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A NEW MOSS GENETICS: Targeted Mutagenesis in *Physcomitrella patens*

Didier G. Schaefer
Institut d'Écologie, Laboratoire de Phytogénétique Cellulaire, Bâtiment de Biologie, Université de Lausanne, CH-1015 Lausanne, Switzerland;
e-mail: Didier.schaefer@ie-pc.unil.ch

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■ **Abstract** The potential of moss as a model system to study plant biology is associated with their relatively simple developmental pattern that nevertheless resembles the basic organization of the body plan of land plants, the direct access to cell-lineage analysis, their similar responses to plant growth factors and environmental stimuli as those observed in other land plants, and the dominance of the gametophyte in the life cycle that facilitates genetic approaches. Transformation studies in the moss *Physcomitrella patens* have revealed a totally unique feature for plants, i.e., that foreign DNA sequences integrate in the genome preferentially at targeted locations by homologous recombination, enabling for the first time in plants the application of the powerful molecular genetic approaches used routinely in bacteria, yeast, and since 1989, the mouse embryonic stem cells. This article reviews our current knowledge of *Physcomitrella patens* transformation and its unique suitability for functional genomic studies.

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INTRODUCTION

A major goal of modern biology is to understand the relationship between biological systems and the presence and activity of genes. Model systems of biology have provided both the experimental material and the biological context for framing

questions that explore this relationship. The investigator's ability to use several different model systems allows the function of related genes to be tested in a variety of biological and/or methodological contexts. Genomics, along with the conservation of gene structure and function found among diverse organisms, represents a "golden thread" that links model systems and allows the comparison between them.

During the past 15 years, an enormous effort by the biological community has led to the completion of nucleotide sequences of genomes from both prokaryotic and eukaryotic model systems such as *Escherichia coli* (9), *Bacillus subtilis* (62), *Saccharomyces cerevisiae* (20), and more recently *Caenorhabditis elegans* (15) and *Drosophila melanogaster* (2). The goal of these efforts has been to establish gene catalogues for particular organisms and to provide insight into the function of the corresponding proteins. In plants, the recently completed genome sequence of *Arabidopsis thaliana* (3a) will soon be followed by the completion of a genome sequence for rice (*Oriza sativa*) (6), and we can expect that further genomic sequences will be produced for other plants in the future. The next challenge for the biological community is to assess the precise function of the sequenced genes, a general approach that is referred to as functional genomics.

Several strategies have been applied to identify new genes and decipher protein function in plants. The most basic approach is sequence comparison of a gene or a protein with existing databases. Homology to another protein whose function has been previously established can provide important clues as to cellular function and biochemical properties. However, beyond this, plant functional genomics is essentially performed by the two strategies described below. The first approach, like homology comparisons, provides only an indirect indication of gene function. It makes use of global gene expression profiles and is based either on the study of populations of cDNAs or expressed sequence tags (ESTs) deposited on DNA microarrays (34, 98) or on the study of populations of proteins separated by two-dimensional gel electrophoresis (proteomics) (122). Transcriptomic and proteomic approaches identify known or new genes or proteins that are specifically up- or downregulated and give valuable information on the expression profile of gene networks in response to experimental conditions. Analysis of such expression data by high-throughput computational methods is useful for assigning genes to particular regulatory networks and for identifying new networks and interactions. However, this is nonetheless a descriptive approach and alone does not tell the investigator what function a gene performs in a cell or in an organism. Similar considerations apply to the emerging areas of metabolomics and phenomics.

The most widely used approach for performing true, genome-wide functional genomics has been insertional mutagenesis. Insertional mutagenesis by transgenesis and the phenotypic and molecular characterization of tagged mutated lines has been used extensively to study plant gene function (128). Several collections of T-DNA and transposon-tagged transformants of *Arabidopsis* (86) and rice (51) have been generated and successfully used to identify and characterize genes involved in different biological processes. Tagged mutagenesis usually generates loss-of-function mutations and rarely identifies genes that act redundantly.

However, activation tagging, based on the random insertion of enhancers in the genome, allows the identification of gain-of-function mutations (129), whereas gene trap screens (117) allow gene activity to be monitored in planta by generating fusion between endogenous genetic elements and a reporter gene. These approaches significantly complement the tools available to plant functional geneticists. Recent collections generated in *Arabidopsis* have generated more than 100,000 insertional lines, and this saturation of the genome essentially ensures that at least one insertional event can be recovered for any given gene. This is particularly useful where fully sequenced genomes are available because the location of an element within the genome can be determined simply by sequencing genomic sequences that flank each insertional element.

Nonetheless, insertional mutagenesis has several conceptual disadvantages: (*a*) It is based on random integration of tags in the genome and thus can only generate stochastically distributed allelic mutations in the genome; (*b*) it requires the generation of large numbers of transformants to saturate the genome with tags; (*c*) it only identifies detectable viable phenotypes and does not generate conditional mutations, which is a prerequisite for the identification and the characterization of essential genes; (*d*) the identification of the tagged mutant or gene can be very difficult and time consuming because multiple insertion or major chromosomal rearrangements can be associated with the insertion of the tag (78); and (*e*) it does not enable the generation of specific point mutations in a gene, which is a prerequisite for detailed functional analysis and for generating an allelic series of mutations within a specific gene that allow a full range of possible phenotypes to be explored.

Gene targeting (GT) is the generation of specific mutations in a genome by homologous recombination-mediated integration of foreign DNA sequences. GT circumvents the limitations associated with stochastic mutational approaches and represents the ultimate genetic tool for functional genomics. GT provides the methodological core of functional genetic studies in bacteria, yeast, and several filamentous fungi because the integration of foreign DNA into their genome occurs efficiently at targeted locations by homologous recombination (HR). It reaches its maximal efficiency in *S. cerevisiae*, and this accounts for an important part in the extensive use of budding yeast as a model system (100, 120). Yet, GT is not routinely used in animals and plants because the integration of foreign DNA in their genome occurs orders of magnitude more frequently at random locations by illegitimate recombination, thus preventing the identification of the rare targeted mutant in a large population of transformants. In 1989, the observation that the ratio of targeted to random integration events upon transformation of a mouse embryonic stem (ES) cell line ranges from 0.1–10% (16, 123) accounts for its exponential development as a model system in animal biology (77). Since 1988, the feasibility of gene targeting in plants has been demonstrated, following either direct gene transfer to protoplasts (87) or *Agrobacterium*-mediated transformation (65, 80) into either artificial (80, 87) or natural loci (65). Yet, despite numerous studies, the ratio of targeted to random integration events observed so far in plants

hardly reaches 10^{-4}, which prevents the general use of gene targeting approaches for plant functional genomics (74, 89, 126).

In contrast to all other plants tested so far, integration of foreign DNA sequences in the genome of the moss *Physcomitrella patens* occurs predominantly at targeted locations by HR (107, 110). This methodological progress represents a true revolution in our strategies to address gene function in plants and places *Physcomitrella patens* in a unique position among model systems in multicellular eukaryotes (108). This ability has been exploited in a number of studies to directly address gene function, and these are discussed here for both the technical lessons that can be learned and for the value of these studies for understanding basic biological processes. I review our current knowledge of genetic transformation and gene targeting in *Physcomitrella patens* and emphasize how this moss can be used as a model system that advantageously complements other tools available for functional genomic approaches.

THE *PHYSCOMITRELLA PATENS* MODEL SYSTEM

The major features of the life cycle of *Physcomitrella* are indicated in Figure 1. *Physcomitrella* has a relatively simple plant architecture that is nevertheless composed of the same elements as those present in other land plants. Its life cycle is dominated by the haploid gametophyte, which presents two distinct developmental phases: the protonema that displays characteristic one-dimensional filamentous growth and the gametophore that develop by three-dimensional caulinary growth into a typical small plant. The former allows the study of basic biological processes with microbiological culture technology, whereas more complex developmental processes such as organogenesis and the establishment of a body plan are adequately addressed in the latter. Most of the biological studies have been performed on the protonema, which is ideal for following biological processes and developmental patterning at the single cell level.

The biology of *Physcomitrella* and its potential as a model genetic system to study plant biological processes have been discussed in several excellent reviews and are not presented here (21–28, 33, 56, 57, 92, 94, 111, 113, 114). These reviews cover most of the work that has been conducted in *Physcomitrella* over the past 20 years and highlight the similarity of its biology with that of higher plants. These studies have shown that this moss provides an adequate system to study a broad range of biological processes including cell division, cell growth, cell polarity, cellular ultrastructure, photomorphogenesis, photo- and gravitropic responses, hormone-mediated responses, signal transduction pathways, chloroplast development, filamentous growth, organogenesis, and plant development.

The wild-type strain of *Physcomitrella patens* grown in different laboratories derives from a single spore isolated in England, thus ensuring genetic homogeneity among studies conducted in different laboratories. The size of the genome has been estimated by flow cytometry to be approximately 480 Mbp, and karyotypic analysis has shown that it is composed of 27 very small chromosomes (94, 95).

To date, no physical or genetic map of the *Physcomitrella* genome has been developed. Recently, the *Physcomitrella* EST Program (90) conducted by the University of Leeds (U.K.) and the Washington University in St. Louis (U.S.A.) has provided the scientific community with approximately 16,000 ESTs in the database, while the German agrochemical company BASF has generated an EST database of *Physcomitrella* that contains approximately 120,000 entries representing more than 20,000 genes. Preliminary analysis of genes and ESTs strongly indicate that these sequences are highly similar to those of the corresponding higher plant genes. These similarities extend to codon usage (68, 96), intron-exon structure (18, 61, 67), and multigene family complexity (18, 61, 95). It is clear that the number of *Physcomitrella* genes and EST sequences accessible in the database will increase rapidly, and each entry directly enables the generation of the corresponding moss mutant by targeted transgenesis.

Increasing interest to study mosses also comes from the field of evolutionary developmental genetics. Experimental model systems in animal developmental biology include organisms from contrasting taxonomic groups, such as worm, fly, and mammal. By contrast, model systems in plant biology have essentially focused on flowering plants. The phylogenetic basal position of Bryophytes among land plants places them in an ideal situation to address fundamental questions such as how have plants evolved from simple to complex forms (29). In this respect, functional analysis of the recently identified *Physcomitrella* gene homolog to key players in plant development such as the MADS (61) and homeobox genes (18, 102) of higher plants may give us valuable information about the ancestral mechanisms that govern the development of land plants (121). One can predict that this is only the beginning.

GENETIC TRANSFORMATION OF THE MOSS *PHYSCOMITRELLA PATENS*

Transformation With DNA Without Homology

METHODOLOGICAL CONSIDERATIONS Protoplasts of *Physcomitrella* provide the ideal material for developing methods in genetic transformation owing to the ease with which they can be isolated and to their high physiological and regenerative capacities. The first successful genetic transformation of *Physcomitrella* was achieved by polyethylene glycol (PEG)-mediated direct gene transfer into protoplasts (112). Since then, the protocol has been optimized (107, 109) and used routinely for transforming *Physcomitrella* and for transient gene expression assays (4, 41, 42, 46a, 47, 48, 52, 66, 79, 97, 101, 110, 119, 130, 131). Transient gene expression was also reported following biolistic delivery of DNA-coated microbeads into protonemal tissue (58, 106), but this method seems to be less efficient than PEG-mediated transformation as there has been only one report of a transgenic strain produced this way (58). Genetic transformation of *Physcomitrella* using *Agrobacterium*-based methods has also been attempted without success in several

laboratories (28, 94, 109). It is nevertheless possible that *Physcomitrella* might be transformed with hyper-virulent *Agrobacterium* strains (63) or upon transformation with T-DNA carrying moss genomic sequences.

To date, the neomycin resistance gene *nptII* (7), the hygromycin resistance gene *aphIV* (45), and the sulfadiazine resistance gene *sul* (52) have been used as positive selectable markers in *Physcomitrella*. The resistance genes are usually driven by the CaMV 35S promoter (4, 41, 42, 47, 48, 58, 79, 107, 110, 112, 119) or the NOS promoter (4, 66, 97, 101, 112), although it seems that the level of resistance achieved by NOS-driven resistance cassettes is lower than that observed with the 35S promoter. Selection conditions for antibiotic resistant clones are stringent and usually use antibiotic concentrations that kill untransformed protoplasts within a week of selection and correspond to 10 times the lethal dose 50 (LD_{50}, i.e., 50 mg/L geneticin sulfate, 25 mg/L hygromycin B, or 150 mg/L sulfadiazine). Meanwhile, the cytological markers encoding β-glucuronidase (GUS) (58, 107, 130) and green fluorescent protein (GFP) (17, 55, 57) were shown to be suitable for transformation experiments in *Physcomitrella*.

Survival rates of *Physcomitrella* protoplasts after PEG-mediated transformation range from 15–30%, and selection for antibiotic resistance is initiated one week after transformation, when protoplasts have regenerated colonies of 5–10 cells. Initial relative transformation frequencies (RTF; the percentage of antibiotic resistant colonies in the regenerating population) monitored 14 days after transfer to selective medium are extremely high, ranging from 3–30% (107). These values correspond to the percentage of protoplasts expressing a cytoplasmic GFP observed 48 h after PEG-mediated transfection with a 35S-GFP plasmid (17). A typical petri dish after 15 days of selection shows two classes of resistant clones (57, 107). The majority of the resistant colonies display poor growth and altered regeneration and are episomal transformants, whereas a small percentage display growth and regeneration comparable to protoplasts plated on nonselective medium and represent integrative transformants.

EPISOMAL REPLICATIVE TRANSFORMATION Episomal antibiotic resistant clones have been obtained with every transforming DNA tested so far and have been routinely referred to as unstable clones. Their phenotypic and molecular characteristics are as follows: (*a*) Unstable clones are recovered at extremely high frequencies (RTF 10–30% and 1–5% with supercoiled and linearized DNA, respectively) that correspond to up to several hundred clones per μg DNA (107). (*b*) They can be propagated for years as protonemal cultures as long as constant selective pressure is maintained but lose their resistance phenotype and the transforming DNA following a 14-day growth period on nonselective medium (4, 107, 109). (*c*) They display reduced growth and altered regeneration on selective medium, both of which are positively correlated with the antibiotic concentration (107). (*d*) Unstable clones are formed by a mosaic of transformed and untransformed cells, as observed by the presence of resistant and sensitive sectors and of GUS- or GFP-labeled and unlabeled cells in protonema regenerating on selective medium (4, 107).

(*e*) Phenotypic and molecular analyses show that maintenance of the transgene is tissue specific, being restricted to the filamentous protonema, and is not transmitted to the gametophore (4, 107, 109). Consistent with this observation, no resistant spores have been recovered from strains regenerated under nonselective conditions (107). (*f*) At the molecular level, analysis of uncut plant DNA by Southern blotting reveals that the transgene sequences comigrate with high molecular weight DNA and that these high molecular weight arrays are formed by direct head-to-tail repeats of the transforming plasmid (4, 107). (*g*) Genomic reconstruction indicates that for DNA isolated from protonema the number of plasmid copies per haploid genome ranges between 3 and 40 (4, 107). This is probably an underestimate of the real copy number per resistant cell given the strong mosaicism observed in protonema. These values fall to below one copy per haploid genome when DNA is isolated from older cultures that have differentiated into gametophores (107). (*h*) Direct evidence that transforming sequences are effectively replicated in moss cells was provided by Southern blot analyses that showed that the bacterial Dam methylation pattern of the transfected DNA was lost in DNA isolated from these clones (D. G. Schaefer, unpublished data).

These characteristics demonstrate that in episomal transformants the transforming sequences are concatenated to form high molecular weight extrachromosomal elements that are replicated in moss cells but poorly partitioned during mitosis (4, 107, 109). Replicative transformation in *Physcomitrella* provides the first example of the successful episomal maintenance of bacterial plasmids in plant cells. Though rare, a similar pattern of behavior has also been observed for plasmid DNA introduced into *Xenopus* early embryos (70), *Caenorabditis elegans* (73, 118), and several other animal eukaryotes. That bacterial plasmids can be concatenated and replicated in moss cells raises very interesting questions as to the mechanisms underlying this process. It also represents the first step toward the development of episomal vectors and moss artificial chromosomes in *Physcomitrella*. Episomal transformation vectors provide extremely valuable tools for molecular genetic approaches in yeast. Their development has been achieved by the combined addition of functional elements, such as origins of DNA replication (yeast ARS element), centromere (yeast CEN), and telomere from yeast or *Tetrahymena* into bacterial plasmids, to generate a palette of tools ranging from ARS plasmid to yeast artificial chromosome (YAC) vectors (13, 120). A similar approach should be tested for vectors used to transform *Physcomitrella*, and the mitotic stability and the developmental partition of these sequences during its life cycle should be assessed. The observation that replicative transformants in *Physcomitrella* obtained with a YAC-derived vector (11) appear to display improved mitotic stability and occasional transmission of the transgenes to the gametophore supports the potential for such approaches in the near future (107). Furthermore, the development of episomal vectors with improved mitotic stability [moss ARS-like plasmids (MARS)] would directly enable the general use of high frequency replicative transformation as an approach to complement mutations in *Physcomitrella*.

In practice, these replicative transformants can be distinguished from true integrative transformants after transformation by adopting a selection screen that alternates periods of growth on selective and nonselective medium. Integrative transgenic strains display unrestricted growth during all stages of selection, whereas replicative transformants either die or show antibiotic sensitive sectors when transferred back to selective medium (56, 107, 109).

ILLEGITIMATE INTEGRATIVE TRANSFORMATION Foreign DNA sequences can integrate by illegitimate recombination in the genome of *Physcomitrella*. Efficiencies are low (58, 66, 97, 101, 107, 112), and RTF range around 0.001% (1 in 10^5 regenerating colonies) after transformation with supercoiled plasmid and are increased approximately five times (RTF 0.005%) when linearized DNA is used (4, 97, 107, 109, 112). In comparison with the high replicative transformation frequencies observed during the same experiments, these values most likely represent the true level of illegitimate integrative transformation in *Physcomitrella*. These transgenic strains can complete their life cycle normally on selective medium and maintain antibiotic resistance when grown on nonselective medium. Compiled data from experiments performed with antibiotic resistance and cytological markers demonstrate that the new trait is mitotically stable and is expressed in every cell and at all stages of development (4, 58, 107, 109, 112). At the molecular level, Southern blot analysis shows that between 1 and 50 direct head-to-tail repeats of the transforming plasmid are integrated in the genome, usually at a single locus (4, 97, 107, 112, 130), although integration at two loci has also been observed (97). Finally, genetic analysis shows that the transgene is meiotically stable and segregates in a Mendelian way as a single locus character that is independent from strain to strain (52, 107, 112).

Transformation With DNA Carrying Homologous Sequences

CONCEPTS OF TARGETED MUTAGENESIS The basic principles and strategies of gene targeting (GT) and allele replacement in eukaryotes have been defined in budding yeast (100, 120) and applied to develop targeted mutagenesis in the mouse ES cell system (77). This methodology has been further improved when associated with the use of site-specific recombination systems (such as bacteriophage P1 Cre/lox), which facilitates the subsequent elimination of undesired sequences integrated in the genome upon transformation. This allows very accurate targeted transgenesis to be performed in the yeast and mouse genome (104, 105). These approaches are based on the use of two main types of targeting vectors: insertion and replacement vectors. Each type generates different types of mutations, and their basic characteristics and the different patterns of integration observed so far in *Physcomitrella* are schematized in Figure 2.

The three main criteria used to assess the efficiency of both gene disruption by targeted insertion and point mutagenesis by targeted allele replacement are (*a*) the ratio of targeted to random integration events observed upon integrative

transformation, (*b*) the extent of sequence homology required to efficiently target chromosomal loci, and (*c*) the ratio of insertion versus replacement events monitored upon targeted integration of replacement vectors. Each of these parameters has now been assessed in *Physcomitrella* and is reviewed in detail below.

TRANSFORMATION WITH INSERTION VECTORS The efficiency of GT with insertion vectors has been assessed in three sets of experiments designed to target six artificial loci (107), three independent single copy genomic sequences (110), and a specific member of the highly conserved multigene family encoding for chlorophyll a/b binding proteins (cab) (47). Taking advantage of the fact that pUC-derived transformation vectors share approximately 3 kb of sequence homology, I have retransformed independent kanamycin- or hygromycin-resistant transformants carrying several repeats of either plasmid pHP23b (35S-neo) (88) or pGL2 (35S-hygro) (8) with the other plasmid to assess GT efficiency to artificial loci (107). The successful targeting of artificial loci with a 90% efficiency was supported by the following experimental evidence: (*a*) Retransformation frequencies were on average one order of magnitude higher in transgenic strains as compared to wild type, with a maximum RTF value of 0.1% (107); (*b*) cosegregation of the hygromycin and kanamycin resistance markers occurred in progeny of 90% of tested clones (107); and (*c*) PCR and Southern blot analyses provided molecular evidence for the integration of direct repeats of the second plasmid by HR within the tandem repeats of the previously integrated plasmid (S. Vlach & D. G. Schaefer, unpublished data). Independently, cosegregation of positive selectable markers used for sequential transformation was also observed in transformants generated to develop transposon-mediated mutagenesis in *Physcomitrella* (25, 52). The successful targeting of three independent single copy genomic loci ranging in size from 2.4 to 3.6 kb definitively demonstrated that targeted integration by HR was the dominant path of integration of foreign DNA sequences in the genome of *Physcomitrella* (110). This conclusion was supported by the following experimental evidence: (*a*) Transformation rates with vectors carrying moss genomic sequences were on average one order of magnitude higher (RTF up to 0.1%) than those observed with nonhomologous control plasmids, (*b*) targeted integration of tandem repeats [2–30] of the vectors by HR was confirmed by Southern blot analyses in 75–100% of the plants analyzed, and (*c*) integration of the targeting plasmids in single loci and meiotic stability of the targeted loci was demonstrated by segregation analyses.

The specificity of GT in *Physcomitrella* was further assessed in experiments designed to disrupt the *ZLAB1* gene (67), one of the 15 members of the cab multigene family (95). Using an insertion vector carrying 1 kb of the ZLAB1 genomic sequence, disruption of only the true homologous gene family member by targeted integration of tandem repeats of the vector occurred in 30% of the transformants analyzed, although nucleotide sequence homology between different members was as high as 87–93% (47).

Taken together, these data demonstrate that the efficiency of targeted insertional mutagenesis with insertion vectors sharing more than 2.0 kb of sequence homology

with the moss genome is in the range of 90%, which is unique in the plant kingdom, is much more efficient than it is in mouse ES cells (32), and is comparable with efficiencies observed in budding yeast (108, 110). Targeted insertion of several [1–50] direct repeats of the transforming plasmid in the corresponding target locus occurs by a single HR event, allowing the direct generation of insertional mutations such as loss-of function (see Figure 2*AI*). The specificity of the recombination process enables the disruption of a specific member within a gene family. The data also indicate that integrated transgenes provide accessible targets for the subsequent integration of additional transforming vectors, a feature that must be considered in designing experiments for the sequential transformation of strains.

TRANSFORMATION WITH REPLACEMENT VECTORS The minimal and optimal amounts of sequence homology required to target genomic sequences and the pattern of targeted integration of replacement vectors have been defined in experiments designed to target the *Physcomitrella* adenine phosphoribosyl transferase gene (*Ppapt*) (D. G. Schaefer, M. Chakhparonian, S. Vlach, K. von Schwartzenberg, N. Houba-Hérin, C. Pethe, J.-P. Zrÿd & M. Laloue, unpublished data). The *apt* gene provides an ideal model locus for GT studies in haploid organisms as its loss of function confers resistance to adenine analogues such as 2,6-diaminopurine (DAP) and can thus be directly selected (76). Using both cDNA- and gDNA-based replacement vectors, we have been able to establish the following parameters: (*a*) Targeted integration by HR is possible using stretches of continuous sequence homology ranging from 50–200 bp (48) and reaches a maximum when targeting sequences extending over 1–2 kb are used; (*b*) under optimal conditions, RTF for the *PPapt* locus are in the range of 0.1%, and targeting efficiencies are above 90% (i.e., more than 9/10 transformation events involve HR); (*c*) targeted replacement of genomic sequences mediated by two HR events (i.e., allele replacement) (Figures 2*BII,III*) and targeted insertion mediated by a single HR coupled with a nonhomologous end-joining reaction (NHEJ) (Figure 2*BV*) occur at similar rates when replacement vectors are used; (*d*) replacement of the genomic sequence by a single copy of the replacement cassette is observed in approximately half of the replacement events (Figure 2*BII*), whereas the structure of the other half of replacement events is characterized by the presence of direct repeats of either the replacement cassette (Figure 2*BIII*) or the entire transforming plasmid (not illustrated); (*e*) targeted insertion of replacement vectors is characterized by the integration of tandem direct repeats [1–30 copies] of either the replacement cassette or the entire plasmid (Figure 2*BIV*); (*f*) NHEJs that occur between direct repeats of the transforming DNA or at the junctions between plasmid sequences and the genome are identical within one set of direct repeats but vary in different targeted transformants. These reactions follow the main features described in other plants for NHEJ events, i.e., deletion of plasmid sequences followed by rejoining within short stretches of microhomology (43).

Taken together, these data demonstrate that targeted mutagenesis with replacement vectors is as efficient in *Physcomitrella* as in *S. cerevisiae*. Sequence

homology requirements and the ratio of targeted to random integration events are comparable to those observed for targeted mutagenesis in budding yeast (100) and are more favorable here than for any other multicellular eukaryotic system amenable to GT (108). Yet the integration pattern of replacement vectors in *Physcomitrella* is different from that observed in budding yeast, where replacement vectors integrate essentially by allele replacement, and is instead highly similar to targeted integration events observed in Chinese hamster ovary cells (1). This suggests that the mechanisms accounting for targeted integration in moss are different from the models proposed for budding yeast (85). The synthesis-dependent-strand-annealing model or the recently proposed break-induced replication model (60) account for the observations described above, but clearly further studies on the mechanisms of GT in wild-type *Physcomitrella* and in strains mutated in recombination genes will be needed to clarify our understanding of the mechanisms of HR in this moss.

Analysis of Gene Function by Targeted Disruption

Efficient GT to natural loci prompted several groups to initiate functional studies by targeted disruption of genes involved in four completely unrelated biological processes: the division of chloroplasts, the biosynthesis of unsaturated fatty acids, the ubiquitin-mediated proteolytic pathway, and the purine salvage pathway. Using a cDNA-based replacement vector carrying approximately 1 kb of discontinuous homologous sequence, Reski's group in Freiburg successfully disrupted the *ftsZ1* gene of *Physcomitrella* in 14% of the transgenic plants analyzed (119). In bacteria, the ftsZ protein plays a major role in the formation of the dividing ring during cytokinesis, and nuclear-encoded eukaryotic ftsZ homologues have been found in plants (55, 82). The observation that cells of *ftsZ* knock-out moss strains contain a single giant chloroplast instead of the 50 lens-shaped chloroplasts usually found in wild-type cells provided the first functional evidence that FtsZ proteins are essential for chloroplast division. This observation was subsequently confirmed in *Arabidopsis*, using antisense approaches (81, 82). Subsequently, a second ftsZ gene was isolated from *Physcomitrella* and shown to be functionally nonredundant to the first one (55). More remarkably, Kiessling and coworkers used transient expression of *ftsZ*-GFP fusion cassettes to demonstrate that both FtsZ proteins form a permanent, highly organized filamentous scaffold within moss chloroplasts. This newly identified subcellular structure is reminiscent of cellular cytoskeleton, and the authors proposed that it may function to maintain plastid shape and named it the plastoskeleton (55). Whether similar structures also exist in chloroplasts of vascular plants awaits further studies, but the work conducted in moss provided new insights into our understanding of the mechanisms of plastid division.

Lipids of *Physcomitrella* are composed of up to 30% arachidonic acid (44), a polyunsaturated fatty acid (PUFA) found only in lower plants. Searching for new genes involved in the biosynthesis of this PUFA, the group of E. Heinz in Hamburg isolated a novel Δ6-acyl-group desaturase gene from *Physcomitrella* (*PPDES6*)

and disrupted it with a genomic DNA-based replacement vector carrying approximately 2 kb of sequence homology (41). Disruption of *PPDES6* was supported by PCR and by Southern and Northern blot analyses in 5/5 randomly chosen transformants. These knock-out plants displayed no visible developmental phenotype, but analyses of their fatty acid composition revealed a clear biochemical phenotype. Plants displayed reduced levels of arachidonic acid and other PUFA whose biosynthesis involves a Δ6-desaturation step and a concomitant accumulation of putative precursors. That *PPDES6* encodes for a real Δ6-desaturase was further confirmed by feeding experiments in moss and expression studies in yeast. Thus, this work showed that targeted disruption in *Physcomitrella* can be used to identify novel plant genes and to describe new biochemical pathways involved in the biosynthesis of metabolites.

One major route for the selective degradation of proteins in eukaryotic cells is the ubiquitin-proteasome pathway. The 26S proteasome, an ATP-dependent protease complex that degrades ubiquitin-tagged proteins, is composed of a core proteinase, the 20S proteasome, associated with a pair of regulatory complexes known as the 19S complex (127). The RPN10 protein is a subunit of the 19S complex that has affinity for multiubiquitin chains in vitro and may function in recognizing and recruiting ubiquitinated proteins to be degraded by the proteasome. Yet, its function is still a matter of debate because disruption of the *rpn10* gene in *S. cerevisiae* did not cause any obvious phenotype (such as growth defects) except for an increased sensitivity to amino-acid homologues (124). However the same disruption did lead to an embryonic lethal phenotype in mouse (53). In our group, Girod and colleagues isolated and disrupted the *rpn10* gene of *Physcomitrella* (*PPrpn10*) with a cDNA-based replacement vector carrying 1300 bp of discontinuous homologous sequence (42). Successful disruption of the gene in 2 out of 55 transgenic strains was confirmed by PCR, Southern blot analysis, and immunological analyses. The knock-out moss strains were viable but displayed a dramatic developmental phenotype characterized by altered protonemal development and the impairment of bud formation. This developmental phenotype was complemented following expression of *PPrpn10* gene at ectopic locations, but mutated versions of the gene failed to fully complement the phenotype, providing insight into the role of the different domains within the *PPrpn10* gene. Thus this work provides a telling example of the need to use different model systems for the functional characterization of complex regulatory mechanisms such as the ubiquitin/proteasome proteolytic pathway. The experiments performed in *Physcomitrella* revealed an interaction between this pathway and development that was not observed in yeast and that could not be further investigated in mouse owing to the embryonic lethality of the knock-out phenotype.

The adenine phosphoribosyl transferase gene (*apt*) encodes for a maintenance enzyme of the purine salvage pathway that recycles adenine into AMP. In *Arabidopsis*, *apt* genes form a small family that contains at least three expressed members (35), and the *apt* mutant BM3 (75) carries a mutation in the *Atapt1* gene (35). This mutant has approximately 1% APRTase activity, is resistant to DAP, displays

reduced growth rate and male sterility due to abortive pollen development (91), and is unable to form callus or regenerate in vitro (64), but whether the observed phenotype could be attributed to impaired purine or cytokinin metabolism was unclear (35, 75). *Physcomitrella* has only one *apt* gene (*Ppapt*) that closely resembles the *Arabidopsis Atapt1* gene at the level of genetic structure and biochemical activity of the encoded protein. *Physcomitrella apt* null alleles display a strong developmental phenotype characterized by abortive gametophore development. In addition, these mutants display increased sensitivity to exogenously supplied adenine, which prompted us to reanalyze the *Arabidopsis* mutant and to observe that BM3 also displayed hypersensitivity to adenine, an observation that had not been made previously. Finally, we could show that expression of the *Arabidopsis Atapt1* or *Atapt2* genes at ectopic locations fully complements the *apt* null phenotype in *Physcomitrella*, giving rise to fertile plants that are sensitive to 2,6-diaminopurine and resistant to adenine (D. G. Schaefer, M. Chakhparonian, S. Vlach, K. von Schwartzenberg, N. Houba-Hérin, C. Pethe, J.-P. Zrÿd & M. Laloue, unpublished data). Thus, disrupting the *apt* gene in *Physcomitrella* provided two novel pieces of information: First, *Arabidopsis* homologues can functionally complement mutations in *Physcomitrella*. Second, the developmental phenotype observed in moss *apt* null strains was associated with the inability of the plant to recycle adenine, an observation that was correlated in *Arabidopsis*.

The studies reviewed above are the first examples of the use of efficient GT in *Physcomitrella* to provide rapid access to valuable new information about genetic networks that control diverse biological processes.

Additional Methodological Development

FUNCTIONAL STUDIES OF PROMOTERS IN *PHYSCOMITRELLA* The generalized use of *Physcomitrella* for targeted transgenesis will require the use of several well-characterized promoters for the construction of constitutive and inducible expression vectors, as a result of the following features of *Physcomitrella* transformation: (*a*) Promoter sequences integrated in the moss genome provide target sites for the subsequent integration of an expression cassette driven by the same promoter. (*b*) Preliminary evidence indicates that when two transgenes are driven by the same promoter, they may be stochastically silenced by a mechanism that remains to be determined (D. G. Schaefer, unpublished observation; 52). Two types of promoters, endogenous moss promoters and heterologous promoters, are discussed below. Their use in transformation experiments will enable very different types of studies to be performed.

The presence of an endogenous moss promoter in a transformation vector will result in the predominant targeted integration of the vector into the homologous promoter locus. This provides a straightforward tool for monitoring the activity of the corresponding promoter in a native chromosomal regulatory context and for characterizing promoter functional elements after replacement of the native sequence with an in vitro mutated one. Such efficient and accurate promoter-trap

strategies will certainly be broadly applied to characterize moss promoters and to manipulate expression levels of endogenous genes.

Yet the use of endogenous moss promoters is clearly not ideal for functional studies in which investigators must be able to alter gene expression levels, and this requires the use of several different heterologous promoters to drive expression cassettes that are to be introduced in the same transformed strain. So far, the rice actin-1 gene promoter (72) and the maize ubiquitin-1 gene promoter (19) have been shown to promote GUS expression levels following transient expression in protoplasts that were comparable to those observed in rice and maize (17, 131; D. G. Schaefer & M. Uzé, unpublished data). In contrast, the 35S-GUS construct pBI221 (50) promoted only weak GUS activity (17, 107, 131). Yet transfection with an improved version of pBI221 [pNcoGUS (12)] carrying an optimized translation initiation context as defined by Kozak (59) resulted in a 10-fold increase in GUS activity (17, 107, 130, 131), whereas the introduction of the 5′ untranslated intron of the rice actin-1 gene promoter between the 35S promoter and the *uidA* gene [pBCGA4 (72)] resulted in an additional 5-fold increase in GUS activity as compared to pNcoGUS, activities that were in the same range as those observed with constructs driven by the rice actin-1 gene promoter (17). These observations indicate that optimizing the expression of transfected genes in moss follows the same basic principles as those observed in other plants and that characterized heterologous promoters from higher plants may provide valuable tools for the future construction of constitutive expression cassettes for *Physcomitrella*.

HETEROLOGOUS CONDITIONAL PROMOTERS The development of a tightly regulated conditional gene expression system for *Physcomitrella* is essential for switching transgenes on and off and for the generation of conditional mutations. This is especially important for the study of essential genes in the haploid background of the moss gametophyte because such experiments can then directly be performed by replacing the natural promoter with a conditional one that can be turned on during the selection period and subsequently switched off for functional studies. Chemically inducible gene expression systems are well suited for such approaches and should ideally meet three objectives: (*a*) The inducible promoter should not be leaky under noninduced conditions; (*b*) the induction factor should reach at least two orders of magnitude, and its modulation should be positively correlated with the concentration of the chemical inducer; and (*c*) the chemical inducer should not interfere with plant biological processes (38).

Knight and colleagues have studied the regulation of the abscissic acid (ABA)– and osmotic stress–induced wheat *Em* gene promoter (69). In transgenic strains, *Em*-driven GUS activity was readily detectable upon noninduced conditions (probably in response to low concentrations of endogenous ABA) and was induced by factors of 5- and 30-fold following treatment with 0.44 M mannitol or 10^{-4} M ABA, respectively (58). The Em-GUS cassette was strongly expressed in transient gene expression assay, most likely in response to the presence of mannitol in the protoplast culture medium, and could be induced fourfold further upon ABA

treatment. Detectable GUS activity under noninduced conditions suggested that some of the molecular mechanisms involved in ABA and stress responses might be conserved between mosses and cereals (58). This assumption was supported by gel retardation assay and DNAseI footprint analysis, which showed that factors present in moss nuclear protein extracts bind specifically to the same abscissic acid response element (ABRE) previously identified from studies with wheat nuclear extracts (46). Thus, although the wheat Em promoter shows strong inducible activity in *Physcomitrella*, this promoter is clearly not ideal for modulating gene expression levels in this moss as it will be continually induced by endogenous fluctuations in ABA levels in the plant.

Tetracycline-regulated gene expression in *Physcomitrella* was reported using the tetracycline-repressible system developed by Gatz et al. (130). In the presence of 1–10 mg/L tetracycline in culture medium, GUS activities measured both by transient gene expression assays and in transgenic strains were hardly detectable. In the absence of tetracycline, GUS expression was induced approximately 100-fold within 24 h to reach specific activities in the range of those observed with GUS reporter cassettes driven by the rice actin-1 gene promoter (130). Expression of the GUS gene could subsequently be repressed upon transfer to tetracycline-supplemented medium, but the kinetics of repression could not be determined owing to the relatively long half-life of the GUS protein in vivo. Although continuous growth on tetracycline may be deleterious to moss growth (M. Laloue, personal communication), tetracycline-regulated gene expression provides a very promising system for conditional expression of genes in *Physcomitrella*.

The two-component glucocorticoid-inducible gene expression GVG system developed by Aoyama & Chua (3) is composed of a constitutive cassette expressing the glucocorticoid receptor fused to a transcriptional activator and of a reporter construct that carries the inducible promoter driving a reporter gene. This system has great potential as it should not, a priori, interfere with plant biological processes. Moreover, wild-type *Physcomitrella* grown on medium supplemented with the synthetic glucocorticoid dexamethasone at concentrations of up to 0.5 mM did not display any obvious alteration of growth or development. Using transient gene expression assay in protoplasts, Chakhparonian (17) showed that GUS expression can be induced up to 50-fold in protoplasts grown in the presence of 30 μM dexamethasone. Yet, transfection with the reporter construct alone yielded detectable GUS activities, indicating that the inducible promoter was leaky, an observation that was previously reported in tobacco (3). Nevertheless, glucocorticoid-regulated gene expression represents a potentially useful system for modulating gene expression in *Physcomitrella*.

Conditional expression systems such as those based on the Gal4 promoter in budding yeast (54) or the *nmt1* promoter in fission yeast (5, 71) have provided essential tools for the functional dissection of genetic networks in these model organisms. Preliminary data obtained in *Physcomitrella* indicated that both the tetracycline-regulated and the glucocorticoid-inducible systems could be used to modulate the expression of genes in this moss, a picture that is similar to that

observed in higher plants (39). Additional studies are required to further improve conditional gene expression in *Physcomitrella*, and the recently developed TGV system that combines dexamethasone induction and tetracycline repression (10) as well as the oestrogen-inducible XVE system that appears less leaky than GVG (132) remain to be tested.

CRE/LOX-MEDIATED SITE-SPECIFIC RECOMBINATION IN *PHYSCOMITRELLA* The site-specific recombination Cre/lox system of bacteriophage P1 is used to introduce site-specific mutations in a genome. It is composed of the Cre recombinase and a small DNA-recognition target site, the *loxP* site, that consists of two 13-bp inverted repeats flanking an 8-bp asymmetric spacer that defines the orientation of the site. Recombination events mediated by the Cre recombinase lead to the excision or the inversion of DNA sequences located between two direct or inverted repeats of *loxP* sites, respectively. In plants, the Cre/lox system was successfully used to generate mutations as diverse as translocations, deletions, or inversions and to regulate conditional gene expression or site-specific integration (83, 84, 126). One of its direct applications in plant biotechnology is the excision of selectable markers and plasmid sequences in genetically modified crops (31, 133). Yet, it is in combination with efficient GT that this system realizes its full potential because the latter allows *loxP* sites to be introduced at specific locations within the genome. The utility of this approach is well illustrated by the extensive use of the Cre/lox system to manipulate the genomes of yeast (103) and mouse (105). In this respect, setting up methodologies for accurate targeted mutagenesis in *Physcomitrella* necessitates the development of an efficient site-specific recombination system.

The possibility of using the Cre/lox system to excise plasmid repeats inserted in the genome of *Physcomitrella patens* was recently demonstrated by Chakhparonian (17) in two independent strains carrying one or five copies of a plasmid containing a 35S nptII cassette flanked by two *loxP* sites in direct orientation. Following transient expression of a 35S Cre cassette [pMM23 (30)], protoplasts of these strains were screened for plasmid excision, based on loss of the resistance marker. Loss of plasmid repeats by Cre-mediated site-specific recombination was observed in 3–10% of protoplast-derived colonies and was confirmed by PCR, Southern blot analyses, and sequence analysis. Such an efficient excision is in the range of that observed in other plant systems following transient expression of Cre. This methodology permits the rapid and easy identification of recombined clones in a small population. This finding has one immediate consequence for strategies of targeted mutagenesis in *Physcomitrella*: *loxP* sites should be built into the design of every vector to be used for transformation. Their presence allows the generation of point mutations by gene conversion (as illustrated in Figure 2*B*), the recycling of selectable markers for sequential transformation, or the elimination of integrated plasmid repeats. Ultimately the combination of efficient GT with site-specific recombination will enable the study of plant biological processes in *Physcomitrella* with the type of accurate targeted mutagenesis strategies that have been developed in budding yeast and mouse (77, 103).

TAGGED MUTAGENESIS AND GENE- AND ENHANCER-TRAP SCREENS Thus far, I have discussed the potential of targeted transgenesis in *Physcomitrella* for the functional analysis of plant genes. The unique opportunity that this offers in the plant kingdom contrasts singularly with the limited genetic knowledge we have of the biology of *Physcomitrella*. This prompted Hasebe's group to use large-scale insertional mutagenesis approaches in combination with efficient GT to generate collections of tagged and trapped lines of *Physcomitrella.* In these collections, new phenotypes and gene expression patterns can be identified and immediately further characterized (46a, 79). The first report from Nishiyama et al. (79) described the construction of two moss genomic libraries mutagenized by shuttle mutagenesis (116) with a mini-transposon designed either to tag moss genes with a *nptII* marker or to trap them with a *uidA* marker. Following transformation of *Physcomitrella* with these libraries, tagged lines displaying a visible developmental phenotype and trapped lines identified by GUS histochemical staining were recovered at frequencies of 4% and 3%, respectively (79). A preliminary analysis of these transformants indicated that such an approach was suitable for generating mutants that displayed a broad range of developmental phenotypes and for identifying gene expression patterns that cover many of the different developmental stages or cell types found in *Physcomitrella*. Using the same overall experimental strategy, Hiwatashi et al. (46a) developed further *uidA*-tagged gene-trap and enhancer-trap elements and used them to transform *Physcomitrella* under both homologous and nonhomologous conditions. Transformation rates were one order of magnitude higher under homologous conditions, suggesting that the use of mutagenized genomic libraries enhanced the yield of tagged and trapped lines. GUS-positive gene-trap and enhancer-trap lines were recovered at frequencies of 4% (235/5637) and 29% (1073/3726), respectively, which represent very high frequencies for the application of such strategies. More importantly, this work demonstrates that the trapped gene can be efficiently identified using RACE PCR with *uidA*-specific primers even though multiple copies of the mini-transposon were inserted in a trap line (46a). To account for the presence of multiple copies of the mini-transposon in tagged and trapped lines, the authors proposed that mini-transposon-tagged sequences concatenate extrachromosomally to generate large tandem arrays of mutagenized genomic sequences that subsequently integrate by HR in a single locus within the terminal genomic sequence of the array (46a), a hypothesis that fits well with the structural feature of replicative and integrative transformation of *Physcomitrella* reviewed here. This enables the study of moss genes or enhancers trapped upon shuttle mutagenesis without the simultaneous disruption of their original locus in the plant, a situation that presents advantages in the haploid gametophyte of *Physcomitrella*.

The two studies reviewed above represent major progress in the development of *Physcomitrella* for plant functional genomics. They provide the first large-scale collection of tagged insertional mutants in *Physcomitrella* available to the public in which a broad range of new phenotypes and new gene expression patterns in vivo can be identified. They also show that subsequent isolation of the mutated gene is

facilitated by the presence of the mini-transposon tag and provide the first example of a complete reverse genetic analysis successfully completed in *Physcomitrella*. Finally, they show that, in a manner similar to its application in budding yeast (14), shuttle mutagenesis can be used to undertake large-scale analysis of gene expression, protein localization, and gene disruption in *Physcomitrella*.

CONCLUSION

The data reviewed above demonstrate that GT efficiency in *Physcomitrella* is amazingly high and can only be compared with that observed in budding yeast (108). Under optimal conditions, such efficiency enables within five weeks the isolation of dozens of targeted mutants from a single transformation experiment (41, 107, 110). The efficiency of GT in *Physcomitrella*, combined with the possibility of using the site-specific Cre/lox system, allows the application of the sophisticated strategies of targeted mutagenesis, which so far have been applied only in yeast and mouse ES cells. Thus, at the methodological level, *Physcomitrella* fulfills the ultimate requirement for its development as a model system because the generation of predetermined point mutations at any location of its genome is directly feasible.

Prospects for improving gene targeting frequencies in animals (125) and plants (74) (126) have been reviewed recently. The ideal situation would be to achieve a useful rate of GT in a wild-type background, as this would allow unambiguous interpretation of the data. This requirement is completely fulfilled by the *Physcomitrella* system. The strategy based on the generation of a recombination substrate in vivo as described by Rong & Golic in *Drosophila* (99) has yet to be tested in higher plants. The use of promoter trap positive-negative screens as applied in mammalian cells (115) also looks promising. Although it will not increase the yield of targeted integration events, it will mainly strengthen the screen for such events. Current strategies also include attempts to improve the ratio of targeted to random integration events by modulating the activity either of endogenous genes involved in DNA recombination and repair or following the overexpression of heterologous genes involved in these pathways (74, 126). Yet, as emphasized by Vasquez et al. (125), such strategies should be adopted with great caution and ideally should be performed only by transient expression of advantageous genes rather than by their inactivation or constitutive expression, as such mutations may seriously impair the maintenance of genome integrity. This is dramatically illustrated by the observation that loss of function of the Rad50 gene in *Arabidopsis* stimulates intrachromosomal recombination in planta (40) but simultaneously leads to plant sterility (36) and accelerated senescence due to telomere instability (37). In this respect again, *Physcomitrella patens* provides an ideal model system.

Why is gene targeting so efficient in *Physcomitrella*? We (108, 110, 111) previously proposed that it could be correlated with the dominance of the haploid gametophyte in its life cycle, whereas Reski (93) proposed that efficient GT could be correlated with the fact that protoplasts are highly synchronized and blocked in G2 during transformation. Both hypotheses can be tested experimentally.

In budding yeast, extensive genetic and biochemical studies have shown that efficient GT is tightly associated with the repair of DNA double-strand breaks (85). Yet a mutation that would specifically alter the ratio of targeted to random integration events observed upon integrative transformation has not yet been identified, indicating that the mechanisms that control this ratio are not easy to unravel (85). In this respect, *Physcomitrella* provides a valuable alternate system to study this process. The identification and functional analysis of moss genes involved in DNA repair and recombination may provide insight into these mechanisms and ultimately facilitate the development of strategies to target genes efficiently in higher eukaryotes.

In my opinion, *Physcomitrella* is poised to become an extremely valuable model system in biology. The efficiency of GT in *Physcomitrella* can and should be used immediately, as it fills a major methodological gap and advantageously complements the tools available for the study of plant functional genomics. The suitability of the *Physcomitrella* system to study plant biology is supported by the structural and functional similarities observed between mosses and higher plants both in biological processes and at the genetic level. The plant scientific community could advantageously benefit from a whole genome sequencing program to gain the required information about *Physcomitrella* genes for future functional genomic studies. The field is moving fast, and we have all the tools in our hands to develop the *Physcomitrella* system as a "green yeast" for addressing plant biological questions. Furthermore, the use of GT technology in *Physcomitrella* allows for the study of complex developmental processes such as organogenesis or the establishment of a body plan, processes that cannot be studied in budding yeast. As such, this plant may prove to be in a unique position to unravel the genetic networks controlling complex developmental processes in multicellular Eukaryotes.

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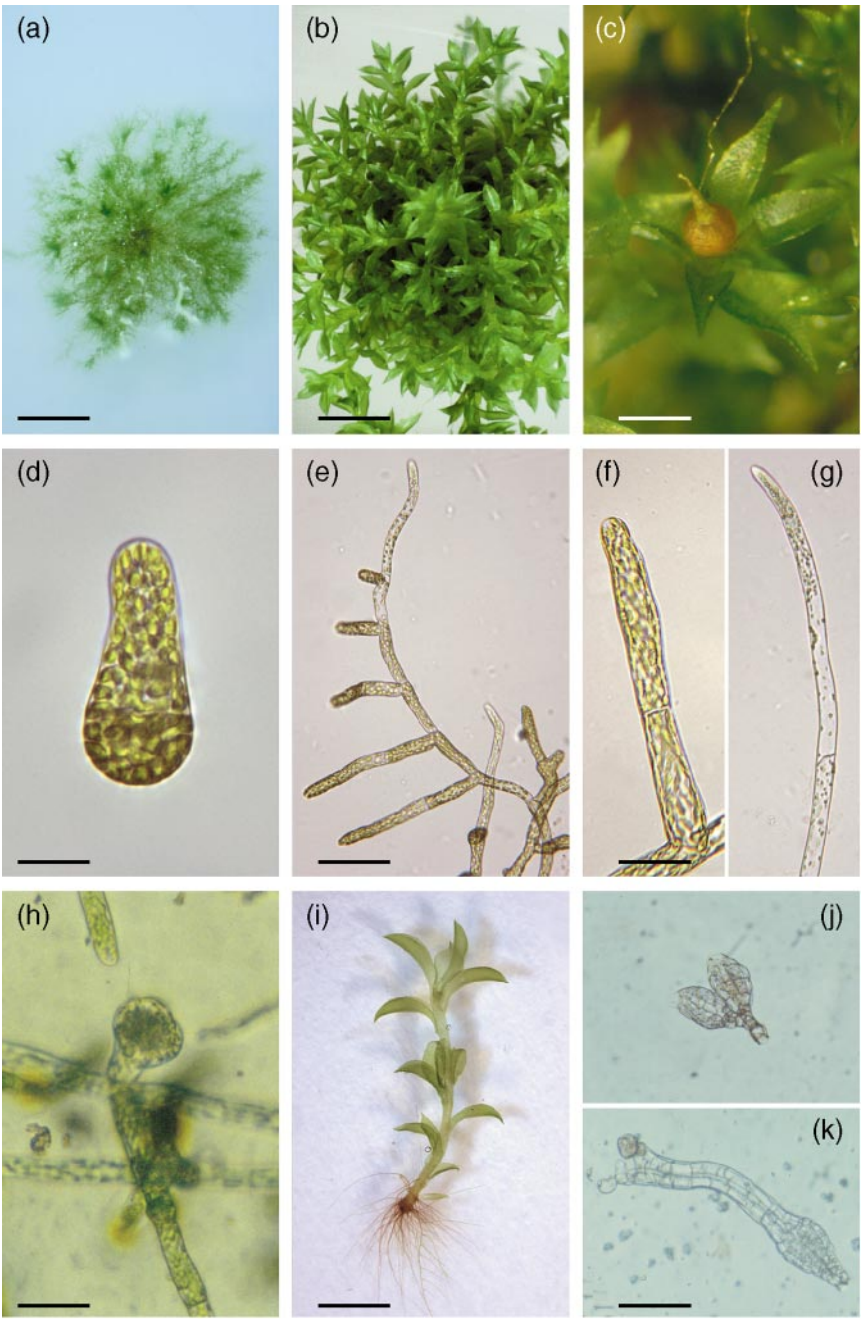

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Figure 1 (See figure on previous page) The life cycle of the moss *Physcomitrella patens* (*a–c*). The three developmental stages of its life cycle: (*a*) the juvenile gametophyte or protonema displaying characteristic filamentous growth (bar = 1 cm); (*b*) the adult gametophyte composed of a colony of gametophores developing by caulinary growth (5 mm); (*c*) the parasitic diploid sporophyte that develops at the apex of a gametophore to form a spore capsule where spores are differentiated (1.5 mm). (*d–h*) Closer view of the different stages of protonema development. (*d*) A germinating protoplast 72 h after isolation displays a highly polarized structure; it has already divided once, and the daughter cell has resumed apical growth (25 μm). (*e*) Typical filamentous growth pattern of a branched chloronemal filament with its tip cell undergoing a developmental transition to form a caulonema (100 μm). (*f–g*) Two cell types with distinct ultrastructural and biological characteristics form the protonema: The photosynthetic chloronema (*f*) is filled with chloroplast and has cell walls perpendicular to the axis of the filament. The adventitious caulonema (*g*) contains less chloroplasts and forms cell walls that are oblique with respect to the axis of the filament (40 μm). (*h*) Transition from filamentous growth to caulinary growth can be pinpointed to a single cell, the caulonemal side-branch initial that differentiates into a primitive meristem, the bud (50 μm). Caulonemal side-branch initials can also form chloronemal or caulonemal cells, and the relative ratio of these different developmental fates can be modulated by chemical and environmental factors and accounts for the general morphology of the protonema. (*i–k*) The gametophore displays a body plan that is simpler but analogous to higher plants. (*i*) A fully differentiated gametophore is composed of a nonvascularized photosynthetic stem carrying simple leaves formed by a single layer of photosynthetic cells and displaying phyllotaxis along the stem axis and of filamentous pigmented rhizoids that radiate from the base of the stem (2 mm). Reproductive organs differentiate in the apical part of the gametophore: the male antheridia (*j*), borne in the leaf axils, and the female archegonia (*k*), borne terminally (100 μm). Ciliated antherozoids formed in the antheridia swim and fuse with the egg cell located in the archegonia to give rise to a diploid cell that further differentiates into the sporophyte (*c*).

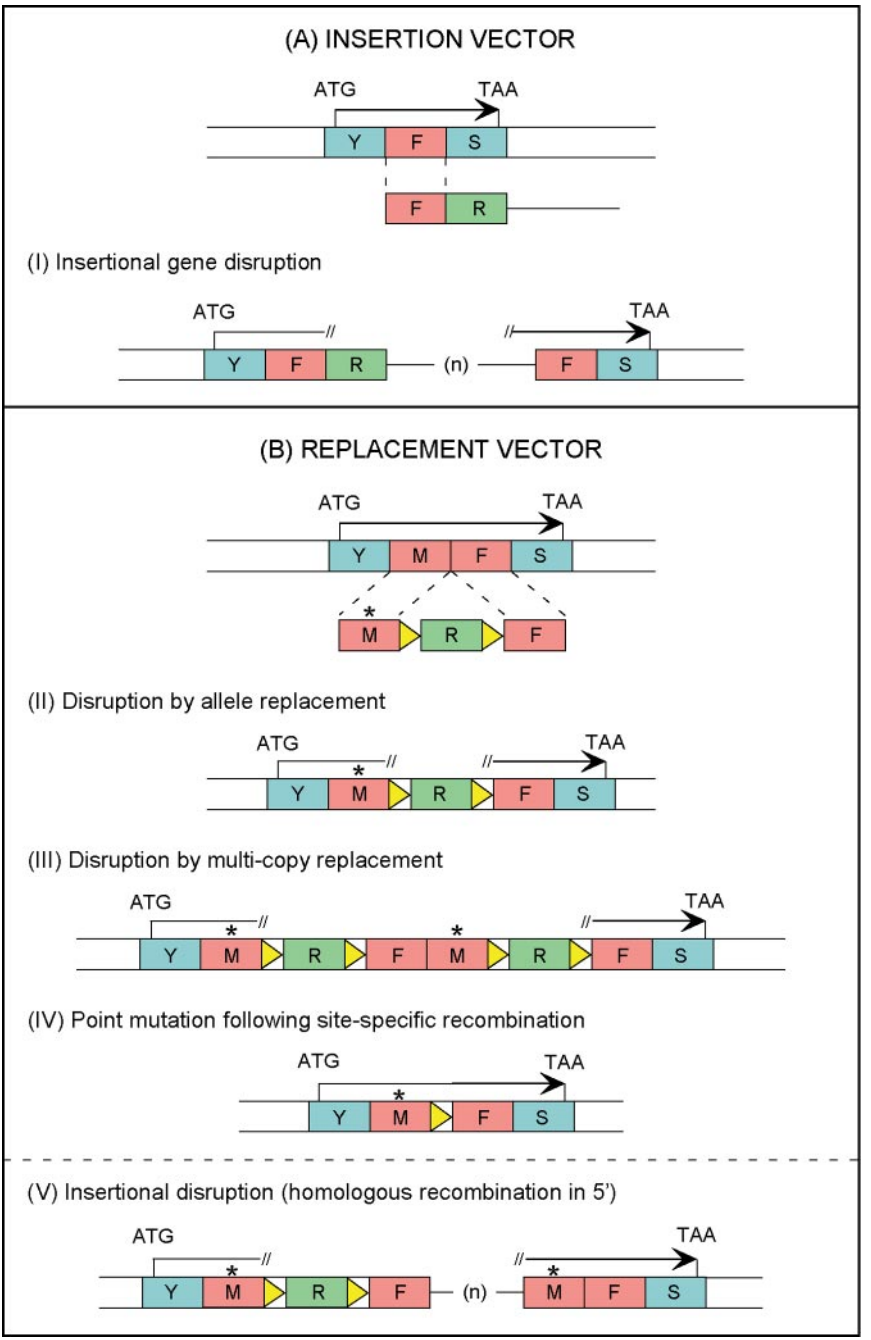

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Figure 2 (See figure on previous page) (*A*) Insertion vectors contain one homologous targeting fragment (*F box*) of your favorite sequence (YFS) cloned next to a marker encoding a resistance gene (*R box*). Targeted integration occurs by a single HR event (*dashed lines*) and results in the insertion of one or several copies of the vector flanked by two repeats of the targeting sequence (*F*). Insertion vectors are suitable for generating insertional gene disruption (*AI*) but not for generating point mutations. (*B*) In replacement vectors, the resistance gene (*R box*) flanked by 2 *loxP* sites in direct orientation (*yellow triangle*) is inserted between two homologous targeting fragments (*M** and *S boxes*, where M* carries a point mutation) of your most favorite sequence (YMFS). Targeted integration occurring by two HR events within M and F leads to the replacement either of the endogenous chromosomal sequence by one (*BII*) or several repeats of the replacement cassette (*BIII*), or of the entire plasmid (*not shown*). When the selectable marker flanked by two *loxP* sites is cloned in an intron, subsequent expression of the Cre recombinase eliminates the selectable marker and the plasmid repeats to generate accurate targeted point mutations (*BIV*) ($M \rightarrow M^*$). Yet, replacement vectors can also integrate by a single HR event within either the M or F sequence leading to an insertional disruption, as described with insertion vectors (V for HR in M).