Gene targeting in Physcomitrella patens
Didier G Schaefer

Gene-targeting efficiency in the land plant Physcomitrella patens (Bryophyta) can only be compared with that observed in Saccharomyces cerevisiae. Sequencing programs and microbiological molecular genetic approaches are now being developed to unravel the precise function of plant genes. Physcomitrella patens, as the new ‘green yeast’, might well become a major tool for functional genomic studies of multicellular eukaryotes.

Introduction
The extensive DNA sequence data sets that have been made available to the scientific community provide an outstanding tool for the investigation of the precise function of genes. The next decade will be devoted to functional genomics and several approaches are currently being applied to unravel gene function in plants [1]. Gene expression profiles in response to abscissic acid and environmental signals as those acting in the same growth substances (i.e. auxin, cytokinin and abscisic acid) and their studies have revealed that, unlike in angiosperms, GT is the predominant pathway for functional genomics in mammalian biology over the past 10 years [9,10]. In plants, despite the fact that the feasibility of GT was demonstrated more than 10 years ago [11], the ratio of targeted to random integration events observed remains too low to enable systematic GT approaches [12,13••,14••] (Table 2).

The moss Physcomitrella patens (Funariales, Bryophyta) is being developed as a model system for plant biological studies. The dominance of the haploid gametophyte in the life cycle of this moss facilitates genetic analysis. Both apical and caulinary growth can be studied at the single-cell level during gametophytic development. The metabolism and development of P. patens are controlled by the same growth substances (i.e. auxin, cytokinin and abscisic acid) and environmental signals as those acting in other land plants. However genetic transformation studies have revealed that, unlike in angiosperms, GT is the predominant pathway of genetic transformation in P. patens. In this review, we discuss the implications of this finding for functional genomic studies in plant biology.

Efficient GT is correlated with the dominant pathway of DNA double strand break repair
Genetic and biochemical studies conducted in S. cerevisiae provide a general model for the high efficiency of GT in yeast [15,16••]. Integration of foreign DNA sequences in the genome by HR or IR seems to be tightly correlated with the dominant pathway used by the cells to repair DNA double-strand breaks (DSB). In S. cerevisiae, DSB repair by HR is the predominant mechanism and the genes involved in this repair pathway have been assigned to the RAD-52 (radiation-sensitive-52) epistasis group. In contrast, DSB repair by non-homologous end joining (NHEJ) in S. cerevisiae is a minor pathway involving genes belonging to the RAD-50/Ku-70 epistasis group [16••]. Genes homologous to those of the S. cerevisiae RAD-52 and RAD-50/Ku-70 epistasis group have been identified in plant [17,18•] and mammalian systems [19], and their studies by homologous recombination (HR), resulting in either gene knock-out by insertion or point mutations by gene conversion (Figure 1).

Although GT is the standard method for the study of functional genetics in microbiological systems such as bacteria, yeast and some filamentous fungi, it can be applied to higher eukaryotic model systems only rarely. This is because the frequency of integration at random locations by illegitimate recombination (IR) in the higher eukaryotes is several orders of magnitude higher than that of targeted integration by HR (Table 1). The high ratio of targeted to random integration events (i.e. 0.1–1%) in murine embryonic stem (ES) cells relative to that in other mammalian systems accounts for the extraordinary development of the mouse ES cell system for functional genomics in mammalian biology over the past 10 years [9,10]. In plants, despite the fact that the feasibility of GT was demonstrated more than 10 years ago [11], the ratio of targeted to random integration events observed remains too low to enable systematic GT approaches [12,13••,14••] (Table 2).

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Figure 1

(a) Insertion vectors

Target locus

\[
\begin{array}{c}
\text{YMFG} \\
\text{YMFG} \\
\end{array}
\]

M+ next to homologous sequence

(i) Conversion

\[
\begin{array}{c}
\text{YMFG} \\
\text{YMFG} \\
\end{array}
\]

(ii) Insertion

\[
\begin{array}{c}
\text{YMFG} \\
\text{YMFG} \\
\end{array}
\]

(iii) 'Hit and run'

\[
\begin{array}{c}
\text{YMFG} \\
\text{YMFG} \\
\end{array}
\]

(b) Replacement vectors

Target locus

\[
\begin{array}{c}
\text{YMFG} \\
\text{YMFG} \\
\end{array}
\]

M+ inserted in homologous sequence

Conversion

Point mutation or deletion after site-specific recombinase action

Insertion (5')

(c) Positive/negative screen with promoterless M+

ATG

p\text{\textsuperscript{Y}}

ATG

p\text{\textsuperscript{Y}}

ATG

p\text{\textsuperscript{Y}}

Current Opinion in Plant Biology
Current gene targeting strategies use either insertion or replacement vectors. (a) An insertion vector carries, as a targeting fragment, an internal part of ‘your most favourite gene’ (YMFG) that lacks the amino- and carboxyl-terminal coding sequences cloned next to the positive selectable marker. The generation of a DSB in the homologous sequence generates an ‘ends-in’ vector, a structure that favours gene conversion events. Three types of homologous interactions have been observed with insertion vectors. (i) Conversion events are characterised by reciprocal or non-reciprocal transfer of genetic information from the vector to the target gene without integration of vector sequences. Conversion events can only be identified if mutations in the gene can be directly selected. This is the case for genes encoding acetolactate synthase, hypoxanthine phosphoribosyl transferase or adenine phosphoribosyl transferase: mutations in their coding sequences confer resistance to chlorosulfuron, hypoxanthine or adenine analogues, respectively. (ii) Insertion events are characterised by the integration of one or several copies of the transforming DNA in YMFG, generating an insertional disruption (i.e. a knock-out [YMFG\textsuperscript{KO}]) and conferring resistance to the positive selectable marker (M\textsuperscript{+R}). (iii) ‘Hit and run’ represents a third class of homologous interaction events observed in plant and animal cells. HR between transforming and genomic DNA results in reciprocal or non-reciprocal transfer of genetic information observed during integrative transformation has not yet been identified in yeast. Obviously, the identification of these genetic determinants will be an important advance in biology as it would not only allow GT approaches to be broadly applied in functional genomic studies, but also enable accurate gene therapy and the evaluation of engineered genes in their own chromosomal environment (i.e. it will facilitate protein design).

Table 1

<table>
<thead>
<tr>
<th>Species*</th>
<th>GT efficiency per µg DNA</th>
<th>GT efficiency per living cell</th>
<th>Size of homology (kb)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Yeasts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>95</td>
<td>1–10</td>
<td>nd</td>
<td>0.1–0.5</td>
</tr>
<tr>
<td>Schizosaccharomyces pombe</td>
<td>10–90</td>
<td>1–10</td>
<td>nd</td>
<td>1–2</td>
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<tr>
<td><strong>Filamentous fungi</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Aspergillus nidulans</td>
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<td>1–10</td>
<td>nd</td>
<td>4–6</td>
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<tr>
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<td>nd</td>
<td>nd</td>
<td>2–9</td>
</tr>
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<td><strong>Protozoa</strong></td>
<td></td>
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<tr>
<td>Trypanosomatidae</td>
<td>95</td>
<td>100</td>
<td>10\textsuperscript{−4}</td>
<td>1–10</td>
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<tr>
<td>Dictyostelium discoideum</td>
<td>15</td>
<td>1</td>
<td>10\textsuperscript{−5}</td>
<td>5.3</td>
</tr>
<tr>
<td><strong>Animals</strong></td>
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<td></td>
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<td></td>
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<tr>
<td>Caenorhabditis elegans</td>
<td>No reports</td>
<td>No reports</td>
<td></td>
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</tr>
<tr>
<td>Zebra fish</td>
<td>nd\textsuperscript{†}</td>
<td>nd</td>
<td>nd</td>
<td>1</td>
</tr>
<tr>
<td>Drosophila melanogaster\textsuperscript{†}</td>
<td>nd\textsuperscript{†}</td>
<td>nd</td>
<td>nd</td>
<td>10\textsuperscript{−4}</td>
</tr>
<tr>
<td>Mouse ES cell</td>
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<td>nd</td>
<td>10\textsuperscript{−4}</td>
<td>8–13</td>
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<td>Chicken B cell lines</td>
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<td>0.2–1</td>
<td>nd</td>
<td>4</td>
</tr>
<tr>
<td>CHO cells</td>
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<td>0.05</td>
<td>5×10\textsuperscript{−6}</td>
<td>2–8</td>
</tr>
<tr>
<td>Mammalian cells \textsuperscript{‡}</td>
<td>0.1–40</td>
<td>0.01</td>
<td>10\textsuperscript{−6}</td>
<td></td>
</tr>
</tbody>
</table>

Species with GT efficiencies above 1% are in bold. \(\textsuperscript{†}\) Size of homologous sequence required to achieve GT at the mentioned efficiencies. \(\textsuperscript{‡}\) Ratio of GT events monitored in a population of integrative transformants (i.e. GT/GT+IR [%]). \(\textsuperscript{§}\)Targeted integration occurred in vivo after illegitimate integration of the targeting sequence and its subsequent excision by FLP recombinase and linearisation by I-SceI endonuclease. This linear substrate generated in vivo subsequently integrates in the target locus by homologous recombination. \(\textsuperscript{‡}\)These high GT/IR ratios were obtained by a positive/negative selection screen in which the promoterless coding sequence of the selectable marker is fused to the promoter of the target gene (see Figure 1c). This strategy is a very efficient method of counter-selecting illegitimate integration events but does not change the low yield of GT events. \(\textsuperscript{‡}\)nd, not determined.
Genetic transformation of Physcomitrella

Two classes of antibiotic-resistant colonies are observed following polyethylene-glycol-mediated direct transfer of bacterial plasmids carrying a plant antibiotic-resistance cassette into protoplasts [20]. The first class is characterised by resistant colonies displaying reduced growth rates on antibiotic-supplemented medium and a rapid loss of the resistance gene following a period of growth on non-antibiotic-supplemented medium and a rapid loss of the resistance gene. These 'replicative transformants' are recovered at RTF in the range of 10^-5 (circa one clone per 10–50 µg DNA) [20]. Considering the efficient replicative transformation frequencies observed in these experiments, these data suggest that integration of foreign DNA sequences at random locations of the genome by illegitimate recombination is possible but not very efficient.

GT is as efficient in Physcomitrella as in S. cerevisiae

Experiments designed to target artificial loci previously generated in the moss genome by illegitimate recombination provide the first evidence to suggest that homologous recombination is the predominant pathway of integration of DNA sequences in the genome of P. patens. Two plasmids that are homologous except for the antibiotic resistance gene (i.e. carrying a kanamycin or a hygromycin resistance gene, respectively) have been used independently to generate kanamycin- and hygromycin- mono-resistant transgenic strains. These strains have subsequently been retransformed with the other plasmid to generate kanamycin and hygromycin resistant transgenic strains. These experiments revealed that RTF are one order of magnitude higher in mono-resistant transgenic strains than those in the wild-type strain and that this increase is correlated with co-segregation of both antibiotic selectable markers in 87% of the tested plants ([21,22,24]; Table 2). Subsequent molecular analyses have confirmed integration of the second plasmid into each target artificial
locus by homologous recombination (DG Schaefer, S Vlach, unpublished data).

To rule out the possibility that such high GT efficiency could be associated with unusual features connected with artificial loci, subsequent GT experiments have been performed on three single-copy unsequenced moss genomic sequences [25]. RTF are again one order of magnitude higher with plasmids carrying moss genomic sequences as compared to those observed with plasmids lacking sequence homology to the moss genome. Southern blot analyses have confirmed targeted integration by HR between the transforming DNA and the genomic homologous sequences at every tested locus and in 66–100% of the plants analysed (Table 2). Consequently, it has been concluded that HR is the predominant pathway used by Physcomitrella to integrate foreign DNA sequences in the genome [25,26]. Subsequently, the optimal and minimal extents of sequence homology required to target genes in Physcomitrella have been assessed, as has the specificity of GT in this moss. The specificity of GT in Physcomitrella was determined in experiments designed to target a specific member of the highly conserved chlorophyll a/b binding protein multigene family (i.e. the cab ZLAB1 locus). Although sequence homology between 11 members of the cab multigene family is as high as 88-93% at the nucleotide level, successful targeting of the ZLAB1 locus has been achieved in 30% of the transgenic plants analysed (Table 2). Sequence analysis of the hybrid junctions between chromosomal and plasmid sequences has confirmed the fidelity of HR-mediated targeted integration at the base-pair level [27•].

The minimal and optimal extent of sequence homology required for GT was determined in experiments designed to target the adenine phosphoribosyl transferase gene of Physcomitrella (Ppapt). These experiments took advantage of the fact that for the ap! gene can be directly selected as it confers resistance to the adenine analogue 2,6-diaminopurine (DAP; DG Schaefer, M Laloue et al., unpublished data). Using cDNA-based replacement vectors, conversion of the ap! gene mediated by HR events occurring within stretches of continuous homology as short as 49 bp 

<table>
<thead>
<tr>
<th>Biological process</th>
<th>ESTs</th>
<th>Species*</th>
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<tbody>
<tr>
<td>Plant development</td>
<td>MADS box genes</td>
<td>P</td>
</tr>
<tr>
<td></td>
<td>Homeobox genes</td>
<td>P</td>
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<tr>
<td>Hormone-mediated responses</td>
<td>Auxin binding protein ABP1</td>
<td>C</td>
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<td></td>
<td>Auxin resistance protein AXR1</td>
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<td></td>
<td>ABA-responsive protein</td>
<td>P, C</td>
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<tr>
<td>Transduction pathways</td>
<td>Ser/threo phosphatase 2A, 2C</td>
<td>P, C</td>
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<td></td>
<td>G-proteins subunit</td>
<td>P</td>
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<td></td>
<td>GTPase rac1, rac4</td>
<td>P</td>
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<td></td>
<td>Protein kinase C, MAP, cAMP</td>
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<td>Plant–pathogen interactions</td>
<td>CF-9/4 haplotype resistance gene cluster</td>
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<td>Cellular ultrastructure</td>
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<td></td>
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<td>FtsZ1 and FtsZ2</td>
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<tr>
<td></td>
<td>Cellulose synthase</td>
<td>P, C</td>
</tr>
</tbody>
</table>

*The moss EST database (URL http:www.ncbi.nih.gov) contains mainly sequences from Physcomitrella patens (P) but also some sequences of another species from the Funariales family, Ceratodon purpureus (C). C. purpureus is currently used in studies of phototropic responses and of the mechanism underlying the establishment of cell polarity. ABA, abscisic acid; CF-4/9, Cladosporium fulvum-4/9; MAP, mitogen-activated protein.

Why is GT so efficient in Physcomitrella?

Obviously, achieving GT in eukaryotes is not a ‘just so story’ and two hypotheses have been proposed to account for the high efficiency of GT in P. patens. First, efficient GT may be correlated with the dominance of the gametophytic haplotype in the life cycle of Bryophytes [21,25]. This proposition is based on the observation that a ratio of targeted to random integration events above 1% seems to be restricted to primitive and/or haploid eukaryotes, such as protozoa, yeast, some green algae and filamentous fungi (Table 1). Yet such a ratio is not sustained in wild-type S. cerevisiae, which is naturally diploid. The proposition is also supported by the assumption that DSB in a haploid genome have to be repaired by HR in order to maintain the integrity of the genome. GT experiments performed on the gametophytic cells of vascular plants may validate this correlation.
The second hypothesis is based on the observation that *P. patens* chloronemal cells, from which protoplasts are isolated for transformation experiments, divide synchronously every 24 hours and are arrested for most of the day at the G1→M boundary [29,30]. Reski [30] proposed that efficient GT could be correlated with this cell cycle arrest but this assumption is not supported by controversial data from other biological systems. Experiments conducted in *S. cerevisiae* have not shown any correlation between GT efficiency and cell cycle stages, except that DSB repair by NHEJ induced by the site-specific HO endonuclease drops nearly 100-fold during G1 [16••], which would argue against Reski’s proposition. In contrast, in vertebrate cells, HR contributes to DSB repair predominantly during S→G2 interphase and NHEJ is the dominant pathway during G1/early S phase [31]. The possibility of a correlation between GT competency and cell cycle stages in plants can be tested experimentally in different synchronised plant cell lines that have been arrested at different stages of the cell cycle. Finally, functional analyses of moss homologues to the genes of the RAD-50/Ku-70 complex suggest that their absence would allow the investigation of homologous and illegitimate recombination processes in *P. patens*.

**Gene disruption in Physcomitrella generates predicted and unpredicted phenotypes that may be complemented by genes from other plant species**

Strepp and co-workers successfully disrupted the *ftsZ1* gene of *P. patens*, which encodes an ancestral tubulin that is thought to be involved in chloroplast division [29]. As predicted, the *ftsZ* knock-out phenotype is characterised by the presence of a single giant chloroplast per cell instead of the 50 chloroplasts that are usually found in wild-type cells. Girke and co-workers have generated Δ6 desaturase knock-out strains whose phenotype is a severe alteration of the normal *P. patens* fatty-acid pattern [32]. Even more interestingly, Girod et al. [33,34••] have shown that disruption of the multi-ubiquitin chain binding gene *mcbl* (encoding a protein of the 26S proteasome) leads to developmental arrest in *P. patens*. This results in the generation of strains that have abnormal filamentous caulonemata and are unable to progress through the developmental transition from uni-dimensional apical growth to three-dimensional caulinary growth. This phenotype cannot be observed in unicellular yeast in which Δmcbl strains display normal growth on rich medium but are more sensitive than the wildtype to amino-acid analogues. These findings emphasise the fact that loss-of-function mutations of genes involved in general biological processes may have more dramatic effects in multicellular eukaryotes than in yeast. Finally, *apt* knock-out strains of *P. patens* display, in addition to resistance to DAP, a developmental phenotype characterised by altered development of the leafy shoot (i.e. gad for gametophore altered development) (DG Schaefer, M Laloue et al., unpublished data). *Arabidopsis apt1* mutants are male sterile but do not otherwise display such a strong developmental phenotype [35], probably because *apt* genes form a small multigene family in *Arabidopsis* and because *apt* null mutants have not yet been obtained. Nevertheless, complementation of the moss developmental phenotype with the *Arabidopsis Aapt1* and *Aapt2* genes has been successful, providing evidence that angiosperm genes can effectively complement mutations in *P. patens* (DG Schaefer, M Laloue et al., unpublished data). Thus, the characterisation of loss-of-function mutations in *Physcomitrella* can provide additional information to that previously obtained in yeast and *Arabidopsis*.

**Physcomitrella, a simple plant model system with modern molecular genetic features**

It is now well recognised that bryophytes and angiosperms display the same basic biological processes [36,37,38•]. The initiation of a large-scale EST sequencing program in *P. patens* [39••] has so far provided almost 10,000 ESTs, representing the same number of directly accessible putative knockouts (Table 3). Angiosperms’ genes and EST sequences available in the database can be used either as heterologous probes or to design degenerate PCR primers in order to identify and isolate the moss homologues and immediately initiate fine functional studies of these genes by GT in *P. patens*. Even more promising, the successful development of tagged mutagenesis and gene-trap approaches in *P. patens* by shuttle mutagenesis [40••] allows the use of a molecular genetic methodology in plants that, up to now, has only been applied in yeast. The data reviewed here clearly support the idea that *Physcomitrella* advantageously complements *Arabidopsis* and rice as a model system for plant biological studies.

**Conclusions**

Efficient GT in *P. patens* finally provides the missing genetic tool required for fine functional genomic studies in plants. Research with *P. patens* is entering an expansion phase. This is the appropriate time to consider the opportunity to initiate a genome-sequencing program for *P. patens*, which has a genome size only twice that of *Arabidopsis* and similar to that of rice, that would provide the scientific community with information indispensable for future research. Considering the importance of efficient GT for functional genomics and the developmental complexity of *P. patens* compared to the few other eukaryotes in which this technology is possible, further progress may place this plant at the forefront of biological studies in multicellular eukaryotes.

**Acknowledgements**

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

• of special interest
•• of outstanding interest


The authors of this study describe progress towards the isolation of gain-of-function that complements of *Arabidopsis*. A collection of plants transformed with T-DNA carrying multimerised transcriptional enhancers from the cauliflower mosaic virus 35S promoter was produced. Dominant mutated phenotypes resulting either from constitutive ectopic or enhanced expression of a gene induced by the 35S enhancers are observed in circa 0.1% of the transformants. The subsequent identification of the mutated gene is facilitated by the T-DNA tag.


The first description of a large collection of T-DNA tagged insertional mutants in rice. The T-DNA used in this work also carries a promoterless β-glucuronidase (GUS) gene that functions as a gene trap, enabling detection of fusion products between GUS and the endogenous tagged gene.


A detailed and comprehensive review of the different strategies used for gene trapping in plants. The advantages and disadvantages of different types of vectors (i.e. T-DNA or transposon) and reporter genes (i.e. GUS or green fluorescent protein [GFP]) are highlighted.


This review summarises the current status of gene targeting and homologous recombination in plants. Special emphasis is given to the biochemical models of DSB repair and to progress using genetic approaches, which involve the isolation of radiation-sensitive mutants, aimed at identifying genes involved in recombination processes in plants.


An extensive review on homologous recombination and gene targeting in plants that complements [19]. This review also provides information on putative problems associated with *Agrobacterium*-mediated targeted transformation, extrachromosomal and intrachromosomal recombination in plants, site-specific mutations induced by the Cre/lox system from bacteriophage P1 and attempts to improve GT efficiency by overexpressing recombination enzymes such as RecA, a major recombination protein from *Escherichia coli*.


An extremely detailed and comprehensive review on DSB repair in *S. cerevisiae*. A number of topics are discussed including the mechanism of DSB repair by HR; proteins involved in HR and NHEJ; the stimulation of mitotic recombination in relation to DNA topology, structure and replication; meiotic recombination; the role of mismatch repair in recombination; and DSB repair check points. This review is a must for everybody who wishes to have a comprehensive view of recombination in eukaryotes.


Recent studies showing that the predominant pathway of DSB repair in plants involves NHEJ are reviewed. The authors also discuss the fact that error-prone DSB repair may be an important driving force in plant genome evolution.


The results reported here confirm those obtained in [21] suggesting that plasmids can be concatenated to form high molecular weight extrachromosomal structures that are replicated in moss cells. This finding provides the starting point to the development of moss artificial chromosomes and moss shuttle vectors. The discrepancies in transformation frequencies described in this report and in [21] are probably caused by differences in the transformation protocols.


The accuracy of GT in *Physcomitrella* is demonstrated on a specific member of a highly conserved multigene family, and HR events are documented at the sequence level.


32. Takata M, Sasaki MS, Sonoda E, Morrison C, Hashimoto M, Utsumi H, Yamaguchitai Y, Shinohara A, Takeda S: Homologous recombination and non-homologous end-joining pathways of DNA double-strand break repair have overlapping roles in the


This work demonstrates that disruption of a subunit of the 26S proteasome leads to developmental arrest in P. patens; an observation that was not feasible in yeast. Partial complementation of the developmental-arrest phenotype by plant hormones is reported, suggesting that the ubiquitin/26S proteasome proteolytic pathway is triggered by hormones.


Several aspects of molecular genetics in Physcomitrella patens are discussed. Data on nuclear, plastidian and mitochondrial genomes and on the codon usage of this moss are included.

39. Quatrano R, Bashiardes S, Cove D, Cuming A, Knight C, Clifton S: Mutated phenotypes are recovered in 4% of the transformants, gene (for gene trapping). The mutagenised library is used to transform Physcomitrella patens by targeted integration. The minitransposons used for shuttle mutagenesis in mutagenised moss genomic library that was designed to tag or trap genes by targeted disruption of the 9th Conference on Arabidopsis Research, 1998 June 24–28, Madison, Wisconsin.


Shuttle mutagenesis was applied for the first time in plants to generate a mutagenised moss genomic library that was designed to tag or trap genes by targeted integration. The minitransposons used for shuttle mutagenesis in E. coli carry either a selectable neo cassette (for tagged mutagenesis) or a GUS gene (for gene trapping). The mutagenised library is used to transform P. patens. Preliminary data indicate that single-locus-targeted insertions are predominant. Mutated phenotypes are recovered in 4% of the transformants, whereas 3% of the transformants exhibit cell-specific or constitutive GUS expression.


The first report of successful gene targeting in Drosophila. This success was obtained with a sophisticated experimental design that combines the use of site-specific recombinase and site-specific endonuclease. The targeting sequence is initially introduced at ectopic locations by standard transformation via P elements. It is subsequently excised in vivo as a circular molecule by the FLP recombinase (from the FLP/frt site-specific recombination system from S. cerevisiae) and linearised within targeting homologous sequences by the I-SceI site-specific endonuclease from S. cerevisiae. This linear substrate finally reintegrates at the targeted location by HR. This approach might be applied to other species and may be recollected to GT but the general validity of such a strategy is still a matter for discussion (see [60]).


The authors of this article discuss the potential of positive/negative screens using a promoterless positive selectable marker fused to promoter sequences of the target gene. Selection against illegitimate integration is efficient with such vectors. Yet, the generation of a specific knock-out with such a screen in Arabidopsis, for example, would require an experiment of the size of that performed to generate a large collection of T-DNA tagged mutants.


