytokinins are predominantly synthesised by the tRNA route. As genes similar to adenylate *IPTs* can neither be detected in green algae (e.g. *Volvox cateri*, *O. tauri*) nor in *Selaginella moellendorffii*, it seems likely that the adenylate IPT pathway was developed relatively late and arose during evolution of the seed plant lineage (see Figure 10.7) (Yevdakova and Schwartzenberg, unpublished; see also Sakakibara et al., 2006).

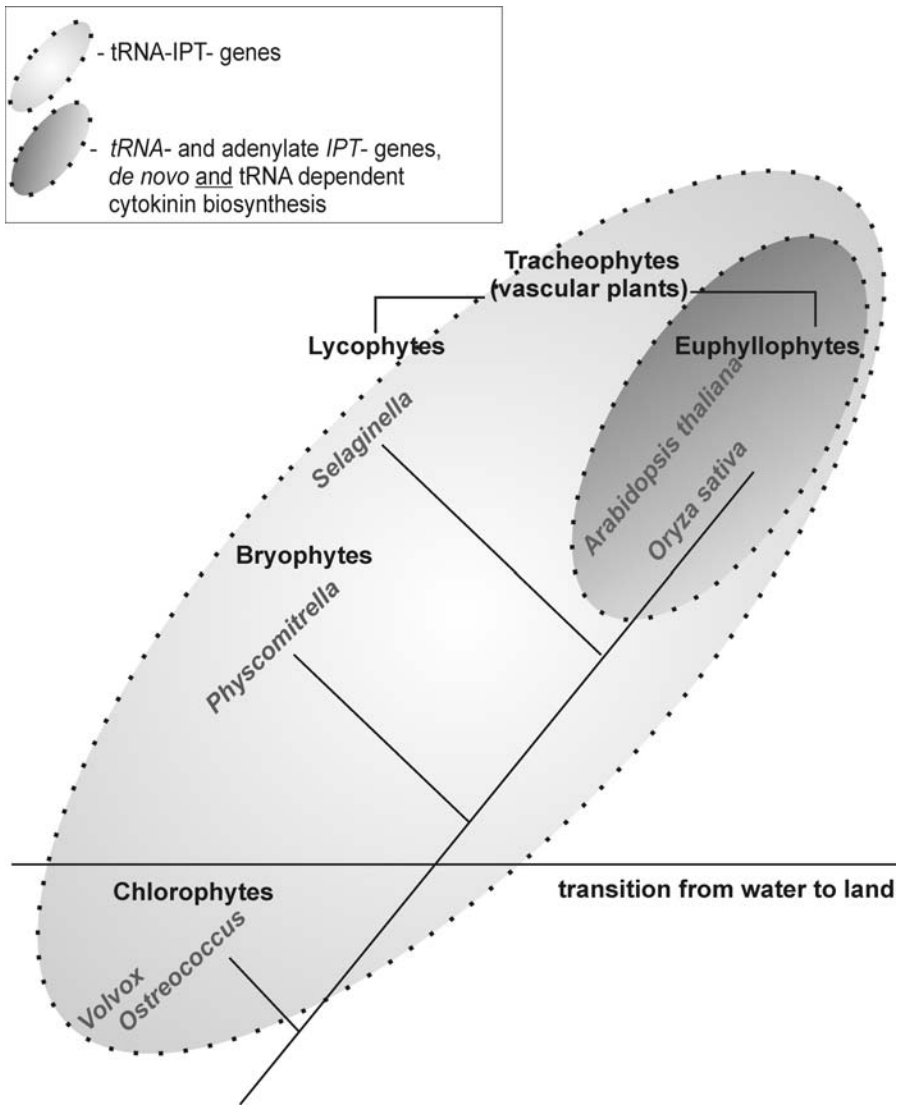

Figure 10.7 Hypothetical scheme depicting occurence of *tRNA*- and adenylate-*IPT* genes in evolutionarily different plant lineages (established on basis of available sequenced genomes). In chlorophytes (*Chlamydomonas rheinhardtii, Ostreococcus tauri, Volvox cateri*), moss (*Physcomitrella patens*) and lycophytes (S*elaginella moellendorfii*), the exclusive presence of tRNA-isopentenyltransferase-like genes (*tRNA-IPT*) suggest tRNA-mediated cytokinin biosynthesis (see Yevdakova and Schwartzenberg, 2007; Yevdakova et al., 2008), whereas in the lineage of flowering plants, additionally adenylate-*IPTs* have evolved catalysing *de novo* biosynthesis of free isoprenoid cytokinins (Yevdakova and Schwartzenberg, unpublished; see also Sakakibara et al., 2006).

The probable tRNA origin of cytokinins in *P. patens* is supported by the fact that with the exception of aromatic forms, all major free cytokinins detected in *P. patens* can also be detected in its tRNA, where cZ and iP also dominate (Yevdakova et al., 2008). Although the substrate specificity of adenylate and tRNA-IPTs was shown to be very high in *A. thaliana* (Kakimoto, 2001), it cannot be completely ruled out that tRNA-IPT-like proteins in *P. patens* accept free adenylic substrates such as ATP/ADP/AMP. Further biochemical studies have to prove in detail whether the tRNA-dependent biosynthetic pathway is indeed exclusive to *P. patens* as suggested by genome analyses.

10.4 Auxin and cytokinin interaction

The cytokinin:auxin ratio appears to play an important role in the differentiation of caulonema from primary chloronema as a typical auxin-dependent proliferation, as Thelander et al. (2005) could repress caulonema development by the addition of cytokinin (see Figure 10.1, Plate 14).

Evidence that internal factors other than cytokinins are necessary to obtain cytokinin-induced budding was concluded from isolated chloronema, which lost the capacity to form buds when cultured on a fresh hormone-free medium, prior to transfer to the cytokinin medium (Bopp and Diekmann, 1967). A factor necessary for the bud initiation leached out of the caulonemata within a few hours. In later work, it was shown that the presence of auxin is essential for buds to develop (Sood and Hackenberg, 1979; Ashton and Cove, 1990).

When culturing wild-type *P. patens* under constant medium replacement (drip-feeding), it was shown that buds only develop if both cytokinins and auxin are present at the same time in the drip-feed medium, thus underlining the fact that cytokinin-induced bud formation is an auxin-dependent process (Cove and Ashton, 1984). In agreement with this is the conclusion made by Cove (2005), based on auxin and cytokinin treatments of mutants as well as on drip-feed studies, which suggests that effects triggered by either cytokinin or auxin generally require the availability of both hormones. Thus, at least in *P. patens*, processes underlying protonemata and bud differentiation do not seem to be governed by a strict sequential interaction of auxin and cytokinins (see Bopp, 1979). However, there is convincing evidence for auxin and cytokinin being required simultaneously in order to assure proper developmental progression.

The auxin–cytokinin crosstalk is also present at the level of cytokinin homeostasis as auxins have been shown to enhance the concentration of iP in *F. hygrometrica* (Bopp and Atzorn, 1992b) and it is remarkable that the concentrations of IAA, NAA and 2,4-D, which caused the greatest increase in budding in mutant 87.13, were also optimal for inducing the accumulation of iP. 2,4-D was about two orders of magnitude less effective in promoting the budding response and in inducing the iP increase. The obvious effect of

IAA on cytokinin content represents one of numerous examples of the close interaction between auxin and cytokinins (see also Chopra and Rashid, 1969; Nyman and Cutter, 1981; Lehnert and Bopp, 1983; Bopp, 2000), which needs to be further characterised on a molecular level.

There is also evidence that the interaction between auxins and cytokinins is involved in the coordination of apical dominance in gametophores as shown for *Splachnum ampullaceum* (Maltzahn, 1959) and for *Plagiomnium cuspidatum* (Nyman and Cutter, 1981). Removing the apex of gametophores resulted in the outgrowth of lateral buds and in the formation of side branches. The inhibitory effect of the apical region could be replaced by the application of an agar block containing IAA (Maltzahn, 1959).

However, when kinetin was simultaneously administered, the suppression of lateral bud formation by auxin was antagonised and lateral buds developed to leafy side branches. Obviously, the auxin–cytokinin-mediated control of apical dominance is similar in the analogous organs of moss gametophores and seed plant shoots. Despite the absence of polar auxin transport in moss gametophores (Fujita et al., 2008), it can be speculated that during seed plant evolution, ancient parts of the machinery necessary to form the regulatory network of auxin- and cytokinin-controlled apical dominance, which were previously established in the gametophytes of ancestral plants, were adopted and modulated in the sporophytes of seed plants.

10.5 Other growth regulating substances

10.5.1 Salicylic acid

The report of Christianson and Duffy (2003) demonstrating that salicylic acid and acetylsalicylate can inhibit the late stages of bud formation in *F. hygrometrica* in a dose-dependent manner indicates that mosses might use these substances as developmental signals. However, further information is lacking on their distribution and signal transduction mechanisms.

10.5.2 Gibberellin

The presence of gibberellin (GA) in mosses, as well as its capability to cause morphogenetic changes, is largely unclear. Although single reports on GA-mediated growth changes have been published (Chopra and Mehta, 1987; Maltzahn and Macquarrie, 1958), there is no strong experimental support for GA causing a specific morphogenetic action in mosses.

It is possible that the molecular structures of putative GA-like compounds in mosses differ strongly from those known from seed plants so that neither the compounds nor clear hormonal effects of exogenously applied GAs have been discovered.

Studies on *Saelania glaucescens* and *P. patens* have revealed a high concentration of a tetracyclic diterpene, 16-hydroxykaurane (Nilsson and Martensson, 1971; Schwartzenberg et al., 2004). This indicates that the first enzymatic steps to form precursors for GAs are present in moss; the gene responsible for its synthesis, encoding for a bifunctional *ent*-kaurene synthase PpCPS/KS, has been cloned and characterised from *P. patens* (Hayashi et al., 2006). However, the search in 13 further moss species including *F. hygrometrica* revealed no 16-hydroxykaurane, leaving the possibility that tetracyclic diterpenes in *S. glaucescens* and *P. patens* are secondary substances without relevance for GA biosynthesis (Schwartzenberg et al., 2004).

Recently, Vandenbussche et al. (2007) reported that high concentrations of gibberellic acid (>10 μM, GA3) can affect gravitropism and elongation growth in *P. patens* under certain growth conditions. In a comparative study of homologues of GA-signalling components, these authors found that the corresponding elements in *P. patens* share only a limited homology with the *A. thaliana* GA-signalling proteins. In homologues of the GA-receptor GID1, the typical amino acid Arg265 usually essential for GA-binding is missing, thus indicating that these proteins are probably not functional GA-receptors in *P. patens* (Vandenbussche et al., 2007). *P. patens* homologues of other components of the GA-signalling pathway (DELLA-like transcription factors and SLY/GID2-like F-box proteins) also show important divergences from corresponding seed plant proteins and Vandenbussche et al. (2007) therefore conclude that GA-signalling, which may have its molecular onset at the evolutionary state of mosses, functions differently as in vascular plants.

A functional study on GA signal transduction components was carried out by Yasumura et al. (2007), including homologues of the GID1 GA receptor and the DELLA transcription factors of *P. patens*. It was shown that the interaction of GID1 and DELLA protein, which is a prerequisite in GA-signalling (Ueguchi-Tanaka et al., 2005), does not take place with the *P. patens* proteins. Yasumura and co-workers conclude, therefore, that the GA signal transduction chain is not yet completely established in bryophytes and arose in the land plant lineage after the bryophyte divergence (ca. 430 million years ago).

10.5.3 Ethylene

Ethylene production in moss has been demonstrated for the liverwort *Pellia epiphylla* (Thomas et al., 1983) and the moss *F. hygrometrica*, where its release and the concentration of its precursor 1-aminocyclopropane-1-carboxylic acid (ACC) coincide with the differentiation of chloronema to caulonema (Rowher and Bopp, 1985, reviewed in Bhatla and Dhingra-Babbar, 1990). Although the physiological role of ethylene in mosses and non-vascular plants remains to be clarified in detail, two putative genes coding for ACC-synthases, catalysing

a critical step in ethylene biosynthesis, were detected in the *P. patens* genome (Rensing et al., 2008). Furthermore, six putative ETR-like ethylene receptors are encoded in *P. patens* and Wang et al. (2006) have shown that at least one of these is able to bind ethylene. Wang and co-workers conclude from a broad binding assay with putative ethylene receptors from many organisms, including several bryophytes, that ethylene binding is confined to land plants, *Chara spp*. and a group of cyanobacteria.

The analysis of the assembled *P. patens* genome and comparative genomic analyses revealed and confirmed that ABA, auxin and cytokinin signalling are well established in *P. patens*; however, other signalling components and transduction pathways such as the ones for ethylene, gibberellic acid, jasmonic acid and brassinosteroids are most likely only fully functional in the lineage of vascular plants (Rensing et al., 2008).

Further comparative genomic approaches accompanied by functional studies will elucidate the steps plants have evolved to establish their regulatory networks of hormonal control. The increasing number of papers published in this area demonstrates that the model system *P. patens* occupies an important site for studies of the developmental evolution of land plants.

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Dedication

This review is dedicated to Professor Heinz Hahn (University of Hamburg/University of Bonn) on the occasion of his 70th birthday.

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Chapter 11

THE ROLE OF ABSCISIC ACID IN STRESS TOLERANCE

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Abstract: Mosses have evolved complex mechanisms to overcome abiotic stresses. The phytohormone abscisic acid (ABA) plays an important role in the establishment of stress tolerance in vascular plants as well as in bryophytes. ABA pre-treatment greatly enhances tolerance to dehydration, cold temperature and osmotic stresses in the moss *Physcomitrella patens*. The response to abiotic stresses and ABA in *P. patens* is accompanied by the accumulation of sugars along with morphological and physiological changes. Some of the genes regulated by ABA in *P. patens* are also controlled by the ABA-signalling pathway in vascular plants. However, there are genes expressed in response to ABA and abiotic stress in *P. patens* that are not found in seed plants. With the ability to easily delete and replace genes by homologous recombination, as well as a fully sequenced and assembled genome, *P. patens* will provide an excellent model for studying stress biology in plants.

Keywords: ABA signalling; abiotic stress; abscisic acid; cold stress; dehydration; heavy metal; osmotic stress; stress tolerance

Abbreviations:

ABA	abscisic acid
ABRE	ABA-responsive element
APD	aparticulate domain
bHLHZip	basic helix-loop-helix-zipper domain
CHX	cycloheximide
DRE	dehydration-responsive element
ERD	early responsive to dehydration
EST	expressed sequence tag
FJL	fracture-jump lesion
FTIR	Fourier transform infrared spectroscopy
GCR	G protein-coupled receptor

GSH	glutathione
GSK3/Shaggy	glycogen synthase kinase 3 or shaggy kinase
LEA	late embryogenesis abundant
MDHAR	monodehydroascorbate reductase
MT	metallothionein
PC	phytochelatin
PEG	polyethylene glycol
PSII	photosystem II
ROS	reactive oxygen species
VP1	viviparous1
γ-ECS	γ-glutamyl cysteine synthetase

11.1 Introduction

Plants lead a sessile life and as a result must have protective responses to tolerate the stresses that they encounter in their natural environment. To overcome stress conditions, plants have evolved complex mechanisms to perceive and transduce external signals that result in the synthesis of different sets of proteins, including transcription factors, enzymes and transporters. Although many genes have been identified that are differentially expressed in response to environmental stresses (Zhu, 2001a, b), it is also possible in the post-genomic era to study networks of responsive genes in a more global and integrated manner which will lead to a more complete understanding of the mechanisms involved. Furthermore, with relatively well-characterized experimental systems such as the moss *Physcomitrella patens*, we can begin to approach the question of how land plants evolved the regulatory circuitry to survive the stresses associated with their habitat.

Mosses and other bryophytes have evolved mechanisms to tolerate many of the same stresses as seed plants, not only dehydration, but also exposure to osmotic stress, to heavy metals and to temperature (Oliver et al., 2000; Cove et al., 2006). As in seed plants, the phytohormone abscisic acid (ABA) has clearly been implicated in many of the responses of bryophytes to stress, but a number of publications also describe morphogenetic effects (see Table 11.1), such as the induction of specialized cells, brachycytes (see Figure 11.1). These cells are formed by intercalary divisions of protonemal cells concomitant with the repression of bud formation (Schnepf and Reinhard, 1997; Decker et al., 2006). Brachycytes, which may function as vegetative spores, are induced under stress or exogenously applied ABA, as is the repression of cytokinin-induced bud formation. With respect to the fact that fecundity and gamete motility in mosses are strictly dependent on the availability of water, the repression of bud growth and gametophore initiation in favour of protonemal growth (and the subsequent formation of brachycytes as durable propagules) is an example of the coordinated response to ABA for protection against stress at various levels of organization (Christianson, 2000).

Table 11.1 Examples of ABA effects on growth and development of mosses

Effect	Species	References
Reduction of protonemal growth and thickening of cell walls	*F. hygrometrica* *P. patens*	Werner et al., 1991; Tintelnot, 2006
Induction of brood cells	*P. patens* *Dicranoweisia cirrata* *Rhytidiadelphus loreus* *Splachnum ampullaceum*	Goode et al., 1993
Induction of tmema cells and brachycytes	*P. patens*	Decker et al., 2006
Inhibition of cytokinin-induced bud formation on protonema	*F. hygrometrica* *P. patens*	Valadon and Mummery, 1971; Christianson, 2000; Schwartzenberg and Yevdakova, unpublished

ABA has been detected immunologically in liverworts and hornworts (Hartung et al., 1987), while Werner et al. (1991) detected ABA in the moss *Funaria hygrometrica* using an immunoenzymatic quantification method. Based on the work of Valadon and Mummery (1971) as well as of Christianson (2000), the inhibition of cytokinin-induced budding can also be used as a bioassay for ABA. The interfering site in the cytokinin-signalling pathway does not appear to be perception of the ABA signal, but rather at a point further downstream of the signalling pathway. Finally, a gas chromatography–mass spectrometry (GC–MS)-based protocol reported by Minami et al. (2005) resulted in the detection of ABA in *P. patens*.

The presence of ABA in various species suggests that ABA is generally present as a growth regulator in bryophytes. It is assumed that ABA, which is known to play important roles in the response to drought, freezing and osmotic stress in seed plants, already took over the regulation of important functions in adjustment to various stresses in bryophytes (Kroemer et al., 2004). The establishment of a coordinated stress adaptation programme seems essential for the water to land transition but is not necessary in a strictly aqueous environment.

Coupled with the clear effect of ABA on stress tolerance in mosses, significant genomic resources are now available in *P. patens* for investigating the molecular mechanisms involved (Frank et al., 2005; Quatrano et al., 2007). Within the last 5 years, exceptional progress has been made to develop *P. patens* as a model experimental system, which includes a sequenced and assembled genome (Quatrano et al., 2007; Rensing et al., 2008), and the development of techniques such as gene targeting (Cove, 2000; Schaefer, 2001) and RNA interference (Bezanilla et al., 2005) to study gene function. In addition, the *P. patens* transcriptome showed that about a quarter of its over 25 000 genes represent unique sequences which could possibly lead to the discovery

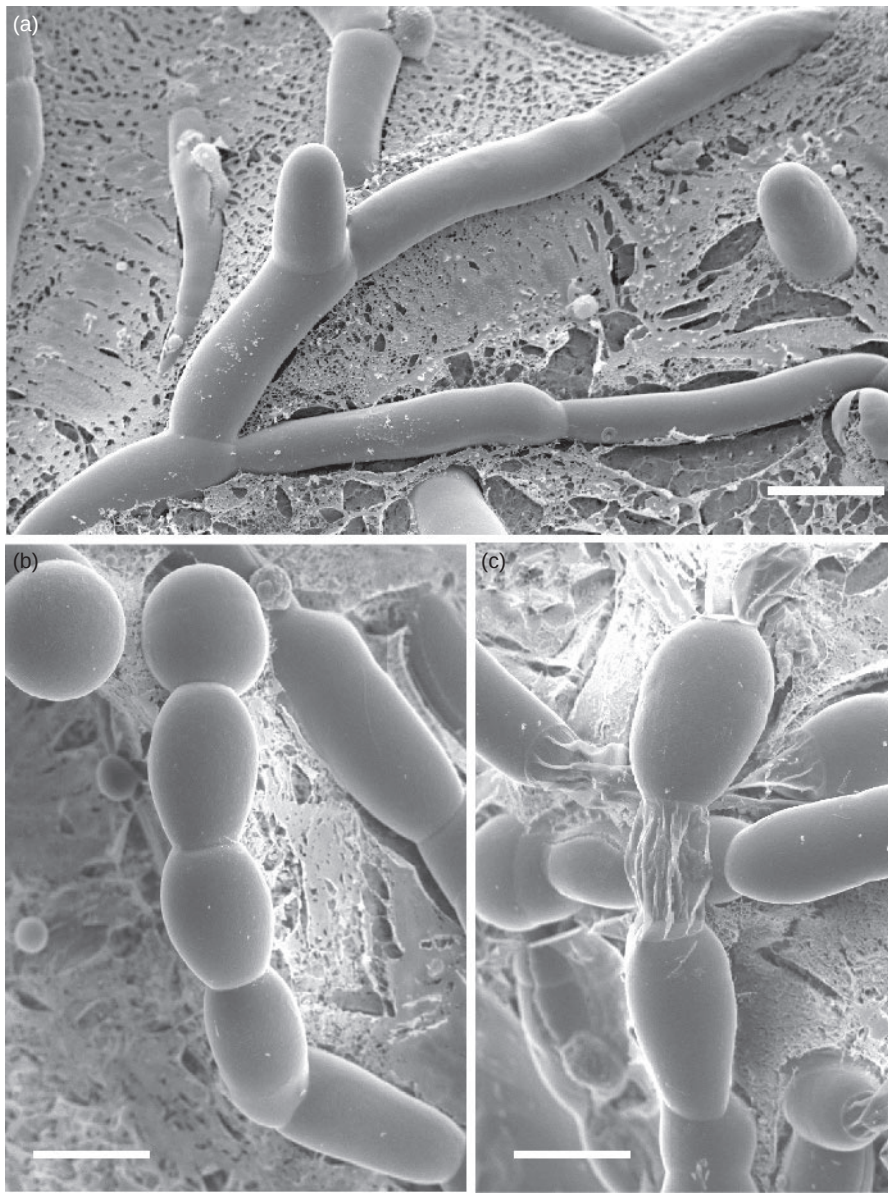

Figure 11.1 ABA induces morphological changes in *P. patens* protonemata. Scanning-electron microscope images of *P. patens* protonemata untreated (a) and after treatment with 100 μM ABA (b), (c). ABA-treated moss cells differentiate into chains of brachycytes (b) or brachycytes flanked by tmema cells (c). Bars, 30 μm. (*Source*: Decker et al., 2006, printed with kind permission of Universität Freiburg.)

of novel stress-related genes (Rensing et al., 2002). Since *P. patens* is highly tolerant of drought, salt and osmotic stress (Frank et al., 2005), this chapter will now focus on research using this model system to study the physiological changes that occur during abiotic stress responses, and to characterize the function of genes that are differentially expressed.

11.2 Tolerance of *P. patens* to abiotic stresses and the effects of ABA

The biochemical and molecular steps leading to stress tolerance are the best-studied aspect of ABA research in mosses. The group of Bopp (Werner et al., 1991; Bopp and Werner, 1993) initially described the relationship between ABA and the acquisition of drought tolerance in *F. hygrometrica*. They showed that treating protonemata of moss with ABA (10 μM) for 16 h induced tolerance to rapid drying. Furthermore, the content of endogenous ABA continuously increased during slow drying over a period of 24 h, thus rendering the protonemata tolerant to subsequent rapid drying. For the moss *Atrichum androgyne*, the ABA protective effect on photosynthesis was analysed by Mayaba et al. (2001) who showed that with ABA pre-treatment of *A. androgyn*e before desiccation, the rate of recovery of photosystem II (PSII) activity is increased. Hence, PSII activity appears to be protected from damage as a result of ABA treatment. This might partially explain why ABA can generate a general protection against several types of environmental stresses since an intact and functional photosystem is likely to be required for recovery from any stress. Furthermore, ABA pre-treated *A. androgyne* reached a positive carbon balance 2 h after rehydration compared with 8 h for the untreated controls. The establishment of the ABA protective effect in *A. androgyne* requires the presence of light during ABA treatment (Beckett et al., 2000), but seems to be phytochrome independent as irradiation with red light did not have the same effect as white light.

P. patens, which shows high tolerance of various environmental stresses, is well suited for studies on mechanisms underlying ABA-induced stress tolerances. For example, *P. patens* can tolerate 350 mM NaCl, and although growth is reduced at 600 mM NaCl, there are no signs of any toxicity or chlorosis (Benito and Rodriguez-Navarro, 2003) at this concentration. By comparison, *Arabidopsis thaliana* displays a severe phenotype at a concentration of 100 mM (Sunkar et al., 2003). However, *P. patens* is not uniformly more tolerant of stresses in general when compared with angiosperms, since Rother et al. (2006) showed that *P. patens* exhibited a greater sensitivity to heavy metal stress (i.e.10 μM Cd^{2+}) in comparison with *A. thaliana*.

More detailed studies have been done on the unique ability of *P. patens* to tolerate severe water deficit and abnormally cold temperatures. For example, there was no effect on growth of *P. patens* at a 92% loss of fresh weight during a dehydration treatment (Frank et al., 2005). Hoekstra et al. (2001)

defines a drought-tolerant plant by its ability to tolerate a moisture content below which there is no bulk cytoplasmic water present (approximately 23% water on a fresh weight basis). Survival of *P. patens* protonemal tissue to desiccation (99.8% fresh weight loss) was achieved by exogenous application of ABA coupled with a slow drying condition (Oldenhof et al., 2006). Beckett et al. (2000) also showed that the moss *Atrichum undulatum* survived desiccation after ABA treatment by enhancing the tolerance of PSII to the stress. However, ABA treatment itself was not enough to allow protonemal tissue to survive extreme conditions such as complete desiccation (Oldenhof et al., 2006). When protonemal tissue was dried rapidly by overnight exposure to dry air, the tissue died. In comparison, protonemal tissue survived slow drying by exposure to decreasing relative humidity over a period of 9 days. In these tissues, there were differences of overall protein secondary structure and in the strength of the hydrogen bonding interactions in the glassy state. In the slowly dried ABA-treated tissue, a higher proportion of α-helical protein structures were found when compared to the rapidly dried ABA-treated tissue. The average strength of hydrogen bonding in the cytoplasmic glassy matrix was also increased, which could be ascribed to accumulation of stress-related proteins during the slow drying condition.

Tolerance to cold temperature has also been documented in *P. patens*, some components of which appear to involve an ABA-signalling pathway. For example, the lethal cold temperature for 50% mortality (LT_{50}) for *P. patens* protonemal tissue is around −2°C (Nagao et al., 2005). An adaptive cold treatment at 0°C for 7 days changed the LT_{50} from −2°C to −3.5°C (Minami et al., 2005). However, treatment with 10 μM ABA for 1 day enhanced this freezing tolerance to −10°C (Nagao et al., 2005) and 100 μM ABA resulted in tolerance of −80°C, which was confirmed by re-growth after thawing and the impermeability of trypan blue, an indicator of viability (Oldenhof et al., 2006).

11.3 Physiological and morphological changes during the establishment of tolerance

As discussed above, the degree of tolerance to environmental stresses, such as freezing and desiccation, is greatly enhanced by ABA treatment (Minami et al., 2003a). When ABA-treated protonemal tissue was observed for morphological changes during freezing temperatures, the size and volume of chloroplasts and vacuoles were rapidly reduced (Nagao et al., 2005). In addition, the plasma membrane of the non-treated protonemal cells was found to form a number of aparticulate domains (APDs) and fracture-jump lesions (FJLs) during freezing, which are the features of irreversible injury caused by freezing (Fujikawa et al., 1999). These APDs and FJLs were not observed as frequently in ABA-treated cells. Membrane integrity was also measured by

in situ Fourier transform infrared spectroscopy (FTIR) using membrane phase transition temperature (T_m) to determine the effects of ABA and cryoprotectant on the behaviour of the membrane phase during the freezing–thawing cycle (Oldenhof et al., 2006). When tissue was treated with ABA and frozen in the cryoprotectant, dimethyl sulfoxide, the T_m value was similar to fresh tissue, whereas the T_m value of non-treated tissue was drastically increased, which is characteristic of freezing-sensitive tissue.

ABA treatment also increased the accumulation of soluble sugars, but not of amino acids (Nagao et al., 2005; Oldenhof et al., 2006). Compared to non-treated tissue, these studies found an increase in sucrose content of 2.6- or 5.9-fold, respectively. When the composition of soluble sugars was analysed in mosses grown on ABA-supplemented media, sucrose contributed about 22% to the total fresh weight, whereas fructose and glucose were less than 1% (Oldenhof et al., 2006). Sugar composition was also analysed in moss tissue treated with ABA for 1 day, resulting in the identification of another sugar, theanderose (G^6-α-glucosyl sucrose) (Nagao et al., 2006). The level of theanderose was increased to 13- and 30-fold after 1 and 2 days of ABA treatment, respectively. While the maximum level for sucrose was reached after 1 day, it took 2 days for theanderose to achieve its maximum level, the time at which the cells obtained their maximum level of freezing tolerance (Nagao et al., 2005). Cold-induced freezing tolerance was therefore associated with the accumulation of sucrose and theanderose. The effect of ABA treatment on freezing tolerance was inhibited by cycloheximide (CHX), an inhibitor of nuclear-encoded protein synthesis (Minami et al., 2003b), suggesting a role for newly synthesized proteins during tolerance development. CHX treatment inhibited the accumulation of theanderose, not sucrose, which decreased ABA-induced freezing tolerance. It should be interesting to see the effect of theanderose on freezing tolerance and other stress tolerance responses.

The increase in soluble sugars with increasing ABA treatment coincided with the increase in the osmotic concentration of tissues indicating that accumulation of soluble sugars by ABA treatment reduces cellular shrinkage caused by freezing, due to an increase in osmotic concentrations (Nagao et al., 2005). Cold-acclimated plants were reported to accumulate soluble sugars for freezing tolerance (Orr et al., 1986; Ristic and Ashworth, 1993; Wanner and Junttila, 1999). In addition, soluble sugars were reported to protect cells from damage caused by freezing and drying (Crowe et al., 1987; Leslie et al., 1995). Thus, the accumulated soluble sugars in protonemal cells, induced by ABA treatment, appear to play a role in protection of the plasma membrane from freezing-induced dehydration.

11.4 Expression of stress-related genes

Plants perceive and transduce stress signals leading to the expression of gene networks required for protection. Stress-induced gene products can function

Table 11.2 Genes with enhanced expression under dehydration conditions

EST with protein homology to:	Function	Accession number in NCBI
ABI3	Transcriptional activator	AJ566720 AJ566739
DREB2A	AP2 domain transcription factor	AJ566736
betA	Choline dehydrogenase	AJ566725 AJ566721b
OsCDPK7	Ca2+-dependent protein kinase	AJ566711 AJ566722 AJ566731
COR TMC-AP3	Chloroplastic amino acid-selective channel protein	AJ566742 AJ566708 AJ566743
COR47	Cold-and dehydration-induced protein	AJ566728 AJ566740 AJ566740
HSP17.6	Small heat-shock protein	AJ566737
WPM-1	ABA-induced plasma membrane protein	AJ566724 AJ566715
OsP5CS	Delta1-pyrroline-5-carboxylate synthetase	AJ566703
SODA	Mn superoxide dismutase	AJ566741

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as protectants of stress-induced cellular damage (e.g. antioxidant enzymes) as well as to perceive and transduce the stress signal through a series of intermediates, resulting in the expression of specific sets of genes for these proteins (Yamaguchi-Shinozaki and Shinozaki, 2006). A comprehensive *P. patens* expressed sequence tag (EST) database (Rensing et al., 2002) was first used to search for stress-related genes which are expressed in other plant species, and those genes were then subjected to expression profiling experiments using macroarrays (Frank et al., 2005). Among 45 EST clones tested, 19 showed upregulation by dehydration treatment in *P. patens*. These include genes whose products serve as protectants as well as in signal transduction (see Table 11.2). Among them, transcript levels of *PpCOR47, PpWPM-1, PpCOR PpTMC-AP3, PpCDPK7* and *Ppbet A* were confirmed by RNA blot analysis to be induced following dehydration treatment. Although ABA has a wide range of functions in plant growth and development, it is mainly involved as an internal signal for a perceived external stress, most commonly dehydration and related water and osmotic stresses (Zhu, 2001a). When *P. patens* protonemata were incubated in solutions of ABA, as well as with sorbitol and NaCl, distinct gene expression patterns were observed. For example, *PpCOR47* and *PpCOR TMC-AP3* were upregulated in all treatments, whereas *PpWPM-1* was expressed only after 2 h of sorbitol treatment (and not at 4 and 8 h) nor with

either ABA or NaCl. *PpbetA* was expressed after 2 h of ABA treatment (and not at 4 and 8 h) and at 8 h after incubation in NaCl and not earlier (Frank et al., 2005).

Some other genes predicted to protect cells from stress were isolated and characterized from *P. patens*. Two genes encoding calmodulin-binding proteins, MCamb1 and MCamb2, were identified in *P. patens*, which were shown to be membrane-bound transporter-like proteins (Takezawa and Minami, 2004). Transcripts of *MCamb1* and *MCamb2* were differentially increased by ABA, low temperature, mannitol and salt treatment. *MCamb1* was highly induced by hyperosmotic solutes such as mannitol and salt, whereas *MCamb2* was strongly induced by cold treatment.

Glutathione (GSH) plays an essential role in the detoxification of heavy metals in conjunction with phytochelatin (PC) in plants and fungi (Cobbett and Goldsbrough, 2002). However, since bryophytes have no PC, GSH itself binds to heavy metal ions and sequesters them (Bruns et al., 2001). The impact of cadmium ion (Cd^{2+}) stress on the sulfate assimilation pathway and GSH synthesis was comprehensively analysed in *P. patens* (Rother et al., 2006). Transcripts of all tested genes in the pathway were increased, and enzyme activities of γ-glutamyl cysteine synthetase (γ-ECS) and GSH synthetase were elevated. In parallel, intracellular cysteine and GSH levels were doubled, but no PC production was detected. Cysteine, the precursor of GSH, was also shown to be involved in the metal stress response. The expression of a *P. patens* gene (*PpMT2*) with the potential to code for a metallothionein, a cysteine-rich protein, was upregulated by Cd^{2+} and Cu^{2+} (Cho et al., 2006). In addition to the heavy metal stress response, GSH also participates in the ascorbate-GSH pathway, which detoxifies highly toxic reactive oxygen species (ROS) generated by a range of abiotic stress (Apel and Hirt, 2004). The genes for three isoforms of monodehydroascorbate reductase (PpMDHAR), one of the key enzymes in ascorbate-GSH pathway, were isolated in *P. patens* (Lunde et al., 2006). Among the genes, *PpMDHAR1* and *PpMDHAR3* were induced in response to salt, polyethylene glycol (PEG) and ABA treatment.

Although the works cited above utilize hyperosmotic solutes to mimic water loss, and ABA treatment to mimic an internal signal for the stress response known from seed plants, we know little about the components of the *in vivo* perception apparatus and the signalling pathway from the perception. In addition to the proteins involved in protection cited above, a gene encoding a signalling molecule, glycogen synthase kinase 3 or shaggy kinase (GSK3/Shaggy), a serine/threonine kinase which is implicated in diverse function in plants (Piao et al., 2001; Charrier et al., 2002; Li and Nam, 2002) was identified in *P. patens*. The family of GSK3/Shaggy kinase-coding genes (*PpSK*), consisting of five members, was shown to be monophyletic (Richard et al., 2005). They showed that expression of the *PpSK* genes was induced either by sorbitol, PEG or desiccation, showing conservation of transcriptional regulation responding to osmotic stress. Perhaps, these

are some of the components needed in parts of the stress transduction pathway.

11.5 Global responses to stress at the transcription level

In order to identify a larger gene set or a regulatory network of stress-responsive genes in *P. patens*, an analysis using a custom designed 60 oligomer microarray (17 000 probes) based on Physcobase (`http://moss.nibb.ac.jp`), the EST database of *P. patens*, was performed with various stress-treated samples (Cuming et al., 2007). Protonemal tissues were treated independently with 10 μM ABA, 0.3 M NaCl and 10% mannitol for 2 h. For a desiccation treatment, protonemal tissue was incubated in a controlled humidity environment (75%) for 24 h, resulting in 84% of fresh weight loss. ABA and drought induced the expression of 57 and 101 genes, respectively, but the expression of only ten genes was elevated by osmotic stress and of eight genes by salt stress. The expression of 51 genes was induced by at least two treatments. The upregulated genes were mainly homologues of genes coding for stress-related proteins in seed plants, including the late embryogenesis abundant (LEA) proteins. In particular, the homologues were mostly group III LEA proteins but also included two group II LEA proteins. Their expression was also verified by experimental RNA blot assays.

Another class of highly upregulated genes potentially coded for proteins involved in the transmembrane transport of solutes for osmoregulation, such as membrane channel proteins, sucrose transporters, voltage-dependent anion channel proteins, aquaporins and sugar transporters. Some genes were homologous to those coding for ABA and stress-induced proteins in flowering plants such as dehydration-associated, cold regulated, early responsive to dehydration 1 (ERD1) and ethylene-responsive rice protein genes. Moreover, ten moss-specific genes with coding potential for proteins without homology to other known proteins were identified, including the gene most strongly upregulated by drought stress. The downregulated genes mainly coded for chloroplast proteins, mostly involved in photosynthesis.

When the promoter regions of the most strongly upregulated genes were investigated for over-representation of specific motifs, the G-box elements (Yamaguchi-Shinozaki and Shinozaki, 2006) containing the ACGT core motif of the ABA-responsive element (ABRE) were significantly over-represented. Other classes of abiotic stress-responsive factors, the dehydration-responsive element (DRE) (Zhu, 2002), the basic helix-loop-helix-zipper domain (bHLHZip) *MYC* factors (CANNTG) and helix-turn-helix *MYB* factors (YAACTG) (Abe et al., 2003) were also found in relatively high abundance in the promoter regions. Other than these, 'CCCCCC', 'CCCCCA', 'GGGGGA' and 'GGGCCC' are hexameric sequences that are over-represented. Considering the promoter regions of *P. patens* are significantly

less GC-rich (41%) than the coding sequence (46%), these sequences might be candidates for *cis*-acting regulatory elements.

11.6 The ABA-signalling pathway

In flowering plants, ABA plays a central role in the regulation of seed development and germination, and of water stress responses during the later stage of embryogenesis as well as in vegetative tissue (Leung and Giraudat, 1998). The ABA-response pathway is present in *P. patens* as demonstrated by the change in gene expression pattern by ABA treatment (Knight et al., 1995; Machuka et al., 1999). The ABA-inducible wheat *Em* promoter was activated by exogenous ABA treatment in *P. patens* (Knight et al., 1995). Mutation of the *Em* promoter demonstrated that the same regulatory *cis*-element in the promoter region was recognized in *P. patens* as in wheat, which suggests that there are common regulatory factors in *P. patens* and seed plants.

LEA proteins are known to be important for protection from dehydration, salt and osmotic stress in vegetative tissues (Ingram and Bartels, 1996). A group-1 *LEA* gene, *PpLEA-1*, was reported to be upregulated in response to ABA treatment and osmotic stress (Kamisugi and Cuming, 2005). Expression analysis using a promoter-GUS (β-glucuronidase) fusion construct showed that *PpLEA-1* responds preferentially to ABA in protonemal tissue, and to osmotic stress in gametophores. The level of expression in response to ABA was similar to that obtained with the promoter of the wheat group-1 *LEA* gene, the *Em* gene. The core of the ABRE, the ACGT sequence, was required for transcription as in higher plants (Yamaguchi-Shinozaki and Shinozaki, 2006). The wheat *Em* promoter was active in *P. patens*, whereas the *PpLEA-1* promoter was not sufficient to direct gene expression in barley protoplasts, suggesting that barley cells require additional factors while moss has the minimal requirements for promoter recognition. However, three copies of ABI3 genes (*PpABI3*), a transcriptional regulator in the ABA-signalling pathway, have been reported and PpABI3A enhanced the expression of *PpLEA1* in response to ABA treatment in *P. patens* (Marella et al., 2006). Similarly, PpABI3A enhanced the expression of *Em*-GUS in barley aleurone cells but to a lesser extent than was achieved by Viviparous1 (VP1) from maize and ABI3 from *A. thaliana*. In addition, a group 2 LEA gene, *Ppdehydrin* (*PpDHNA*), was also characterized in *P. patens* (Saavedra et al., 2006). Transcript levels of *PpDHNA* were increased by ABA, NaCl and mannitol treatment, showing a similar expression pattern to dehydrin-coding genes in seed plants (reviewed in Rorat, 2006). When moss returned to normal growth condition, the transcript level rapidly decreased. The removal of the *PpDHNA* gene by homologous gene targeting resulted in severe impairment of the capacity to resume growth after salt and osmotic stress treatments, suggesting PpDHNA plays an essential role during salt and osmotic stress in *P. patens*. Sugar and proline transporters also take part in the protection of cells from stress (Yamaguchi-Shinozaki and

Shinozaki, 2006). Recently, genes with the potential to code for three different classes of ABA receptors have been reported. *P. patens* possesses in its genome, sequences with the potential to code for all the known ABA receptors. A gene coding for an ABA receptor, Mg-chelatase H subunit (MgCH) (Shen et al., 2006) is present in the *A. thaliana* genome as one copy, whereas *P. patens* has two copies. It shares more than 75% of identity with MgCHs from seed plants. Genes which could code for another ABA receptor, a G protein-coupled receptor (GCR2) (Liu et al., 2007), exist in the *P. patens* genome as two copies, compared with three copies in *A. thaliana*, which shares about 50% of identity with GCR2 in the seed plants *A. thaliana, Oryza sativa*, and *Medicago truncatula*. Transcripts for both of these genes are represented in the EST collection. Transcripts coding for the FCA-type ABA receptor, an RNA-binding protein involved in flowering processes in seed plants (Razem et al., 2006), are also present and contain all the components encoding the RNA recognition motif and WW protein interaction domain of FCA in seed plants. *P. patens* possesses two copies of FCA-coding genes, which produce transcripts of about 2.6 and 2.9 kb. The number of exons are 21 and 22 respectively, which is similar to the 21 exons reported in *A. thaliana*. However, the conceptual translations of both *P. patens* FCAs are terminated in the middle of their transcripts, resulting in loss of the proximal ABA-binding domain at the C-terminus. Considering that *P. patens* does not flower, the function of FCA may be different. This raises the interesting possibility that the sequence might have been edited at some point between the bryophytes and seed plants.

11.7 Conclusions

It is clear from this brief review that *P. patens* is an excellent model to study signalling pathways involved with abiotic stress responses in plants, not only because its ABA responses and intermediates are so similar to seed plants, but also because it can be manipulated genetically and molecularly to identify the underlying causes. Pre-treatment with ABA has significant effects in promoting tolerance to high water stress in protonemal tissue, and it appears that some of the same signalling molecules identified to transduce the signal in *A. thaliana* are also present in *P. patens*. Several studies have or soon will be able to exchange specific genes in the pathway between *A. thaliana* and *P. patens* to determine if complete gene function is conserved, and by so doing define the important domains that have a conserved function. Finally, since the phenotypes that arise from exposure of the wild type to ABA stress, or from specific gene deletion lines of ABA-signalling intermediates, are rapidly and easily distinguishable from untreated or wild-type tissue, *P. patens* will serve as an ideal screen for mutants with altered function. With the genome sequenced (Rensing et al., 2008) and a genetic map being assembled, the genes that are associated with the phenotypes should be quickly identified

and the putative target genes can be immediately validated by targeted gene deletion (Quatrano et al., 2007).

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Chapter 12

PATHOGENESIS IN MOSSES

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Abstract: *Physcomitrella patens* is an emerging model system for functional studies of plant–pathogen interactions. The ability to create gene knockouts and site-specific mutations in any gene, coupled with other features, such as dominance of the haploid phase, simple anatomical organization and ease of growth and propagation, allow *P. patens* to be used as a 'green yeast' in experiments with pathogens and pathogen-derived inducers of the defence response. *P. patens* has been shown to be susceptible to a range of necrotrophic bacterial and fungal pathogens, which are able to infect and multiply on the plant. Many of the cellular and molecular responses of *P. patens* to attempted infection are similar to those observed in seed plants, and include the production of reactive oxygen species, the synthesis of secondary metabolites, changes in genes expression and the activation of a programmed cell death pathway (PCD). Pathogen-derived elicitors, toxins and pathogen-associated molecular patterns (PAMPs) can also induce many of these responses. Gene knockouts confirm that this response is under genetic control and show that this model system can be used to examine other aspects of plant–pathogen interactions. We review our current understanding of *P. patens* pathology and defence responses and discuss how this system can be used to study plant defence signaling pathways, and to characterize the functional contributions of defence genes to the expression of disease resistance. We also discuss how genomic and other large-scale approaches might be used for *de novo* gene discovery in this system.

Keywords: defence response; disease resistance; PAMPs; physcomitrella; plant–pathogen interactions; programmed cell death (PCD)

Abbreviations:

HR	hypersensitive response
MAMPs	microbial-associated molecular patterns
PAMPs	pathogen-associated molecular patterns
PCD	programmed cell death
R-gene	resistance-gene
ROS	reactive oxygen species

12.1 Introduction

Physcomitrella patens, though rarely studied by plant pathologists, offers a unique experimental platform for studying plant–pathogen interactions. As has been described in previous chapters of this book, *P. patens* is unrivalled as a multicellular model plant system for reverse genetic studies. The focus of this chapter is on using *P. patens* to carry out basic and applied research in plant pathology, with a particular emphasis on gene discovery. We address the following specific topics and questions:

- *Experimental features of P. patens*. What are the advantages and limitations of studying disease in *P. patens* and how can it contribute to our fundamental understanding of plant–pathogen interactions? We provide an overview of how *P. patens* can be used to characterize plant genes involved in disease susceptibility or resistance. We also indicate the suitability of *P. patens* for high-throughput screens of pathogen genes, as well as chemical compounds that can interfere with infection or suppress disease symptoms.
- *Developing a P. patens pathosystem*. Developing an infection system is a basic requirement for studies in plant pathology. In this section, we discuss two different approaches for characterizing pathogens of *P. patens* for experimental use. The first uses 'off-the-shelf', broad host-range pathogens that infect seed plants. The second involves the characterization and use of pathogens that infect *P. patens* or other mosses. The validity and limitations of these approaches will be discussed, along with the criteria that can be used to assess their significance for particular infection systems. The advantages of using alternatives to pathogens, such as elicitors of the defence response or pathogen-derived toxins, will be discussed.
- *Responses of P. patens to infection*. Since *P. patens* is a new experimental pathosystem, it is important to characterize the course of infection and the responses of the plant to pathogen attack. We discuss some of the cellular, molecular and biochemical responses associated with infection of *P. patens* by pathogens. We also describe how these responses can serve as markers for interpreting different plant–pathogen interactions and for interpreting the phenotypes of gene knockout plants.
- *Selecting P. patens genes for functional studies of disease*. Once an infection system has been developed for *P. patens*, it is possible to assess the contribution of candidate plant genes to disease susceptibility or resistance by creating knockouts of these genes. We describe various approaches and criteria for selecting genes for functional studies. We also discuss how approaches that combine whole-genome analysis with the use of mutants can be used to improve the efficiency of this process.
- *Future directions and opportunities*. The study of *P. patens* pathology is in its infancy. The immediate potential is in defining mechanisms associated with disease susceptibility. We will discuss how *P. patens* might also be used as a system to understand the molecular basis of disease resistance

and to identify the plant cell targets of pathogen effectors. We also indicate the experimental tools that need to be developed so that the advantages of *P. patens* for gene discovery can be fully exploited, and in particular the need for methods that can promote the use of forward genetics.

To place these experimental opportunities in context, we first discuss recent advances in plant–pathogen interactions, with a particular focus on the means by which pathogens are recognized by plants, the steps that pathogens take to subvert this process, and the countermeasures developed by plants to overcome pathogens' subversive mechanisms. This rich and complex interplay between host and pathogen highlights the importance for the plant to activate a defence response as well as the corresponding imperative for the pathogen to prevent this from happening. This framework is useful for highlighting areas that are not yet well understood and for indicating where *P. patens* can make a useful contribution to our understanding of plant–pathogen interactions.

12.2 Recent advances in molecular plant pathology

Molecular genetic approaches have contributed greatly to our understanding of the mechanisms that regulate plant–pathogen interactions, including the molecular basis for pathogen detection, the signaling pathways that control the plant defence response and the nature and mode of action of components of the plant defence response (Dangl and Jones, 2001). In this section, we provide an overview of some of these recent developments, and indicate areas that might benefit from the experimental tools available in *P. patens*. For a more comprehensive review of our understanding of molecular and biochemical phytopathology, the reader is referred to several excellent and recent reviews (Glazebrook, 2005; Chisholm et al., 2006; Jones and Dangl, 2006; Bent and Mackay, 2007; de Wit, 2007; Wise et al., 2007).

12.2.1 Innate immunity and PAMPs

In the last decade, it has become clear that plants can detect pathogens through a mechanism that shares characteristics with innate immunity of animals (Nicholas and Hodgkin, 2002; Ausubel, 2005; Akira et al., 2006; Iriti and Faoro, 2007; Ryan et al., 2007). Plants can recognize and respond to a number of molecules that are characteristic of microbial pathogens (Boller, 1995), such as flagellin (Gómez-Gómez and Boller, 2002; Zipfel et al., 2004), lipopolysaccharide (Silipo et al., 2005), elongation factor EF-Tu (Zipfel et al., 2006), chitin oligomers (Ning et al., 2004; Kaku et al., 2006), β-glucan oligomers (Daxberger et al., 2006) and enzymes (Ron and Avni, 2004) among others (reviewed in He et al., 2007). These pathogen determinants are called microbial-, or pathogen-associated molecular patterns (MAMPs or PAMPs) and are recognized by dedicated plant receptors, many of which share structural features with R-proteins, the products of classical disease resistance, or *R*-genes (Tameling

and Takken, 2007). Recognition of PAMPs induces the plant defence response via a MAP kinase signaling cascade that regulates defence gene expression through the activity of WRKY transcription factors (Euglem et al., 2000; Asai et al., 2002; Journot-Catalino et al., 2006; Tameling and Takken, 2007). This series of events ultimately restricts or kills the pathogen and hence prevents disease.

Because PAMP receptors interact with highly conserved features of pathogens, these receptors are likely to be highly conserved among plants and able to detect a broad range of pathogens. In contrast, the products of plant *R*-genes and pathogen avirulence genes tend to be polymorphic. As a result, R-proteins can recognize only a narrow range of avirulence determinants (Ellis et al., 2000). This latter type of interaction forms the basis for the gene-for-gene interactions that characterize race–cultivar-specific disease resistance. While these two systems of pathogen recognition (one for broadly acting PAMPs that bind with a lower affinity to receptors found in many plant species and the other for effector proteins that bind selectively and with higher affinity to specialized R-proteins, or R-protein complexes) appear to be distinct, there may be some functional and evolutionary overlap between them, especially if we consider the structural similarities between many R-proteins and PAMP receptors. The functional significance of PAMPs has been demonstrated in studies where PAMP receptors have been mutated. The flagellin receptor FLS2 is a transmembrane protein of the LRR-kinase family (Gómez-Gómez and Boller, 2002). *Arabidopsis thaliana* mutants of *FLS2* were more susceptible to *Pseudomonas syringae* pv. *tomato* strains DC3000 (Zipfel et al., 2004). Similarly, gene knockouts of the *A. thaliana* EFR1 transmembrane receptor kinase, which binds the bacterial EF-Tu protein, made plants more susceptible to *Agrobacterium tumefaciens* (Zipfel et al., 2006).

12.2.2 Pathogen effectors of virulence and R-proteins

Our current view is that these different recognition systems came about through an evolutionary game of cat and mouse on the part of the plant and the pathogen (Chisholm et al., 2006), in which pathogens were first recognized by a plant surveillance system that could then detect the broadly conserved yet indispensable pathogenic components we now call PAMPs. This 'primitive' PAMP-inducible plant defence response must have been (and remains) an effective disease resistance mechanism, since pathogens have developed strategies to thwart it. Pathogens produce and deliver a range of effector molecules that can interfere with various steps in the PAMP-induced signaling pathway and defence response. In the last decade, there has been a greater understanding of the importance and mode of action of pathogenic effectors of virulence, the nature of their targets within the plant and the ways in which the plant is able to detect these effectors (Bent and Mackay, 2007; da Cunha et al., 2007; de Wit, 2007; He et al., 2007).

Pathogen effector molecules (which are mostly proteins, but can also be small molecules) promote colonization of the host by interfering with its

normal cellular functions. Effectors and effector targets vary considerably from pathogen to pathogen and have been shown to inhibit diverse aspects of the defence response (Hauck et al., 2003; DebRoy et al., 2004; Fujikawa et al., 2006; He et al., 2006; Greenshields and Jones, 2007). Several effectors inhibit plant cell death that is associated with disease resistance mediated through the hypersensitive response (HR) (Jamir et al., 2004). Other effectors, produced by necrotrophic pathogens (which colonize dead or dying tissues), promote plant cell death and hence enhance pathogen virulence on susceptible hosts. *Pseudomonas syringae* pv. *tomato* AvrPtoB encodes a single protein with separate domains that respectively promote and inhibit host cell death (Abramovitch and Martin, 2005). These opposing activities may provide advantages to the pathogen at different stages of infection, first inhibiting host cell death during the early stages of infection, then promoting it once the pathogen has established itself and is ready to switch from a biotrophic to a necrotrophic phase. Effectors can also inhibit events downstream of PAMP recognition such as signaling through the MAP kinase pathway (He et al., 2006; Shan et al., 2007), or even at the site of expression of the antimicrobial response (Kamoun, 2007).

Effectors of virulence were first characterized in bacterial pathogens, but it is clear that these molecules are also produced by fungal pathogens (Chisholm et al., 2006; Kamoun, 2007). What distinguishes these pathogens is their means for delivering effectors into plant cells. While bacteria have developed specialized secretion pathways that can deliver effectors into host cells such as the type III secretion system, or TTSS (Alfano and Collmer, 1997; Casper-Lindley et al., 2002; Alfano and Collmer, 2004) and other related systems (Cascales and Christie, 2003; Troisfontaines and Cornelis, 2005), secreted effectors of fungal and oomycete pathogens (Rentel et al., 2008) are taken up through an unknown mechanism pathway that occurs at the fungal–plant interface (e.g. at the haustorium (Chisholm et al., 2006; Catanzariti et al., 2007; Kamoun, 2007)).

To prevent pathogen effectors from subverting PAMP activation of the defence response, plants have developed an additional surveillance system to detect effector molecules (Jones and Dangl, 2006). In this secondary system, pathogen effectors are detected through a direct interaction with R-proteins or via an indirect mechanism, in which the interaction of the pathogen effector with its host target is perceived by the R-protein through the perturbation of other receptor components such as proteases and protein kinases (Bent and Mackay, 2007; Tameling and Takken, 2007). An effector that is recognized by the plant and that leads to the induction of the defence response and the restriction of the pathogen also serves as a *de facto* avirulence protein, which is the traditional nomenclature for the pathogen-recognition component of gene-for-gene-type interactions. The molecular details underlying the recognition of pathogen effectors as avirulence determinants by *R*-genes have begun to be elucidated (Kim et al., 2005; Day et al., 2006; Jones and Dangl, 2006; Bent and Mackay, 2007).

12.2.3 Disease susceptibility

Effectors target key points in the regulation and expression of the defence response and one can think of pathogen effectors cumulatively as a natural pharmacopoeia for probing host mechanisms involved in the expression of disease susceptibility. The practical goal of such studies is to understand these interactions to the point where the cellular target can be mutated so that it no longer interacts with the pathogen effector but is still able to perform its inherent cellular functions. *P. patens* may be useful in developing this strategy since it would be easy to test the efficacy of large numbers of mutated targets of pathogen effectors in this system. The hope is that disease resistance based on such strategies might be more durable than *R*-gene mediated resistance. Our experience indicates that *P. patens* represents an excellent system for these types of studies and for studying disease susceptibility in general. If pathogen-recognition mechanisms are conserved in *P. patens* (and our studies and others' indicate that they may well be), then it should be possible to use the unique molecular genetic approaches available in *P. patens* to identify the nature of PAMP and effector targets and assess the consequences of their mutation on the course of infection, the ability to mount a defence response and the expression of disease resistance.

12.2.4 Disease resistance

No gene-for-gene-type interactions have yet been characterized in *P. patens*, and until they are, the biology of *R*-gene mediated disease resistance will not be accessible in this system. This may well change, as pathogens that induce an incompatible resistance response are identified, or if race–cultivar-based recognition systems that function in seed plants can be transferred into *P. patens*. A number of alternative approaches can be used to study the plant defence response in the absence of such pathogens, and these can be used to access mechanisms of disease resistance in *P. patens* experimentally. One approach is to induce disease resistance responses with elicitors, characterize the components of the response and determine its efficacy against virulent pathogens. Another is to induce a non-host resistance response by inoculating *P. patens* with non-pathogens (Heath, 2000b; Mishina and Zeier, 2007). These approaches, when combined with the creation of targeted gene knockouts, can be used to assess the functional contributions of individual defence response genes toward the expression of disease resistance. This is an area in which *P. patens* can make an immediate contribution to our understanding of the nature and function of the plant defence response. While a huge number of genes are induced during infection, at least in seed plants (Eulgem et al., 2004; Glazebrook, 2005; Wise et al., 2007), the functional significance of most them is unknown. In only a limited number of cases is there compelling genetic evidence from gain-of-function or loss-of-function studies that demonstrates how a particular defence gene contributes to the

expression of disease resistance (Glazebrook, 2001; van Loon et al., 2006). Most likely, this is because there is tremendous functional redundancy in the defence response of seed plants.

12.2.5 Evolution of plant defence responses

Our understanding of plant–pathogen interactions is based on a phylogenetically narrow group of host plants that includes many economically important crops such as maize, rice and tomato, as well as experimental systems such as *A. thaliana*. There have been a number of studies on the defence responses and pathogenic interactions of gymnosperms, especially conifers (Davis et al., 2002; Adomas et al., 2007). A recent review of the literature suggests that bryophytes may form a wide range of interactions with fungi (Davey and Currah, 2006) and there is good evidence to show that even multicellular and unicellular algae are subject to biotic stress and can mount a defence response (Potin et al., 2002; Kubanek et al., 2003; Bidle and Falkowski, 2004; Bouarab et al., 2004; Weinberger, 2007). Nonetheless, we know very little about how plants other than seed plants cope with biotic stress and whether they have evolved specific and novel mechanisms for coping with them. The use of *P. patens* as an experimental system should throw light on the functional significance of the defence mechanisms of bryophytes and the degree to which they diverge from the corresponding mechanisms present in angiosperms. These bryophyte-specific mechanisms may even suggest new ways to control disease in crop plants.

12.3 Experimental advantages of *P. patens*

12.3.1 Studying stress responses in *P. Patens*

P. patens has a number of practical and experimental features that make it very well suited as a system for studying fundamental cellular and molecular processes in plant biology (Cove et al., 1997; Reski, 1998a; Schaefer, 2002). In addition to its suitability for studying fundamental processes in plant cell biology, *P. patens* has been used increasingly as a model system to study plant responses to environmental stress (Knight et al., 1995; Russell et al., 1996; Machuka et al., 1999; Oliver et al., 2000; Frank et al., 2004). Examples, developed in more detail in Chapter 11 of this book, include the response to, and tolerance of, drought (Minibayeva and Beckett, 2001; Minami et al., 2003a), salt stress (Frank et al., 2004; Saavedra et al., 2006), heat shock (Saidi et al., 2007), osmotic stress (Knight et al., 1995; Richard et al., 2005) and cold shock and freezing tolerance (Minami et al., 2003a, 2003b; Takezawa and Minami, 2004; Oldenhof et al., 2006). The establishment of infection systems, described later on, in conjunction with other experimental tools, opens up *P. patens* as an experimental system for the similar study of biotic

stress responses. In the following section, we describe how the experimental features of *P. patens* lend themselves to these studies.

12.3.2 Using reverse genetics to study plant–pathogen interactions in *P. patens*

The ability to generate structurally defined mutants in *P. patens* offers enormous potential for reverse genetics focused on plant–pathogen interactions. While tools for obtaining insertional mutants and suppressing gene activity have been developed for seed plants, such as *A. thaliana* (Borevitz and Ecker, 2004), *P. patens* provides the researcher with an unlimited capacity to manipulate the genome in a manner that is comparable to approaches that have been widely exploited in yeast (Fernandez-Lobato et al., 1990). For example, yeast has been elegantly used to explore the function and mechanism of action of the PR-5 class of plant pathogenesis-related proteins (Narasimhan et al., 2005) and to define cellular targets for mycotoxins produced by plant pathogens (Schindler et al., 1974).

Pathologists studying seed plant diseases may benefit from an experimental tour into *P. patens* in order to address questions that are not experimentally amenable to study or simple resolution in seed plants. Examples include genes that exist as large families in seed plants, but are represented in *P. patens* by only a single or two gene copies, or where there is functional redundancy due to the operation of multiple, mechanistically distinct pathways. If *P. patens* represents a simpler plant than *A. thaliana*, we might expect some mutant phenotypes to be more apparent than they are in seed plants. We have seen examples of this in our own work on genes that regulate programmed cell death (PCD). For example, knockouts of the Bax inhibitor-1 (BI-1) gene in *P. patens* show a marked increase in sensitivity to cell death signals such as mycotoxins, compared to the comparable *A. thaliana* T-DNA insertion line, which shows only a quantitative increase in sensitivity to mycotoxins (HS and ML, unpublished data; Watanabe and Lam, 2006). Phenotypic differences between mutants in *P. patens* and *A. thaliana* cannot be predicted but these results remind us that alternative experimental systems help elucidate gene function, especially where no phenotype has been observed for extant mutants in seed plants (Ossowski et al., 2008).

These features of *P. patens* should make it useful for assessing the contribution to infection of putative PAMP receptors, the targets of cellular effectors and toxins, and components of defence signaling and response. In the long run, we expect that *P. patens* will become amenable to additional kinds of investigations in plant pathology, but this will require the development of additional pathosystems (such as those that demonstrate incompatible interactions and invoke an HR (Morel and Dangl, 1997; Heath, 2000a; Mur et al., 2008)) as well as the development of molecular genetic tools that allow the mapping and cloning of genes that are defined from forward genetic screens (Østergaard and Yanofsky, 2004).

12.3.3 Screening for pathogen genes and small molecules in *P. patens*

P. patens is also well suited for performing high-throughput screens of pathogen genes that affect virulence as well as screens for chemical or biochemical agents that can interfere with the course of disease. Its small size and ability to be cultured easily make it well suited for high-throughput screens conducted in multiwell plates. For pathological interactions with a clearly defined end point (e.g. the extensive plant cell death displayed by plants susceptible to necrotrophic pathogens), it is relatively easy to screen large collections of combinatorial chemical libraries or natural product collections to identify compounds that either interfere with the interaction or phenocopy responses of known *P. patens* gene knockouts to infection. These approaches can also be used to screen bacterial and fungal mutant libraries for microbial genes that condition pathogenicity and virulence on this host. Similar approaches have been used in angiosperms (Collmer et al., 2002; Chang et al., 2005), although in those cases, the manual infusion of individual mutants into leaf sectors makes the process labor intensive, while the size of the plant (even as cotyledons) makes demands on greenhouse or growth chamber space. In contrast, a large-scale assay using *P. patens* can be conducted in a minimal amount of space within a controlled growth environment. An additional advantage of using *P. patens* for these types of screens is that the mode of action of any 'hits' (e.g. a chemical that inhibits disease symptoms) can be investigated through the creation and assay of knockouts or other mutations in genes that encode possible targets of the inhibitor. This approach is essentially derived from screens for pharmaceuticals and is subject to similar considerations of cost, scale, efficiency, specificity, redundancy and the need to avoid rediscovery of known agents.

12.3.4 Limitations of *P. patens* in studying plant–pathogen interactions

P. patens, like any model system, has a number of disadvantages. Some of these are inherent, while others simply reflect our present ignorance. For example, we simply do not yet know if all of the signaling molecules that coordinate the seed plant defence response are present in *P. patens* and whether they play a similar role. So far, there are no reports of interactions with pathogens that result in an HR, although this probably reflects a sampling problem, rather than an inherent inability of the plant to mount this response. Nonetheless, it is not yet practical to study this response in *P. patens*, and the search for pathogens (and non-pathogens) that might induce this response as it occurs in seed plants (Mishina and Zeier, 2007; Sanabria et al., 2008) is a major priority. For the time being, *P. patens* remains an excellent system for studying disease susceptibility, but is not yet suitable for studying gene-for-gene-type interactions that condition race–cultivar specificity. The likelihood that such a system exists, and that *P. patens* is capable of recognizing pathogen avirulence

proteins, is suggested by the presence within the moss genome of TIR-NBS-type *R*-genes (Akita and Valkonen, 2002).

P. patens also lacks many anatomical features that are important in the interactions of pathogens with tracheophytes. *P. patens* gametophytes lack stomata, which are a point of entry for many pathogens, a site for the expression of innate immunity and a target for bacterial effectors that prevent stomatal closure (Nomura et al., 2005; Melotto et al., 2006). *P. patens* also lacks the intercellular spaces found in leaf spongy mesophyll tissues, and which provide a protected environment in which bacteria can multiply, sense if there is a quorum for infection and induce virulence mechanisms (von Bodman et al., 2003). These spaces also allow hemi-biotrophic fungal pathogens to ramify throughout host tissues, prior to the expression of the necrotrophic phase. A more obvious difference is the absence of an elaborate vasculature in *P. patens*, compared to tracheophytes. This has obvious consequences for the spread of pathogens within the plant, especially for viruses and bacteria. In angiosperms, localization of pathogens to a limited sector of the leaf can provide effective resistance for the rest of the plant, as long as the pathogen is denied access to the plant's vascular system. However, mosses do contain simple conducting vessels which are important for transporting metabolites and these may similarly facilitate the spread of pathogens (Ligrone et al., 2001).

Differences in the composition of the cell wall, especially the absence of secondary thickening associated with lignification, may also be important (Edelmann et al., 1998; Lee et al., 2005; Fu et al., 2007; Ligrone et al., 2007). Many pathogens have developed an extensive and effective lytic machinery, which they use to break down the cell wall. This provides access into the host cell, and may also serve as a source of nutrients. There may also be critical differences in the composition of pre-existing and inducible chemical and biochemical defences. Other differences may extend to the suite of defence signals that are present in *P. patens*, the nature of their targets, and the composition of the defence response. Examination of the *P. patens* genome indicates the presence of a biosynthetic pathway for ethylene, as well as components that are known to be important for ethylene perception and signaling in seed plants (Rensing et al., 2008). To date, no ethylene-dependent response has been characterized, but it may be possible to reveal the biological role of this signaling molecule in *P. patens* through the creation of targeted knockouts of genes that encode components of ethylene biosynthesis or signaling. Oxylipins are produced upon wounding of *P. patens* (Senger et al., 2005; Wichard et al., 2005) and it is possible that these molecules also play a role in other responses of *P. patens*, such as senescence and defence against pathogens (Farmer et al., 2003). It is also not yet clear if salicylic acid, which is known to be important for defence signaling and the expression of the HR in seed plants (Klessig et al., 2000; Durrant and Dong, 2004), is produced by or plays a similar role in *P. patens*. These factors do not disqualify *P. patens* as an experimental system, but they do need to be borne in mind if *P. patens* is to be used as a model system for pathogens of seed plants.

12.3.5 Developing resources for forward genetics

P. patens currently lacks many molecular and genetic tools for performing forward genetic screens (Østergaard and Yanofsky, 2004). While it is simple to generate point mutants through the use of chemicals or ionizing radiation (indeed, these were among the first types of genetic experiments performed in this system (Engel, 1966, 1968)), it is not yet possible to isolate the corresponding genes routinely. This situation is in stark contrast to the tools that are available for such studies in *A. thaliana*, such as high-density linkage maps and recombinant inbred lines (Singer et al., 2006) that are integrated with the physical sequenced genome, as well as YAC and BAC libraries whose ends are sequenced and mapped onto the genome (Chang et al., 2001; Borevitz and Ecker, 2004). Although these tools are being developed (and are being aided by the availability of a fully sequenced genome (Rensing et al., 2008)), it will be some time before one can routinely and rapidly isolate a gene from a mutant phenotype in *P. patens*. There is nothing inherent about *P. patens* that precludes the development of these tools, and we expect that these tools will soon become available within the *P. patens* research community. One promising development is the application of next-generation DNA sequencing technologies (e.g. the 454, Solexa/Illumina and Applied Biosystems SOLiD technology platforms), which should help identify large numbers of SNP and VNTR polymorphisms for constructing high-density linkage maps.

12.4 Developing a *P. patens* pathosystem

12.4.1 Natural pathogens of *P. Patens*

A recent and comprehensive review by Davey and Curruh (Davey and Currah, 2006) and previously by Felix (Felix, 1988) indicates that interactions between mosses and fungi may be widespread and involve a taxonomically broad range of mosses and fungi (Racovitza, 1959; Felix, 1988; Döbbeler, 1997; Tsuneda et al., 2001). For example, *Nectri mnii* was able to ramify throughout stem tissues of *Plagiomnium medium* (Döbbeler, 1988) while *Scleroconidioma spagnicola* infected and caused disease on *Sphagnum fuscum* (Tsuneda et al., 2001; Davey and Currah, 2006). Some of these interactions are quite specific (e.g. those of *Epibryon interlamellare* and *Bryorella cryptocarpa* with species in the *Polytrichaceae* (Davey and Currah, 2006)) and may not be transferable to *P. patens*, whereas others involve broad host-range necrotrophs that may also be able to colonize and cause disease symptoms on *P. patens*. Interactions of mosses with bacterial pathogens are less well characterized, but this most likely reflects the paucity of studies on this topic rather than any inherent resistance of mosses to bacterial infection. Certainly, we have found it possible to infect laboratory-grown *P. patens* with a range of pathogenic bacteria (see below). *P. patens* plants collected

from the wild (Parisod and Streiff, 2002) display a range of symptoms that are consistent with infection by pathogenic micro-organisms (Figure 12.1a, panels 1–3, Plate 18). Symptoms include the presence of small clear or brown lesions with a discrete border involving one or a few cells that are reminiscent of the HR of seed plants as well as spreading brown lesions with a diffuse border, which are characteristic of susceptible interactions with necrotrophic pathogens. We also observed otherwise healthy leaves of *P. patens* in which fungal hyphae have ramified throughout the infected area (Figure 12.1a, panels 4 and 5, Plate 18). One of these pathogens was identified by DNA sequencing as a near relative of *Fusarium culmorum*, a known pathogen of wheat and other plants (M. Hijri and ML, unpublished data). This strain could reinfect and cause symptoms on healthy plants (Figure 12.1a, panel 6, Plate 18). These studies have prompted us to test related fungi that cause disease on seed plants for their ability to infect *P. patens* (described below).

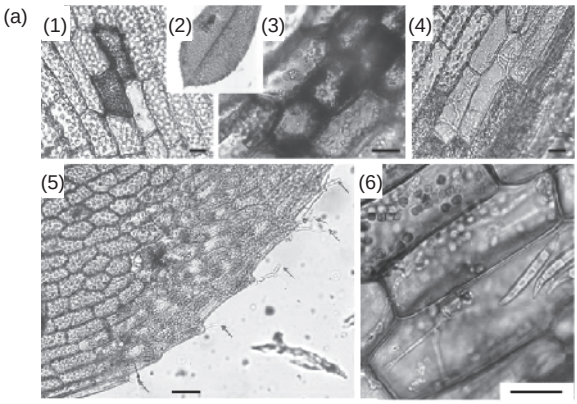
12.4.2 Laboratory infection of *P. Patens*

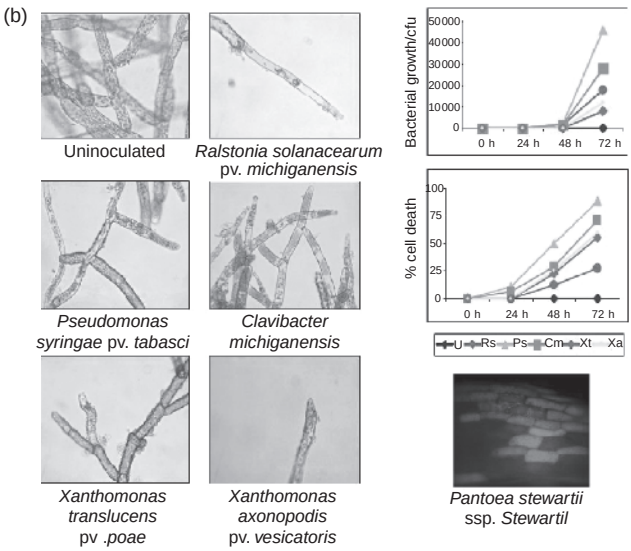
There are two approaches to developing an experimental infection system in *P. patens*. The first makes use of broad host-range pathogens that are known to infect other plants. To ensure maximal relevance for agricultural research, it is helpful if these pathogens cause disease on crop species, so that results obtained from studies in *P. patens* are relevant and applicable to understanding the progression of disease in economically important plants. By focusing on pathogens of crop species, the experimenter can exploit a large body of information on the pathogen and gain access to important experimental resources such as sequenced genomes and mutant collections. A limitation of this approach is that not all broad host-range pathogens of seed plants may be competent to infect *P. patens*, or even mosses in general.

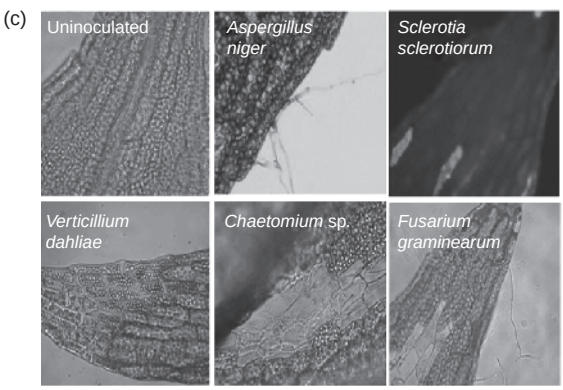
The second approach is the classical plant pathological one of characterizing diseased *P. patens* plants (or other mosses), isolating, culturing and propagating the infectious agents and recreating the disease symptoms by reinoculation onto uninfected plants. The advantages of this approach are that the infectious agent, resulting disease symptoms and plant defence responses are likely to be biologically relevant and this allows *P. patens* mutants to be assayed in a biologically appropriate context. These two approaches are not mutually exclusive and it may be possible to find *P. patens* plants infected in the wild with pathogens that also infect seed plants.

12.4.3 Physiological significance of pathogenic interactions with *P. Patens*

How do we judge the biological significance of infections of *P. patens* with microbial pathogens? Can we distinguish a true pathogen from an opportunistic one? There has been much debate on this subject (Falkow, 1997). Similar concerns have been made concerning the interactions of *A. thaliana* with some


(a)
(1)
(2)
(3)
(4)
(5)
(6)


(b)
Uninoculated
Ralstonia solanacearum pv. michiganensis
Pseudomonas syringae pv. tabasci
Clavibacter michiganensis
Xanthomonas translucens pv .poae
Xanthomonas axonopodis pv. vesicatoris
Bacterial growth/cfu
50000
40000
30000
20000
10000
0
0 h
24 h
48 h
72 h
% cell death
100
75
50
25
0
U
Rs
Ps
Cm
Xt
Xa
Pantoea stewartii ssp. Stewartil


(c)
Uninoculated
Aspergillus niger
Sclerotia sclerotiorum
Verticillium dahliae
Chaetomium sp.
Fusarium graminearum

experimental pathogens (Mur et al., 2008). For example, *Pseudomonas* spp. inoculation on *A. thaliana* is typically performed by infusing an artificially high titer of bacteria into the intercellular leaf spaces. This almost certainly does not reflect the situation in the field, where typically a few bacteria enter a plant at a wound site or through the stomata or are delivered via an insect vector. Nonetheless, by using this approach in *A. thaliana*, it has been possible to characterize mutants and clone genes such as *R*-genes and other downstream regulatory components such as *NPR1* (Cao et al., 1997) and *NDR1* (Century et al., 1997) that are structurally and functionally conserved in other crop plants. In this case, the 'proof of the pudding' (i.e. the validation

Figure 12.1 (a) Examples of disease-like symptoms displayed by gametophytic tissues of *P. patens* plants collected directly from the field. (1) A sharp-bordered, pigmented lesion reminiscent of a typical HR. Cytoplasm in the pigmented cells is highly granular and there are no discernable chloroplasts. One cell is completely empty, possibly the result of absorption of cell contents by a pathogen, or the result of cellular autophagy. (2) A diffuse, pigmented lesion on a leaf. The lack of a sharp lesion boundary is consistent with a spreading lesion. (3) Higher magnification of the lesion shown in (2). Cells contain the degraded products of chloroplasts together with crystalline structures. Movement within these cells during a series of time-lapse photographs is consistent with the presence of bacteria. (4) Leaf tissue colonized by an unidentified fungus. Hyphal strands, clearly visible in cells that have lost their entire contents, appear to pass from cell to cell. Cells at various stages of breakdown can be seen in this view. (5) A portion of a leaf showing extensive ramification of fungal hyphae. The progressive wave of cellular breakdown in response to the fungus is apparent. Red arrows indicate individual hyphal tips, which appear to emerge from the ends of each file of plant cells. (6) Reinoculation of an axenically cultured *Fusarium* spp. isolate on *P. patens*. Note the extensive ramification of hyphae within individual cells together with the presence of canoe-shaped, septated conidia. Scale bars represent 20 μm, except for E, which represents 50 μm. (b) Inoculation of *P. patens* with known bacterial pathogens of seed plants. Bacteria were inoculated onto *P. patens* protonemata. The pictures show typical disease symptoms at 24 and 48 h post inoculation. The graphs on the right show the growth of bacteria *in planta*, determined by bacterial plating assays (cfu = colony forming unit) and the extent of *P. patens* cell death measured by Evans blue staining. U: uninoculated; Rs: *Ralstonia solanacearum* pv. *michiganensis*; Ps: *Pseudomonas syringae* pv. *tabasci*; Cm: *Clavibacter michiganensis*; Xt: *Xanthomonas translucens* pv. *poae*; Xa: *Xanthomonas axonopodis* pv. *vesicatoria*. There is an excellent correlation between cell death and bacterial growth, and pathogens that are more effective in killing the host also grow more rapidly. The panel on the lower right-hand side shows the internalization of GFP-labeled *Pantoea stewartii* ssp. *stewartii* in *P. patens* leaves viewed by epifluorescence. (c) Inoculation of *P. patens* with fungal pathogens of seed plants and mosses. *P. patens* gametophore tissues were inoculated with *Aspergillus niger* (which causes black mold on fruits and vegetables), GFP-labeled *Sclerotia sclerotiorum* (which causes leaf drop on lettuce), *Verticillium dahliae* (which causes wilt on a large number of plants), *Fusarium graminearum* (which causes head scab on wheat and barley) and an unknown species of *Chaetomium* cultured from infected moss plants by the authors. Samples were illuminated under bright-field conditions with the exception of GFP-labeled *S. sclerotiorum*, which was viewed by epifluorescence. Note the difference in the morphology of dying cells in *V. dahlaie*-infected plants compared to dying cells in plants infected with *F. graminearum* and *Chaetomium*. (For a color version of this figure, see Plate 18)

of the experimental system) lies in its ability to uncover and isolate genes that play a similar role in the natural pathogenic interactions of other plants.

For the present discussion, we define a pathogen as a microbe that causes disease on the host and multiplies in host tissues through the application of specialized mechanisms designed to access the host's resources, disarm the host's defence responses and (in some cases) promote pathogen dispersal. In this view, a pathogen has 'an inherent capacity to breach host cell barriers' (quoted from Falkow, 1997), overcome host defences and subvert host pathways toward the requirements of the pathogen. We make use of these criteria in assessing the biological significance and ability of field-collected or 'off-the shelf' (broad host-range) pathogens to cause disease on *P. patens*. Clearly, one needs to assemble a detailed picture of a host–pathogen interaction before its biological significance can be judged, but, one useful measure of the validity of a model pathosystem lies in its ability to uncover cellular, genetic and biochemical mechanisms that also apply to other plant–pathogen interactions.

12.4.4 Viral pathogens

Little is known about the infection of mosses with viral pathogens. Polischuk and colleagues reported evidence for plant viruses infecting *Barbilophozia* spp. and *Polytrichum* spp. mosses of Antartica (Polischuk et al., 2007). Significantly these viruses included members of the Tobamoviruses, which also infect seed plants. *P. patens* has been shown to support the replication of tomato spotted wilt virus (TSWV, a broad host-range viral pathogen), but it is not known whether this virus naturally infects *P. patens* (Huhns et al., 2003). The ability to infect *P. patens* with viruses would present valuable experimental opportunities, including the ability to examine cellular requirements for viral replication, cell-to-cell spread of viral particles or genomes and the genetic basis for host symptom development, such as chlorosis and the HR, where such symptoms occur. Circumstantial evidence for the importance of viruses as potential pathogens of *P. patens* is the presence of micro-RNAs (Axtell, 2008; Chapter 5 of the present book) and a functional system that supports RNA interference (Bezanilla et al., 2003, 2005). While RNAi has been exploited as a means to down-regulate genes, the cellular machinery underlying it is thought to have originated as part of an immune response to viral infection (Hamilton and Baulcombe, 1999).

12.4.5 Bacterial pathogens

The literature on bacterial infection of *P. patens* and of mosses in general is limited. However, it is clear from several recent reports, as well as from our own observations that *P. patens* is susceptible to a range of bacterial pathogens. For practical reasons, most studies have focused on susceptible interactions with necrotrophic pathogens that cause extensive plant cell death. The first

such report was from Valkonen and colleagues who reported that *P. patens* was susceptible to infection with *Erwinea carotovora*, which causes soft rot on a range of economically important crop plants (Andersson et al., 2005). *Erwinea* species produce a potent mix of lytic enzymes, which have been shown to be important for their ability to infect seed plants. The importance of this report is that it showed that lytic enzymes produced by a known pathogen of seed plants could degrade the cell walls of mosses. This, in turn, raised the possibility that pathogens of seed plants, in general, might be competent to breach the physical defences of *P. patens*, and overcome any chemical differences in cell wall composition compared to seed plants, such as the extent of chemical cross-linking, or the nature and abundance of cell wall bound phenolic compounds (Lee et al., 2005). Further studies using *E. carotovora* ssp. *carotovora* (Ponce de León et al., 2007) showed that *P. patens* leaves could be infected by this bacterium. By labeling the bacterium with a GFP reporter, the authors were able to detect its location within the apoplast as well as in the intercellular spaces of leaf cells. Interestingly, these bacteria were able to cause extensive cell death on *P. patens* leaves, whether or not they carried a gene encoding harpin, which is an inducer of the HR in seed plants (Wei and Beer, 1996; Alfano and Collmer, 1997; Xie and Chen, 2000; Krause and Durner, 2004). In this infection assay, *E. carotovora* appeared to enter the plant through wound sites and this made it difficult to quantify plant cell death and bacterial growth *in vivo*. As a result, the rest of this study was performed using culture filtrates produced by the bacterium, as these could be applied uniformly and were able to recapitulate many of the cellular and molecular responses associated with infection (these results are discussed below in the section on the use of elicitors). In contrast, our own studies have shown that *P. patens* can be infected with GFP-labeled strains of *Panteoa stewartii* pv. *stewartii* (synonymous with *Erwinia stewartii*) without any requirement for tissue wounding. This pathogen is able to gain access to both protonemata and gametophore tissues (Figure 12.1b, Plate 18). This might reflect a difference in the ability of these related pathogens to penetrate the *P. patens* cell wall; alternatively, it may reflect the operation in this bacterium of other pathogenicity mechanisms (e.g. the production of toxins or the delivery of type III effector molecules into plant cells). We have found that other bacterial pathogens, including several that infect seed plants, can cause cell death in both protonemal (Figure 11.1b, Plate 18) and gametophore tissues (data not shown) of *P. patens*. Significantly, these bacteria were able to multiply as a result of infection of the host plant (Figure 12.1b, Plate 18). Bacterial growth correlated with *P. patens* cell death, indicating that the ability to cause host PCD contributed to bacterial virulence.

Genetic approaches can be helpful in gauging the significance of these interactions. We have found that several of the interactions of *P. patens* with these pathogens are dependent on classical bacterial pathogenicity mechanisms, such as the ability to produce toxins or to deliver effector molecules into the plant host (HS and ML, unpublished data). These mutants show a

substantially attenuated virulence on wild-type *P. patens* plants. A similar genetic approach can be applied to the host. For example, manipulation of *P. patens* host pathways associated with cell death renders these plants resistant to otherwise virulent bacteria (HS and ML, unpublished data). These results indicate a clear genetic basis for the ability of the pathogen to kill the plant that correlates with the ability of the pathogen to multiply *in planta*. Genetic manipulation that removes either the pathogen cell death signal or the ability of the plant to respond to that signal not only eliminates host cell death but also compromises the ability of the pathogen to multiply *in planta*. These studies do not indicate whether these bacteria are natural pathogens of *P. patens*, but they do establish a firm experimental foundation for using this system as a model for studying the genetic basis of plant–pathogen interactions.

This discussion highlights the advantages of working with pathogens that are known to infect seed plants. The availability of mutant pathogens that display altered virulence on seed plants, provides an immediate entrée into the mechanisms of disease caused by these pathogens on *P. patens*, as well as providing an important experimental reality check on the biological significance of the interaction between the pathogen and *P. patens*. For example, bacterial pathogens that lack the *hrp* gene cluster are unable to deliver effector molecules through the type III secretion pathway into host cells and display reduced virulence on their (seed plant) hosts. A requirement for this pathway during pathogen interactions with *P. patens* would imply the operation of similar mechanisms responsible for disease and bacterial growth *in planta* on both hosts. Similar experiments can be performed using other pathogen mutants that address the role of other virulence mechanisms, such as bacterial quorum sensing, the production and secretion of lytic enzymes, the involvement of toxins in the promotion of host cell death, and even the roles of specific type III effector proteins in evolutionarily diverse hosts.

12.4.6 Fungal pathogens

P. patens is susceptible to a number of fungal pathogens of seed plants that are known necrotrophs. Published reports have shown that *P. patens* can be infected with *Botrytis cinerea*, which results in browning, necrosis and tissue maceration (Ponce de León et al., 2007). Fungal mycelium was observed growing within plant tissues and could be observed spreading through an area of dead and dying tissues. We have seen similar responses when a strain of *B. cinerea* isolated from infected tomato fruit was inoculated onto a lawn of *P. patens* (ML, unpublished data). The resulting infected area will continue to spread until all tissues are consumed, indicating that *P. patens* is fully susceptible to this aggressive pathogen. We surveyed a number of other fungal pathogens for their ability to infect *P. patens*, including *Verticillium dahliae, Aspergillus niger* and an uncharacterized species of *Chaetomium* that was isolated from infected moss plants collected near the authors' laboratory. Each of these fungi infected *P. patens* and caused extensive cell death in the plant (Figure 12.1c, Plate 18).

The characterization of a *F. culmorum*-like fungus from infected *P. patens* plants collected in the wild prompted us to inoculate *P. patens* with authentic *F. graminearum*, which causes head scab on grain crops worldwide (Leonard and Bushnell, 2003; Bai and Shaner, 2004). *Fusarium* spp. are characterized by the production of toxins which enhance virulence (Nelson et al., 1993). These toxins cause cell death ahead of the spread of the fungus, which then enters and feeds on dead or dying tissues (Leonard and Bushnell, 2003). *P. patens* susceptibility to toxins produced by *F. graminearum* (Saidasan and Lawton, 2007) suggests a similar mechanism in moss. This focuses attention on the host plant pathways controlling PCD as important targets for the pathogen during infection and as possible targets for pathologists interested in engineering resistance to these pathogens.

The wide variety of fungal pathogens able to infect *P. patens* under simple laboratory conditions suggests that *P. patens* may be amenable to infection with other fungal pathogens, including those that cause economically important diseases. Obvious candidates for investigation include the oomycetes *Phytophthora infestans* and *P. sojae* as well as *Magnaporthe grisea* and *Rhizoctonia* spp. It may also be worth finding out if *P. patens* is susceptible to pathogens that infect other mosses, particularly those that display broad host-range specificity, such as *Octospora similis* (Kirchstein) Benkert, which infects many species of *Bryum* (Benkert, 1987; Felix, 1988; Davey and Currah, 2006) and *Eocronartium muscicola* (Pers.) Fitzp., which infects representatives of eleven different families of moss (Boehm and McLaughlin, 1988). Other fungal pathogens display a high degree of specialization. For example, the ascomycete *Epibryon interlamellare* Döbbeler is restricted to the interlamellar spaces of leaves of species of the *Polytricaceae* (Felix, 1988; Döbbeler, 1998, 2002). Other interactions between fungal pathogens and their hosts are highly evolved, as is the case for *Bryophytomyces sphagni* (Navashin) Cif., in which the host moss spores are replaced by fungal propagules (Redhead and Spicer, 1981). These observations remind us that successful pathogens not only grow and reproduce at the expense of the host plant but may also exploit the host for their dispersal.

12.4.7 Elicitation and activation of the *P. patens* defence response

Many studies have used elicitors of the plant defence response as a substitute for pathogens (Ebel and Cosio, 1994; Hahn, 1996; Bent and Mackay, 2007). Elicitors are structurally diverse molecules that are able to induce various aspects of the plant defence response. If they represent indispensable features of the pathogen and are also recognized by the plant *in vivo*, then they function as PAMPs, and their use as *in vitro* elicitors of plant defence mimics their role during the interaction with plants. Other elicitors are virulence effectors and are detected by plant R-proteins. Two well-characterized examples of pathogen-derived elicitors are the *Cladosporium fulvum* Avr9 peptide (which is also recognized as an avirulence protein by the *Lycopersicon esculantum*

Cf9 R-protein (de Wit, 1995, 2007)) and the elicitins produced by *Phytophthora infestans* (Kamoun et al., 1993; Kanneganti et al., 2006; Kamoun, 2007). A number of elicitors are released from the pathogen cell wall through the enzymatic action of plant lytic enzymes such as chitinases and glucanases (Joosten and De Wit, 1989; van Loon et al., 2006). Other elicitors are released from the plant cell wall through the action of pathogen-derived polyglacturonases (whose activity is countered by plant polygalacturonase-inhibiting proteins (Federicia et al., 2005)). Many of these cell-wall-derived elicitors can also be produced artificially by exposing pathogen or plant cell walls to enzymatic digestion, chemical treatment or hydrolysis at high temperatures.

While the physiological roles of elicitors vary, their ability to be recognized by the plant provides a useful tool for the experimental biologist. Elicitors can help characterize components of the defence response, and are particularly useful in transcriptomic or proteomic approaches to define induced genes and proteins that comprise the inducible defence response (Navarro et al., 2004; Thilmony et al., 2006; Livajaa et al., 2007). Elicitors can also be used to study biochemical and cellular components of the defence response, such as changes in cellular ultrastructure, the production of reactive oxygen species (ROS) (Apel and Hirt, 2004; Halliwell, 2006) or other biochemical changes including the production of secondary metabolites that may act as phytoalexins (which are defined as pathogen-induced antimicrobial compounds) (Darvill and Albersheim, 1984; Hammerschmidt, 1999). Elicitors may also be used to assess the functional significance of an induced defence response. Thus, pretreatment of plants (including *P. patens*) with elicitors may induce a defence response that allows the plant to withstand challenge with an otherwise virulent pathogen. This induced resistance response has been observed in seed plants and can even be used to enhance field resistance in crop plants (Sharathchandra et al., 2004).

Elicitors have been used to examine the defence responses of *P. patens*. Ponce de León and colleagues used culture filtrates of axenically grown *Erwinia carotovora* as a substitute for the pathogen to induce ultrastructural and molecular responses in *P. patens* (Ponce de León et al., 2007). In seed plants, these filtrates are able to elicit the same symptoms and defence responses as the corresponding bacterial strain (Palva et al., 1993). Culture filtrates induced extensive cell death when applied to *P. patens* protonemata and gametophore tissues. However, cell death in gametophore tissues was enhanced when culture filtrates were prepared from a strain of *E. carotovora* that also produced the protein harpin (Wei and Beer, 1996; Alfano and Collmer, 1997; Krause and Durner, 2004; Livajaa et al., 2007). The presence of harpin in *E. carotovora* culture filtrates accentuated the responses of *P. patens* (Ponce de León et al., 2007). Responses included cytoplasmic shrinkage, the browning and loss of integrity of chloroplasts, and the production of auto-fluorescent compounds. Culture filtrates also induced the expression of several genes associated with the defence response in seed plants. The functional significance of these responses is not yet known. Still, induction of these genes provides markers

for the response to the perception of the pathogen or to the molecules that it secretes (including, but not limited to, harpin). This study is important in that it shows that *P. patens* is physiologically competent to respond to a broad host-range pathogen and to elicitors derived from it. It is also significant that harpin aggravates cell death in *P. patens* and suggests that the plant target for this protein may be conserved in *P. patens*. Clearly, it will be important to determine if *P. patens* can respond to purified harpin, or whether it acts in conjunction with lytic enzymes and other factors present in the culture fluid.

We have used elicitors in our own studies to induce cell death and defence responses in *P. patens*. Chitosan has been widely used as an inducer of defence responses in seed plants (Ramonell et al., 2002) and prior treatment with chitosan can induce immunity against pathogenic attack (Bhaskara Reddy et al., 1999). Chitosan has also been shown to induce PCD in cell cultures (Zuppini et al., 2003; Ning et al., 2004). We have found that chitosan can evoke all of these responses in *P. patens* (HS and ML, unpublished data). At low doses, chitosan induces a defence response characterized by production of ROS and changes in defence gene expression. This response appears to be functionally relevant since it can protect *P. patens* against subsequent infection with otherwise virulent bacterial or fungal pathogens. At higher doses, chitosan induces a cell death response in protonemata and gametophore tissues that has the hallmarks of PCD.

Examination of the *P. patens* genome reveals the presence of homologues of the recently cloned chitosan receptor genes (HS, personal communication), suggesting that the machinery involved in the recognition of this PAMP is quite ancient. Gene knockdowns of the chitosan receptor in *O. sativa* cells partially suppressed responses to chitosan, but did not completely abolish chitosan-induced gene expression (Kaku et al., 2006). More recently, Wan et al. reported that this receptor plays a critical role in the response of *A. thaliana* to fungal pathogens (Wan et al., 2008). It will be interesting to see if *P. patens* responds to other molecules that function as PAMPs in seed plants and whether these responses can be disrupted by pathogen effectors. If *P. patens* can be shown to respond to a particular PAMP, it should be possible to test whether knockouts of genes that encode candidate receptors abolish PAMP perception. This may prove a useful experimental approach as new PAMPs (and MAMPs) are characterized from diverse micro-organisms.

Similar approaches can be used to identify the responses of *P. patens* to toxins. Since toxins can serve as virulence effectors, characterizing the responses of the plant to these molecules can provide information on their mechanism of action by highlighting the kinds of responses they invoke. These responses can provide specific markers for the effects of different toxins and help characterize mutants that are defective in toxin response pathways. In our own work, we have created several gene knockouts in PCD components that show a reduced sensitivity to *Fusarium*-derived toxins (Saidasan and Lawton, 2007). These mutants fail to display almost all of the toxin-induced responses exhibited by wild-type plants, suggesting that the knockouts affect early stages

in the perception and signaling of toxins. Genes that are identified as toxin-inducible in wild-type plants represent excellent candidates for functional study through the creation of additional gene knockouts.

12.5 Responses of *P. patens* to infection

P. patens, like seed plants, reacts to infection or elicitor treatment with an array of responses at the molecular, biochemical, cellular and whole-plant levels. One of the most striking responses of diseased plants is cell death, which may occur during the HR response to incompatible biotrophic pathogens or as a symptom of disease during susceptible interactions with necrotrophic pathogens (Heath, 2000a; Delledonne et al., 2001; Dickman et al., 2001; Lam et al., 2001; Greenberg and Yao, 2004). Cell death in protonemal and gametophore tissues was observed during the infection of *P. patens* by *E. carotovora* (Andersson et al., 2005; Ponce de León et al., 2007), or following treatment with bacterial culture filtrates. The aggravation of this response by bacterial strains that produce harpin suggests that the induction of cell death on *P. patens* may contribute to pathogenic virulence (Ponce de León et al., 2007). We have observed that a number of bacterial and fungal pathogens also kill *P. patens* cells (Figure 12.1b, Plate 18), as do toxins or elicitors such as chitosan. Interestingly, while each of these treatments causes plant cell death, the morphology and ultrastructural features of dying cells are often distinct and characteristic of a particular PCD inducer. Distinguishing features include the degree of cellular browning, the morphology and behavior of dying chloroplasts (e.g. whether they migrate to the cell periphery and whether they fuse), the ultrastructure of the nucleus as it first condenses and then breaks up, and the formation of cytoplasmic vacuoles and other vesicles. These features suggest that different pathogens and elicitors may induce cell death through multiple and distinct pathways. Alternatively, these treatments may invoke a common PCD that operates in parallel with other distinguishing cellular responses.

ROS (specifically hydrogen peroxide; HS and ML, unpublished data) are produced by *P. patens* following treatment with elicitors, toxins and a diverse range of pathogens. This ancient stress response is present in tracheophytes (Lamb and Dixon, 1997; Torres et al., 2006), macroalgae (Weinberger, 2007), yeast (Madeo et al., 1999) and animal cells (Cai, 2005). In seed plants, ROS are thought to be important as signals for the induction of plant defence responses and as positive and negative regulators of hypersensitive cell death (Alvarez et al., 1998; Torres et al., 2005; Van Breusegem and Dat, 2006). ROS also play a role in cell wall cross-linking (Bradley et al., 1992) and may have direct antimicrobial activity. Both ROS and nitric oxide (NO) radicals are thought to be important in orchestrating the induction of the defence response and the expression of the HR (Jabs et al., 1996; Delledonne et al., 2001; del Rio et al., 2006). Interestingly, ROS also appear to be involved in the rehydration

response of desiccated mosses and lichen (Minibayeva and Beckett, 2001; Mayaba et al., 2002). One attractive feature of using ROS as a read-out of the response to PCD inducers and pathogens is that these assays are sensitive, rapid and quantifiable, and can be used to examine the site of ROS production within infected tissues. We have found this approach especially useful to determine the spatial relationship between cells that produce ROS and the location of GFP-labeled pathogens. Histological stains for ROS also allow the site of ROS production within the cell to be determined and this is important for understanding the biochemical mechanisms responsible for ROS production during infection (Halliwell, 2006).

What is the evidence that the cell death observed in *P. patens* is a PCD? In our own studies, we have observed that the response of *P. patens* to several elicitors, as well as bacterial and fungal pathogens, results in cell death that has many of the hallmarks of PCD including a requirement for protein synthesis, induction of gene expression, fragmentation of the nucleus, the production of ROS and the induction of peroxidase and nuclease activities (HS and ML, unpublished data). PCD is an ordered breakdown of the cell and is typically accompanied by a stereotypical fragmentation of the nucleus as well as the organized disassembly of other cellular components. PCD in *P. patens* is typically accompanied by condensation and fragmentation of the nucleus. For chitosan-induced PCD, we were able to detect the DNA laddering characteristic of nucleosomal breakdown. However, we have not been able to detect these discrete fragments during cell death induced by other treatments. It will be interesting to see if earlier events in nuclear breakdown can be detected through the use of terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) staining assays for nicked DNA. Cell death in *P. patens* is also accompanied by the loss of chloroplast integrity, the appearance of cytoplasmic vacuoles and the breakdown of cellular proteins. More convincing evidence for a PCD in *P. patens* comes from the range of active responses that are induced by elicitors, toxins and pathogens. Induction of defence genes was also observed in *P. patens* plants exposed to *E. carotovora* culture filtrates (Ponce de León et al., 2007). Changes in gene expression in the latter study included transcripts that are associated with defence responses in seed plants. These include phenylalanine ammonia-lyase (PAL), which catalyzes the first step in the phenylpropanoid pathway and gives rise to phytoalexins (in some plants), preformed antimicrobial compounds, lignin precursors and antioxidants; chalcone synthase (CHS), which is involved in flavonoid and isoflavonoid biosynthesis in seed plants; lipoxygenase (LOX) which is involved in the biosynthesis of jasmonic acid in seed plants (Glazebrook et al., 2003; Balbi and Devoto, 2008) but which might have additional functions in *P. patens* (Senger et al., 2005); and PR-1 which is a classical pathogenesis-related protein and serves as a marker of the defence response in seed plants (van Loon et al., 2006; Ferreira et al., 2007). In our own studies, we have found that elicitors and pathogens that invoke cell death in *P. patens* also induce the expression of genes typically associated

with the induction of PCD, such as those encoding proteases, ribonucleases and dexoyribonucleases, as well as genes associated with the ER-stress pathway (Schroeder and Kaufman, 2005) and the anti-apoptotic response, characterized by BI-1 (Xu and Reed, 1998). This paradoxical response might be explained if an anti-PCD response is induced early on in the response as the cell attempts to cope with stress. If the cell is overcome by stress, it might then induce a PCD pathway whose components prepare the cell for an orderly disassembly.

Stronger evidence for a genetically determined PCD is provided by manipulating genes involved in PCD. We have found that transgenic *P. patens* plants that over-express the anti-apoptotic BI-1 gene are relatively insensitive to a range of death signals, including elicitors and toxins. These plants are also resistant to infection with necrotrophic pathogens, suggesting that the plant PCD pathway is a critical target for these pathogens. It will be interesting to find out exactly how this pathway is activated by pathogen effectors. We have obtained similar results using knockouts for genes that are required for apoptosis. These results demonstrate that cell death in response to elicitors and pathogens in *P. patens* is genetically programmed and confirm the validity of using *P. patens* as an experimental system to examine the genetic basis of disease susceptibility.

Changes in gene expression can be used to identify components of the defence response and to provide markers for the expression of specific programs that operate within (or alongside) it. This can be a valuable approach for identifying genes for future functional analysis, and will be described in more detail in the following section. Clearly, our understanding of the scope of the defence response and PCD programs in *P. patens* would be aided by the use of large-scale transcriptomic analyses such as EST sequencing, DNA microarrays, SAGE, next-generation DNA sequencing technologies and proteomic approaches (Reski et al., 1998; Machuka et al., 1999; Matsumura et al., 2003; Nishiyama et al., 2003; Rensing et al., 2007). Nonetheless, results from similar experiments performed in *A. thaliana* suggest that even a simple defined elicitor, such as flagellin (Navarro et al., 2004) or chitosan (Ramonell et al., 2002; Kaku et al., 2006), can cause extensive changes in the pattern of gene expression. Clearly, there are ample opportunities to exploit modern analytical techniques to define better the extent and nature of the responses of *P. patens* to elicitors, toxins and pathogens, but these need to be combined with other approaches to help identify early steps in the response pathway.

Exposure of *P. patens* to chitosan induces significant changes in the levels of small molecules (A. Poulev, unpublished data). We have characterized some of these induced compounds and found that they are structurally derived from kaurenes, including a compound that has been previously described in *P. patens* (von Schwartzenberg et al., 2004). This response is accompanied by an induction of transcripts for biosynthetic enzymes associated with this pathway, suggesting that the de novo production of secondary compounds may be an important part of the plant defence responses in *P. patens*, as

it is in seed plants (Dixon, 2001). It has been recognized that bryophytes can serve as sources of novel, biologically active compounds (e.g. see Cassady et al., 2004 and Sabovljevica et al., 2005) and an enhanced biochemical capacity appears to be reflected in the *P. patens* genome. Transcriptomic analysis of (unelicited) *P. patens* plants along with the completion of the *P. patens* genome sequence reveals a significant expansion of gene families devoted to metabolism (Rensing et al., 2008). For example, while *A. thaliana* has only a single CHS gene, *P. patens* has 19 related CHS genes (Quatrano et al., 2007). CHS is a member of the large family of polyketide synthases known for their biosynthetic versatility in synthesizing biologically active compounds (Jez et al., 2001; Austin and Noel, 2003). These compounds may contribute to the resistance of *P. patens* against natural microbial pathogens, as well as insect and invertebrate predators. It will be interesting to examine whether this capacity is constitutive, or inducible, and to elucidate the biosynthetic pathways and the signals involved in their accumulation.

12.6 Selecting *P. Patens* genes for functional studies of disease

What is the best way to select genes for functional studies in *P. patens*? Clearly, there is a practical limit on the number of gene knockouts that can be constructed by a single researcher or facility and the researcher will need tools to help select genes for further study. In many cases, interest in a particular gene or protein is based on prior research carried out in another experimental system. For example, yeast can be used to select mutants and clone genes associated with insensitivity to pathogenic toxins or elicitors. Recreating the corresponding mutants in *P. patens* tells us whether these mutations render plant cells similarly insensitive to toxins. More significantly, the effects of these mutations on disease susceptibility can also be tested. The critical features of this approach are the ability to recreate mutations in *P. patens* that were discovered in other systems and couple these to a biological assay (such as infection) that is not available in the original organism (e.g. yeast is not a susceptible host for pathogenic micro-organisms). This type of experimental tourism is also useful where a gene family in seed plants is represented by only one or two copies in *P. patens*, where no phenotype is apparent in known mutants or RNAi-suppressed lines of seed plants, or where the original system is simply unsuitable for the particular biological assay (e.g. by not displaying susceptibility to a pathogen or toxin).

Recreating mutations in *P. patens* for genes known to affect disease susceptibility and resistance in seed plants is important for establishing *P. patens* as a relevant model for studying interactions with pathogens of seed plants. If mutants give a similar phenotype in both *P. patens* and *A. thaliana*, this suggests functional conservation between the systems and provides confidence about their biological relevance and mechanistic similarities. Furthermore, a

collection of mutants that are defective in individual aspects of the defence response (e.g. in the expression of PCD, the production of secondary metabolites, the production of ROS, and the induction of PR-proteins) can help define the virulence mechanisms employed by a particular pathogen. Hence, if mutation of the PCD pathway confers resistance to a particular pathogen, we can deduce that this pathway is important for disease susceptibility, whereas mutations that show no effect are presumably not involved in the expression of susceptibility. Similar approaches should be very useful for distinguishing the modes of action of biotrophic and necrotrophic pathogens on *P. patens*.

Limitations on the use of forward genetics in *P. patens* mean that other approaches must be used to identify candidate genes for functional studies. One powerful approach is the use of whole-genome expression profiling to identify genes that are induced during infection (Alba et al., 2004). Depending on the type of interaction, as well as on timing and localization, induced genes may be associated with attempted pathogen limitation and activation of the defence response, the production of local and systemic defence signals, or the expression of PCD associated with both hypersensitive resistance and the large-scale necrosis associated with susceptible interactions to necrotrophs. The major problem with these approaches is one of scale since the expression of many genes is altered during infection (Schenk et al., 2000; Ramonell et al., 2002; Eulgem et al., 2004; Navarro et al., 2004). One useful approach is to focus on early changes in gene expression and to further limit the scope of the response by performing experiments in the presence of translational inhibitors (Wang et al., 2006). This allows genes that are the initial targets of pathogenic signals to be identified in the absence of any secondary responses. Another helpful approach is to use elicitors instead of pathogens to identify downstream genes in a particular defence response pathway. This allows *P. patens* cells to be uniformly and coordinately exposed to a single inducer that is recognized by a dedicated receptor, rather than to multiple, sequential signals that may be produced as the interaction between the plant and the pathogen unfolds. This strategy should result in more coherent and focused changes in gene expression in the plant, although even defined PAMPs and elicitors can cause extensive changes in host gene expression (Ramonell et al., 2002; Navarro et al., 2004; Livajaa et al., 2007).

One powerful way to focus on downstream targets of specific genes is to compare the pattern of gene expression in wild-type and mutant plants (Nakamura et al., 2005). We have shown that over-expression of the BI-1 gene confers resistance to PCD inducers (HS and ML, unpublished data). Small-scale transcript profiling revealed significant differences in the steady-state levels of a number of stress-response genes, suggesting that these genes are directly or indirectly regulated by BI-1. Knockouts of several BI-1 responsive genes displayed an altered sensitivity to PCD inducers, suggesting that they too play a role in the expression of PCD in *P. patens* (Saidasan and Lawton, 2007 and HS and ML, unpublished data). The efficiency of creating gene knockouts

in *P. patens* is sufficiently high that these selection strategies do not have to be 100% efficient. If only one gene knockout in five gives a phenotype, this would represent a significant return on the effort invested and be sufficient to identify novel genes involved in plant–pathogen interactions.

Another way to select a smaller group of co-regulated genes combines transcription profiling with *in silico* analysis of the promoter regions of responsive genes. Since a complete genome sequence is available for *P. patens*, one can search for known stress-response regulatory elements in a set of promoters of induced genes. Alternatively, the promoter sequences of co-regulated genes can be directly compared without bias for the presence of related *cis*-regulatory sequences. This approach has been used in *A. thaliana* to identify pathogen-response genes that fall into a common regulatory cluster (Eulgem et al., 2004; Wang et al., 2006). Other approaches for selecting genes for functional studies are similar to those available in other systems. These include defining protein–protein interactions through the yeast two-hybrid assay, mass spectrometry of native protein complexes, epitope tagging and chemical cross-linking and (where antisera are available) immunological pull-down assays. One refinement of this approach is to make use of protein 'interactomes' from other organisms such as yeast (Goll and Uetz, 2006) to find candidates (or archetypes for candidates) that might similarly interact with the protein of interest in *P. patens*. These approaches are perhaps most useful when they are used in conjunction with forward genetic studies.

12.7 Future directions and opportunities

While *P. patens* pathology is just beginning, some of its applications are already clear and we expect to see much activity in this area in the coming years. Yet, much work needs to be done if the full potential of this system is to be realized. Two specific areas demand attention and these involve (i) developing genomic tools for large-scale mutant analysis and forward genetics, and (ii) developing new tools for understanding the responses of *P. patens* to pathogens.

12.7.1 Developing tools for genomics analysis

Although the functional properties of any *P. patens* gene can be studied through the creation of gene knockouts, it is not yet possible to recover genes from classical genetic screens. This situation will change as high-density linkage maps become available. The ability to clone genes based on positional information will allow *de novo* screens to be performed for mutants that show altered responses to toxins, elicitors and pathogens, much in the way that these methods have been used in *A. thaliana*. Perhaps more significantly for *P. patens*, this approach will allow one to screen for suppressors and enhancers of mutants created by gene knockouts. The ability to create gene knockouts

can also be used to determine which of several genes within a mapped chromosomal interval are associated with a mutant phenotype.

In yeast, the availability of a collection of defined knockouts for each and every gene has been an extremely useful resource (Winzeler et al., 1999; Giaever et al., 2002; Hillenmeyer et al., 2008). Because *P. patens* has similarly high frequency of gene targeting, a defined collection of gene knockouts can also be made for this plant (Reski, 1998b; Egener et al., 2002; Holtorf et al., 2002; Schween et al., 2002; Reski, 2003; Hohe et al., 2004; Reski and Cove, 2004; Frank et al., 2005; Schween et al., 2005). In addition to making defined knockouts available to any researcher, the availability of this resource would greatly facilitate *de novo* gene discovery, in which the contribution of every gene is assayed in parallel. This approach would be extremely useful for screens to identify mutants that are non-responsive or hypersensitive to particular PAMPs, elicitors, effectors or toxins, as well as pathogens. This resource would also be useful for chemical genetic screens for small molecules that mimic or inhibit the response to pathogens. However, generating this resource is not trivial and resources are also needed for its maintenance, replication and distribution. Alternative approaches include the creation of a collection of random insertion T-DNA mutants whose positions are located on the genome by TAIL sequencing, but this too faces the same requirements for maintenance and propagation.

It may also be possible to characterize genes from forward genetic screens through physical methods. One promising approach is to use fast neutron radiation to create deletion mutations in *P. patens*. A collection of mutated *P. patens* spores or cells could be screened for altered responses to pathogenic signals. Instead of mapping deletions genetically, they could instead be directly located on the genome by hybridization to whole-genome oligonucleotide tiling arrays or through the use of ultra-high-throughput DNA sequencing. Sequences deleted in the mutant would become apparent as gaps in the hybridization signal on the tiling array or as gaps in the mutant genome sequence (when compared with the existing wild-type genome scaffold). This approach may become practical and cost effective as the capacity for DNA sequencing increases and algorithms for real-time sequence assembly improve. Its major advantage, compared to the use of defined collections of gene knockouts created in a wild-type genetic background, is that it can be used to screen for suppressors and enhancers of extant mutants.

Finally, since the defence response is ultimately expressed at the level of proteins, enzymes and small molecules, we need a better understanding of the metabolic pathways present in *P. patens*, and in particular how these differ from seed plants (e.g. see Reski et al. (1998); Koprivova et al. (2002); Kopriva (2006); Quatrano et al. (2007)). By combining genomic methods with metabolomic methods (Schulte et al., 2006), we will also gain a better understanding of the relationship between genes, proteins and cellular chemistry, as well as a set of powerful analytical tools for interpreting mutant phenotypes and the responses of *P. patens* to microbial pathogens. Lastly, as

computational methods improve and the identity of transcription factors and their DNA binding sites improves, it should become possible to make better predictions about gene regulatory networks in *P. patens*.

12.7.2 Developing *P. patens* pathology

We know that *P. patens* is susceptible to bacterial and fungal pathogens and that it mounts a defence response that has many of the characteristics of the defence response of seed plants. Our own preliminary results using gene knockouts for PCD genes have shown that the response to elicitors, toxins and pathogens is genetically determined. This indicates that *P. patens* is a viable and useful system for understanding the genetic basis of disease susceptibility. It also confirms that the approach of creating gene knockouts in candidate genes is a useful way to characterize the role of genes involved in this process. However, certain areas of plant pathology are not yet accessible in *P. patens*. One critical area is the study of incompatible (resistant) interactions, including those induced by non-host pathogens as well as those conditioned by classical pathogen avirulence and *R*-genes. These types of interactions could also help address the role of the HR in the expression of disease resistance in *P. patens*. If these interactions and responses are present in *P. patens*, they would expand its utility as a system for studying the genetic determinants of disease resistance, as well as susceptibility.

There are several ways to study disease resistance in the absence of a classical resistance response. One approach is to induce resistance using elicitors. This is an experimentally useful way to define the *P. patens* defence response and identify new genes for functional analysis. An alternative, but more risky approach, would be to introduce into *P. patens* a seed plant *R*-gene that is able to recognize a known pathogen avirulence protein. If this system functions in *P. patens*, then one could induce a resistance response by exposing plants to a pathogen carrying the appropriate avirulence gene. This approach would also test the degree to which the cellular machinery involved in R-protein signaling is conserved in *P. patens*.

The other major task is to use these technologies to define the defence response in *P. patens*. One useful approach is to couple the use of elicitors or other inducers of the defence response to analytical methods that sample the entire transcriptome or proteome. Whole-genome DNA microarrays are now available for *P. patens* and these should prove useful for gene discovery and for defining regulatory nodes in defence gene signaling. Recent developments in ultra-high-throughput DNA sequencing can also be used for deep sequencing, especially where a reference genome is available. These methods are quantitative over a wide dynamic range (compared to microarrays) and are comprehensive. They are also useful for discovering transcripts, such as alternate splice products, that may have been overlooked in genome annotation.

In addition to these experimental approaches, we need a better understanding of the fundamental cell and molecular biology that underlies

the interactions of *P. patens* with pathogens. Specific questions that should be addressed in the future include the chemical composition and structural properties of the *P. patens* cell wall; the nature and role of defence signals in *P. patens* (such as ethylene, jasmonic acid, oxylipins, salicylic acid and methyl-salicylate, as well as ROS and NO); the nature of cell–cell signaling and its role in localized responses to infection; whether *P. patens* undergoes any long-range or systemic responses to infection; the role of the ER (Kamauchi et al., 2005) and mitochondrion (Eisenberg et al., 2007) in stress responses and the induction of PCD; the nature of the biochemical machinery involved in PCD; the source and biochemical origin of ROS during the response to elicitors and pathogens (Apel and Hirt, 2004); and the nature and antimicrobial properties of components of the defence responses, including small molecules and proteins. Some of these points are known from studies in seed plants but it will be important to determine if they also hold true for *P. patens*. Others (such as the nature and regulation of PCD in plants) are not well characterized in any system, and we expect *P. patens* will contribute to our understanding in these areas. As is the case for seed plants, we need to understand how *P. patens* and mosses in general are able to distinguish between pathogenic and non-pathogenic microbes. Finally, we will need to understand the relationship between the response to infection and the responses to other stresses, such as wounding, oxidative and environmental stress and to plant senescence.

One final mystery is why *P. patens* and other mosses appear to be so fit in nature yet they are easily infected with necrotrophs in the laboratory. The trivial answer is that this is a sampling error and that we have systematically overlooked infected mosses in nature in favor of greener and healthier specimens. Additionally, *P. patens* is an opportunistic plant and may not be representative of other perennial mosses. More interesting biological explanations are that mosses in the wild either possess formidable preformed (possibly chemical) defences against pathogens or that their continual exposure to potential pathogens induces resistance via a PAMP-mediated signaling pathway. We now have the tools to answer these questions, which may help us understand the ecology of mosses better and find applications for this knowledge in agriculture.

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