The Plant Journal (1997) **11**(6), 1195–1206

Efficient gene targeting in the moss *Physcomitrella patens*

Didier G. Schaefer* and Jean-Pierre Zrÿd
Laboratoire de Phytogénétique Cellulaire, Université de Lausanne, Bâtiment de Biologie, CH-1015 Lausanne-Dorigny, Switzerland

Summary

The moss *Physcomitrella patens* is used as a genetic model system to study plant development, taking advantage of the fact that the haploid gametophyte dominates in its life cycle. Transformation experiments designed to target three single-copy genomic loci were performed to determine the efficiency of gene targeting in this plant. Mean transformation rates were 10-fold higher with the targeting vectors and molecular evidence for the integration of exogenous DNA into each targeted locus by homologous recombination is provided. The efficiency of gene targeting determined in these experiments is above 90%, which is in the range of that observed in yeast and several orders of magnitude higher than previous reports of gene targeting in plants. Thus, gene knock-out and allele replacement approaches are directly accessible to study plant development in the moss *Physcomitrella patens*. Moreover, efficient gene targeting has so far only been observed in lower eukaryotes such as protozoa, yeasts and filamentous fungi, and, as shown here the first example from the plant kingdom is a haplobiontic moss. This suggests a possible correlation between efficient gene targeting and haplophase in eukaryotes.

Introduction

In most eukaryotes, genetic transformation is achieved following the integration of foreign DNA sequences by illegitimate recombination at random locations on the genome. However, the presence of isogenic genomic sequences on the introduced DNA can target it to its own chromosomal locus and facilitate integration by homologous recombination, a process known as gene targeting. Initially observed in *Saccharomyces cerevisiae* (Hinnen *et al.*, 1978), this event is a prerequisite to application of the most sophisticated tools of reverse genetics, i.e. gene disruption and allele replacement (Berg, 1991). With such approaches, virtually any cloned gene, even of unknown function, can be specifically mutagenized *in vitro* and re-introduced to its own chromosomal location in order to study its function (Capecchi, 1989b; Struhl, 1983).

The successful application of allele replacement is dependent on the ratio of homologous to illegitimate recombination events during integrative transformation, and this ratio is extremely variable among different eukaryotes. So far, a targeting efficiency above 10% has only been observed in lower eukaryotes such as yeasts (Grimm and Kohli, 1988; Struhl, 1983), some filamentous fungi (Fotheringham and Holloman, 1989; Kronstad *et al.*, 1989; Paietta and Marzluf, 1985; Shiotani and Tsuge, 1995; Timberlake and Marshall, 1989), *Dictyostelium discoideum* (De Lozanne and Spudich, 1987), Trypanosomatideae (Cruz and Beverley, 1990; ten Asbroek *et al.*, 1990), and in a chicken lymphocyte B cell line (Buerstedde and Takeda, 1991). In most other eukaryotes tested, the ratio of targeted to random integration events falls to very low levels, ranging from 10^{-2} to 10^{-5} in mammalian cells (Bollag *et al.*, 1989) and from 10^{-3} to 10^{-6} in plant protoplasts (Ohl *et al.*, 1994). Thus, although allele replacement is a well-established routine in *S. cerevisiae*, where exogenous DNA integrates into the genome almost exclusively by homologous recombination (Rothstein, 1991), it is not directly accessible in animals and plants, since the gene-targeted mutant has to be identified among the large number of transgenics generated by illegitimate recombination. This problem has been circumvented in animal systems with the use of mouse embryonic stem cells (ES cells) which display a higher gene targeting efficiency than other mammalian cells (10^{-1} to 10^{-3}) (Thomas *et al.*, 1986) and which can be pre-screened in culture for gene targeting events prior to their re-introduction into mouse embryos (Capecchi, 1989a; Joyner, 1991). However, although several reports have clearly demonstrated the feasibility of gene targeting in plants following both *Agrobacterium*- and polyethyleneglycol-mediated (PEG) gene transfer (Halfter *et al.*, 1992; Lee *et al.*, 1990; Miao and Lam, 1995; Offringa *et al.*, 1990; Paszkowski *et al.*, 1988), the ratio of targeted to illegitimate integration has so far been too low to allow the development of allele replacement approaches (Hrouda and Paszkowski, 1994; Lichtenstein and Barrena, 1993; Puchta *et al.*, 1994).

The potential use of bryophytes as a model genetic system to study plant development was already recognized in the 1930s (von Wettstein, 1932), and is associated with (a) the facilitated genetic analyses resulting from the predominance of the haplophase in the life cycle (Cove, 1983), (b) the similar responses of mosses and higher plants to environmental and growth factors (Bopp and Werner, 1993; Cove and Ashton, 1984; Hartmann and Jenkins, 1984; Knight *et al.*, 1995), and (c) the simple

Received 25 November 1996; revised 27 January 1997; accepted 4 February 1997.
*For correspondence (fax +41 21 692 42 55; e-mail Didier.Schaefer@lpc.unil.ch).

morphology of the plant, allowing cellular and developmental processes to be followed at the level of individual cells (Wang and Cove, 1989). Since the first report of successful isolation of biochemical and developmental mutants in the moss *Physcomitrella patens* (Engel, 1968), this plant has been used as a model genetic system for physiological and developmental studies (Cove, 1992; Cove and Knight, 1993). We have previously shown that genetic transformation of *P. patens* following PEG-mediated direct DNA transfer into protoplasts is feasible (Schaefer *et al.*, 1991). This transformation by plasmids that do not contain sequences homologous to the moss genome is accomplished by illegitimate integration of several direct repeats of the plasmid at independent single genomic loci (Schaefer, 1994). We have also presented phenotypic and genetic data strongly suggesting that the integration of exogenous DNA sharing sequence homology with moss artificial loci occurs preferentially by homologous recombination (Schaefer, 1994; Schaefer *et al.*, 1994), and similar results have been obtained independently (Kammerer and Cove, 1996). We report here experiments designed to target three independent single-copy genomic loci of the moss *P. patens*. Our results provide phenotypic, genetic and molecular evidence demonstrating for the first time in a plant that exogenous DNA sequences integrate in the genome predominantly at targeted locations by homologous recombination. The efficiency of gene targeting detected in these experiments is above 90%, which corresponds to the efficiency observed in lower eukaryotes such as yeasts. The development of gene knock-out and allele replacement approaches to study plant biology in the haplobiontic moss *Physcomitrella patens* is therefore feasible.

Results

Experimental strategy

Since the natural variability of different genetic regions affects targeting efficiency, we have performed targeting experiments at three independent single-copy genomic loci to assess the efficiency of gene targeting in *P. patens*. Therefore, three fragments of moss DNA corresponding to single-copy genomic sequences were isolated from independent lambda genomic clones (λ108, λ420, shown in Figure 1; λ213, not shown; D. Schaefer, unpublished data), and these fragments were subcloned into plasmid pGL2 (35S–hygro^r) (Bilang *et al.*, 1991) or pHP23b (35S–neo^r) (Paszkowski *et al.*, 1988) to give rise to the targeting plasmids pGL-108 (Figure 1), pGL-420 (Figure 1) and pHP-213 (not shown). Sequence homology between these constructs and the moss genome extends over 3.6 kb in pGL-108, 2.7 kb in pGL-420 and 2.3 kb in pHP-213. We have transformed wild-type protoplasts with these constructs, and have selected and analysed stable hygromycin- or kanamycin-resistant transgenic plants (further designated as integrative transformants, see Schaefer *et al.*, 1994) to assess the efficiency of gene targeting to single-copy genomic loci.

Transformation frequencies

In wild-type *P. patens*, illegitimate integration of the non-homologous supercoiled plasmids pHP23b and pGL2 in the genome occurs at a mean relative transformation frequency (RTF) of 1 in 10^5 surviving plants (Table 1), corresponding to 0.05 integrative transformants per μg DNA (Schaefer, 1994). The RTF of the wild-type with the targeting constructs ranges from 5 to 22 integrative transformants in 10^5 surviving plants (Table 1), corresponding to 0.3 to 2 transformants per μg DNA, and the highest transformation frequency achieved with plasmid pGL–108 is in the same range as insertion frequencies in *S. cerevisiae* (Struhl, 1983). Higher transformation rates with exogenous DNA sharing sequence homology with chromosomal DNA suggest efficient integration by homologous recombination, since efficient gene targeting is associated with higher transformation rates in yeasts (Grimm and Kohli, 1988; Hinnen *et al.*, 1978). Additionally, the mean transformation frequencies observed in these experiments are approximately 1 order of magnitude higher with homologous vectors, providing an estimation of the efficiency of gene targeting in *P. patens* in the range of 90%.

Phenotypic and genetic analyses

Phenotypic and genetic criteria demonstrating integrative transformation in plants include mitotic stability, meiotic stability and Mendelian segregation of the new character (Potrykus, 1991). Unrestricted growth and differentiation on selective medium, transmission of the antibiotic resistance to the leafy gametophores, and maintenance of the resistance after non-selective growth, which indicate mitotic stability of the new character in integrative transformants of *P. patens* (Knight, 1994; Schaefer *et al.*, 1991, 1994), have been detected in more than 90% of the 130 transformants described here.

The progeny resulting from self-fertilization (designated selfed progeny) of 10 plants obtained with the targeting constructs (three plants obtained with pGL-108, three with pGL-420 and four with pGL-213) were analysed and 100% transmission of the antibiotic resistance was observed in each case (data not shown). The same plants were crossed with the strain *nicB5ylo6* (Ashton and Cove, 1977), and the segregation of the independent yellow marker of *nicB5ylo6* and of the antibiotic resistance markers was analysed. The yellow marker segregates in a 1:1 ratio and independently of the antibiotic markers in the 10 F_1 progeny tested (data

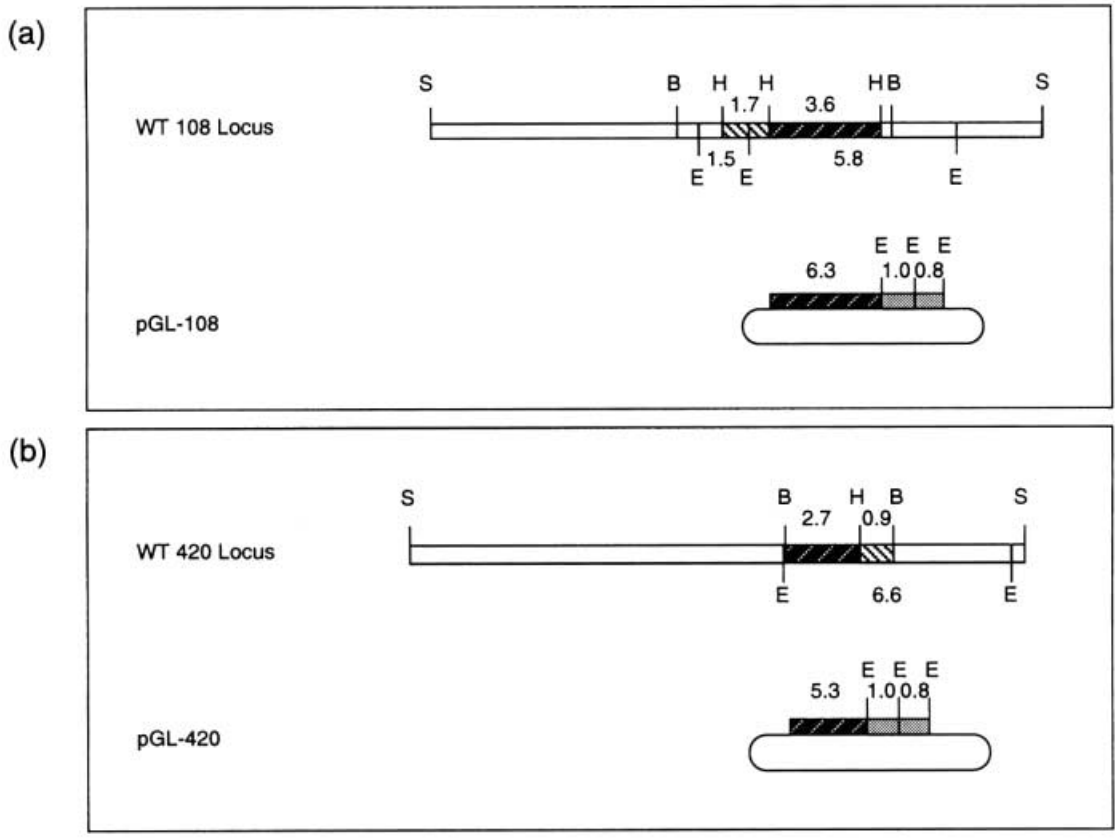


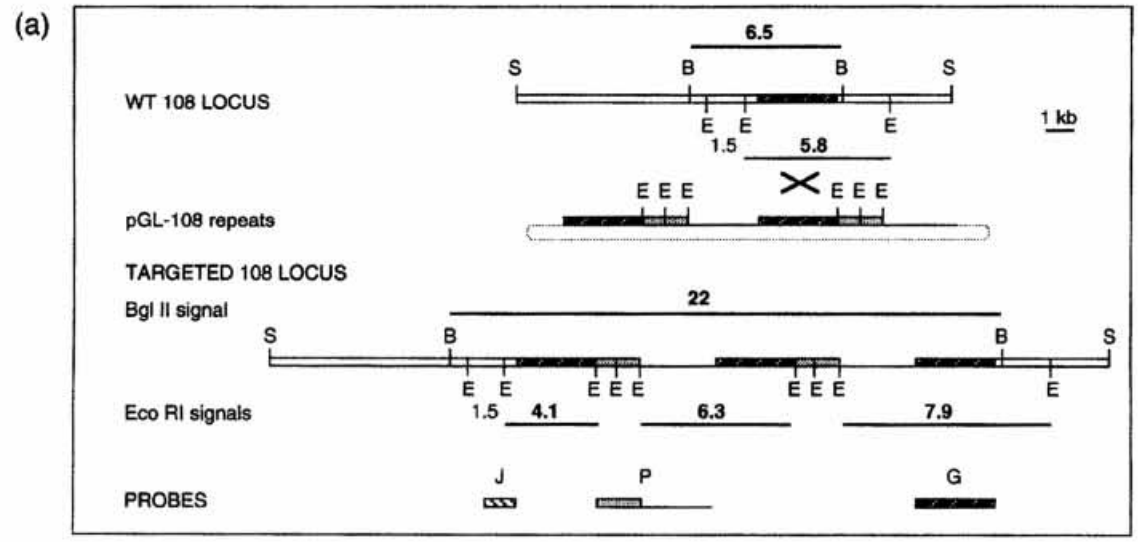
Figure 1. Partial restriction maps of the wild-type genomic locus λ108 (a) and λ420 (b) and of the transforming plasmids pGL–108 (a) and pGL–420 (b). The white boxes represent the moss genomic loci, the black hatched boxes the targeting fragments cloned in pGL2 (*Hin*dIII 3.6 kb for locus 108 and *Hin*dIII-*Bgl*I 2.7 kb for locus 420, probes G) and the white hatched boxes the flanking genomic fragments used as junction-specific probes (*Hin*dIII 1.7 kb for locus 108 and *Hin*dIII-*Bgl*I 0.9 kb for locus 420, probes J). The grey boxes represent the 35S–aphIV cassette coding for hygromycin-resistance and the thin lines the pUC sequences of pGL2 (probe P). The size of the fragments generated upon *Eco*RI digestion of the wild-type loci and of the transforming plasmids are given in kb. Restriction sites are for *Sal* I (S), *Bgl*I (B), *Hin*dIII (H) and *Eco*RI (E).

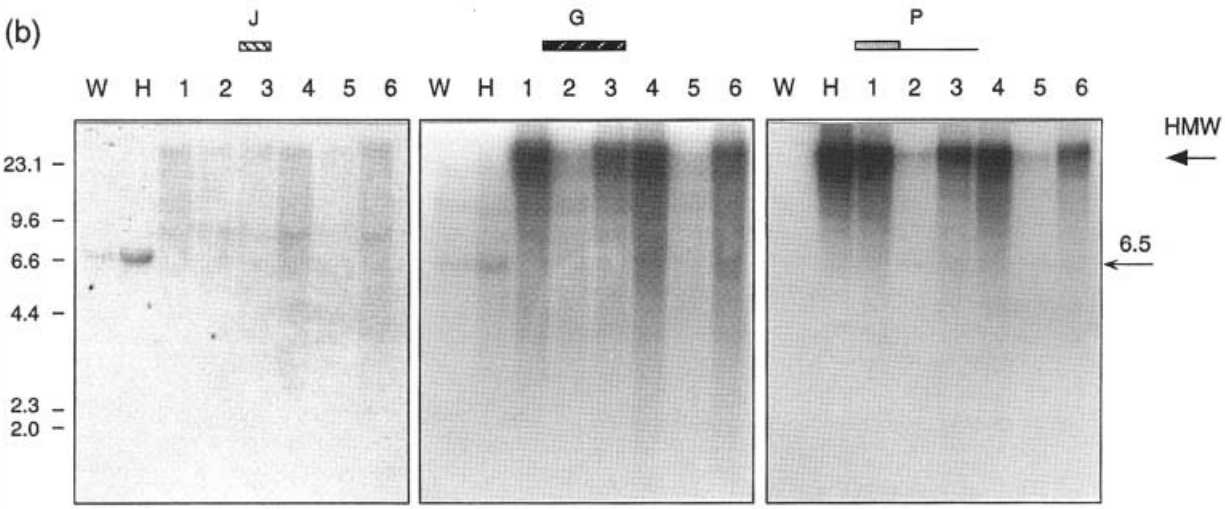
Table 1. Integration of vectors containing *P. patens* single-copy genomic sequences at higher rates than their non-homologous equivalent

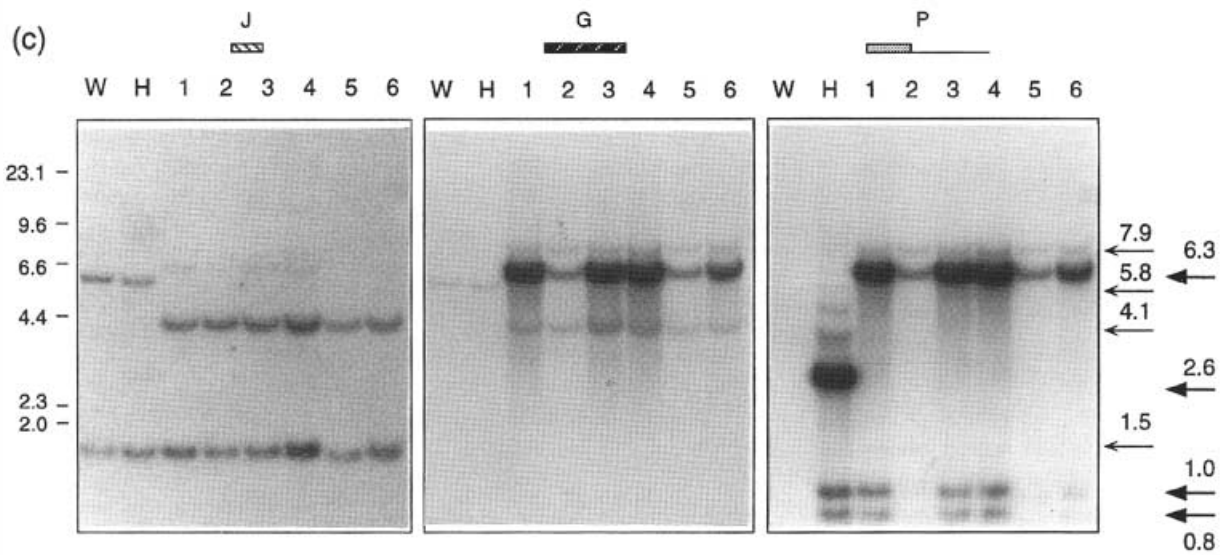
Plasmid (size in kb)[a]	Number of experiments[b]	Regenerating plants ($\times 10^5$)	Transgenic plants	RTF per 10^5 plants[c]	RTF per 10^{12} molecules[d]
pHP23b (4.4 kb)	5	4.2	2	0.4 ± 0.5	0.13 ± 0.16
pHP-213 (6.7 kb)	7	5.3	24	4.9 ± 2.3	1.78 ± 0.78
pGL2 (4.5 kb)	4	2.8	10	2.8 ± 2.3	0.79 ± 0.73
pGL-108 (8.1 kb)	3	2.7	59	21.5 ± 2.0	11.57 ± 5.70
pGL-420 (7.2 kb)	5	4.0	32	8.2 ± 1.4	3.14 ± 0.91

[a] Plasmids pHP23b and pGL2 do not share any homologous sequences with the *P. patens* genome. The targeting plasmids pHP-213, pGL-108 and pGL-420 each contain a single-copy moss genomic sequence.
[b] Each experiment was performed with 15 μg of supercoiled plasmid and the data summarized were obtained with three independent batches of protoplasts.
[c,d] Relative transformation frequencies (RTF) are expressed as c the number of transgenic plants obtained in 10^5 regenerating plants, and as d the number of transgenic plants obtained per 10^{12} plasmid molecules to account for the different sizes of the plasmids (both as mean value ± standard deviation).

not shown). A 1:1 segregation of the antibiotic resistance in the F_1 progeny was scored in nine plants, whereas the F_1 offspring of the last plant (strain pHP-213 #7) were 100% sensitive to the antibiotic (data not shown). These data provide genetic evidence for meiotic stability of the antibiotic resistance and for single-locus insertion of the targeting plasmids in 9/10 plants. In the case of strain HP-213 #7, we postulate, as observed in *S. cerevisiae* (Struhl, 1983), that the loss of resistance in this F_1-offspring results from meiotic recombination events leading to the deletion


(a)
WT 108 LOCUS
6.5
S
B
B
S
E
E
E
1.5
5.8
1 kb
pGL-108 repeats
E E E
E E E
TARGETED 108 LOCUS
Bgl II signal
22
S
B
B
S
E
E
E E E
E E E
E
Eco RI signals
1.5
4.1
6.3
7.9
PROBES
J
P
G


(b)
J
G
P
W H 1 2 3 4 5 6 W H 1 2 3 4 5 6 W H 1 2 3 4 5 6
23.1
9.6
6.6
4.4
2.3
2.0
HMW
6.5


(c)
J
G
P
W H 1 2 3 4 5 6 W H 1 2 3 4 5 6 W H 1 2 3 4 5 6
23.1
9.6
6.6
4.4
2.3
2.0
7.9
6.3
5.8
4.1
2.6
1.5
1.0
0.8

of plasmid sequences and to the restoration of the wild-type locus in both alleles.

Molecular analysis

To demonstrate insertion of the targeting plasmid by homologous recombination, disruption of the wild-type targeted sequence associated with the generation of two new hybrid junctions of predicted size and hybridization specificity was assessed by Southern blot analysis. The predicted structure of targeted locus 108 is shown in Figure 2(a), and the analysis of DNA extracted from six randomly chosen independent transformants obtained with plasmid pGL-108 and digested by *Bgl*II or *Eco*RI in Figure 2(b) and (c), respectively. Insertion of several direct repeats of pGL-108 in the 108 wild-type locus by a single reciprocal cross-over is fully supported in the six plants analysed. In *Bgl*II-digested DNA (Figure 2b), the wild-type 6.5 kb band is detected in the wild-type and the hygromycin-resistant plants, but is replaced by a high-molecular-weight signal of single-copy intensity when the *Hin*dIII 1.7 kb junction-specific probe is used (detected after over-exposure of the membrane, data not shown), and of multi-copy intensity upon hybridization with the 3.6 kb targeting fragment or with the plasmid probe. In *Eco*RI digested DNAs (Figure 2c), use of the genomic probe (G) reveals that the wild-type 5.8 kb band is present in the two control plants, and is replaced by two new fragments of 7.9 and 4.1 kb as predicted. The 4.1 kb junction is detected by the junction probe, as well as a flanking 1.5 kb band which is present in all plants, showing that the sequences flanking the target site have not been altered. Both junctions are detected by the targeting fragment, demonstrating that two copies of this fragment now flank the integration site, whereas the plasmid probe only detects the 7.9 kb junction. These results are totally consistent with the predictions and demonstrate integration of pGL-108 into the 108 locus by a single reciprocal cross-over in the six plants analysed. Furthermore, the use of the plasmid probe (P) reveals the presence of the 6.3, 1.0 and 0.8 kb bands of multi-copy intensity, demonstrating that several direct repeats of pGL-108 have been integrated in locus 108. Comparison of the intensity of the signals with determined amount of the probes (genomic reconstruction not shown) indicates that the number of integrated repeats varies in independent transformants, ranging from five in plants 2 and 5, to 10 in plant 6, and up to 20 in plants 1, 3 and 4.

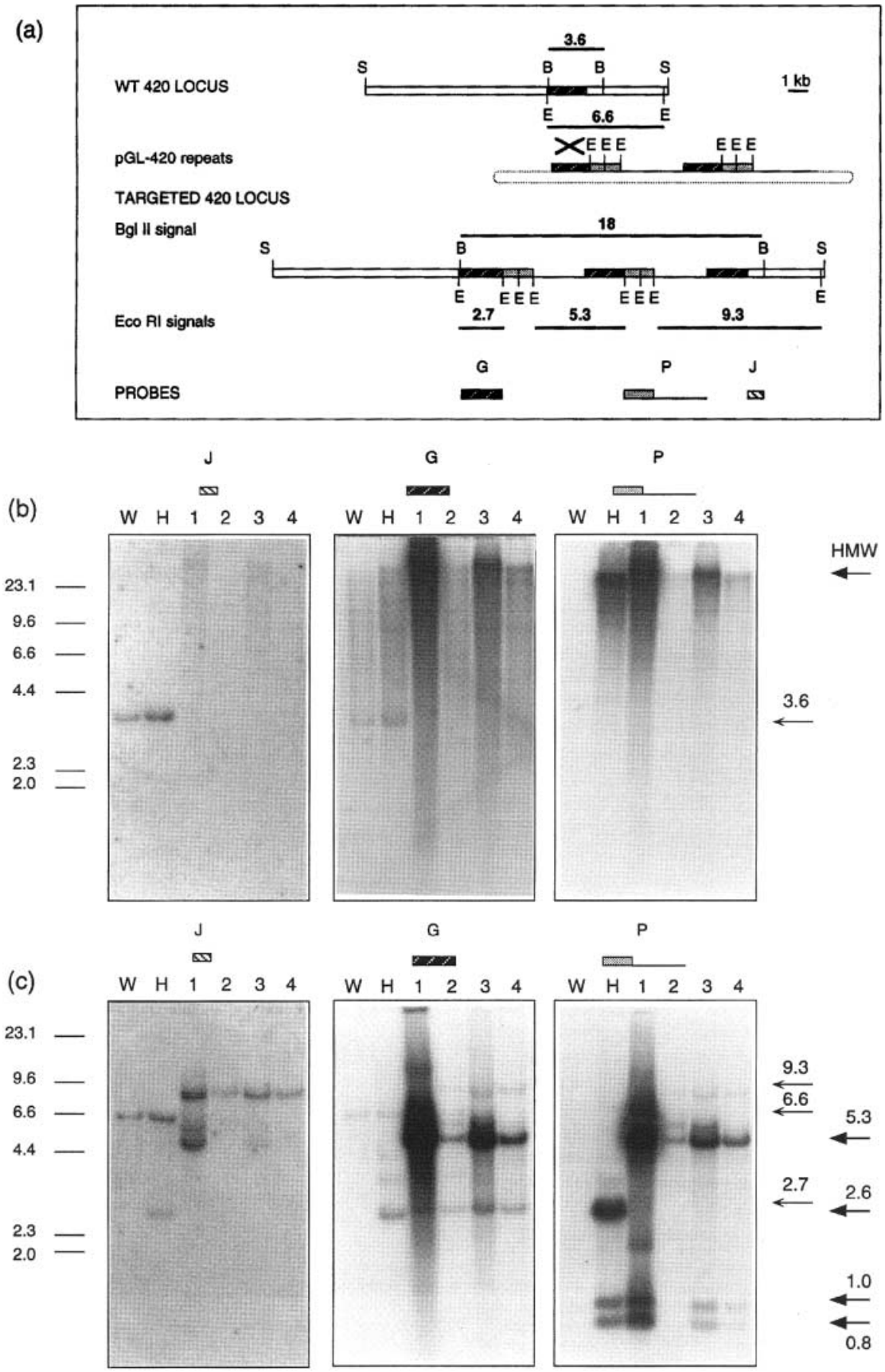
A similar analysis was performed on DNA extracted from four independent transformants obtained with pGL-420 (Figure 3). Integration of several direct repeats of plasmid pGL-420 in the 420 wild-type locus by a single reciprocal cross-over is again fully supported in the four plants analysed, by (a) the replacement of the *Bgl*II 3.6 kb wild-type band by a high-molecular-weight signal (Figure 3b), (b) the disruption of the wild-type *Eco*RI 6.6 kb band associated with the generation of two predicted new junctions of 2.7 and 9.3 kb displaying the expected hybridization specificity (Figure 3c), and (c) the presence of the 5.3, 1.0 and 0.8 kb bands generated by pGL–420 direct repeats (Figure 3c). The weak signal at 2.7 kb detected in lane H is most likely due to hybridization of plasmid repeats with a small amount of pUC sequences present in the purified junction probe. This is further supported by the presence of a 5.3 kb band in the DNA from the high-copy-number plant 1. This analysis also shows the presence of a minor band at 6.3 kb in plants 2 and 3, which is possibly generated by loss or methylation of one of the *Eco*RI sites located in the 35S hygromycin expression cassette in the integrated concatenate. The second minor band detected in DNA from plant 2, although displaying an electrophoretic mobility similar to the wild-type band, is also generated by partial digestion of the integrated repeats since the disruption of the wild-type locus is clearly demonstrated upon hybridization with the junction specific probe. Finally, genomic reconstruction indicates that between 5 and 50 direct repeats of pGL-420 are integrated at locus 420 in independent transformants (data not shown).

Similar results were obtained for four out of six plants obtained with plasmid pHP-213 (data not shown). One plant displayed phenotypic signs of mitotic instability associated with very high copy number of the transforming plasmid, and was thus not analysed further. Integration of the plasmid by illegitimate recombination was recognized in the second one (data not shown). Therefore, these results demonstrate that several direct repeats of the targeting plasmids have inserted at their specific targets by a single

Figure 2. Southern blot analysis of genomic DNA extracted from plants obtained with plamid pGL-108

(a) Predicted structure of targeted locus 108 following insertion by homologous recombination (single cross-over) of two direct repeats of plasmid pGL-108. Symbols are as described in Figure 1 and the dotted line represents the circular status of the transforming plasmid. Restriction sites are for *Sal*I (S), *Bgl*II (B) and *Eco*RI (E). The wild-type and recombinant *Eco*RI and *Bgl*II fragments are represented with thick lines and their molecular weight is given in kb. The three probes used in this analysis are also represented (J; junction probe, G; genomic probe, P; plasmid probe, described in Figure 1)

(b, c) Southern blot analysis of DNA extracted from plants obtained with pGL-108. DNA (2.5 μg) extracted from the wild-type strain (W), from a hygromycin-resistant transgenic plant obtained with pGL2 (H) and from six independent transgenic plants obtained with plasmid pGL-108 (1–6) were digested with *Bgl*II (b) or *Eco*RI (c) and hybridized with a 1.7 kb *Hin*dIII fragment flanking the target sequence (probe J), with the 3.6 kb *Hin*dIII 108 targeting fragment (probe G), or with plasmid pGL2 sequences (probe P). The thin arrows indicate the wild-type band and the new hybrid junctions, the thick arrows the bands generated by direct repeats of the plasmid (sizes in kb).


(a)
WT 420 LOCUS
3.6
S
B
B
S
1 kb
E
6.6
E
pGL-420 repeats
E E E
E E E
TARGETED 420 LOCUS
Bgl II signal
18
S
B
B
S
E
E E E
E E E
E
Eco RI signals
2.7
5.3
9.3
G
P
J
PROBES
(b)
J
G
P
W H 1 2 3 4
W H 1 2 3 4
W H 1 2 3 4
HMW
23.1
9.6
6.6
4.4
2.3
2.0
3.6
(c)
J
G
P
W H 1 2 3 4
W H 1 2 3 4
W H 1 2 3 4
23.1
9.6
6.6
4.4
2.3
2.0
9.3
6.6
5.3
2.7
2.6
1.0
0.8

reciprocal cross-over in all three loci examined and in the majority of plants analysed. The efficiency of gene targeting determined from these analyses is 100% of the tested target loci (3/3), and 93% of the tested plants (14/15). This estimation is consistent with the 10-fold increase in transformation rates observed with the targeting vectors.

Molecular analysis of the progeny

Southern blot analysis of DNA extracted from selfed, hygromycin-resistant and hygromycin-sensitive F_1 siblings of plants 108#3 and 108#5 was performed to correlate genetic and molecular data in the analysis of the progeny. Transmission of the new locus to selfed siblings, and restoration of the wild-type 5.8 kb *Eco*RI band in hygromycin-sensitive F_1 siblings is shown for both plants tested (Figure 4a and b). The new locus is also conserved in hygromycin-resistant F_1 siblings of the low-copy-number plant 108#5. However, we observed recombination events leading to a reduction in the number of integrated copies, to the loss of the new 7.9 kb junction fragment and to the restoration of the wild-type 5.8 kb band in the DNA from hygromycin-resistant F_1 siblings derived from the high-copy-number plant 108#3. Size difference between the artificial locus (150 kb, corresponding to 20 copies of pGL-108) and its wild-type allele (3.6 kb) may be responsible for these meiotic recombination events. In this case, we postulate that duplication, deletion by intramolecular homologous recombination and translocation might be involved in such recombination. Duplication is required to restore the wild-type locus and maintain the new 4.1 kb junction, deletion by intramolecular homologous recombination can account for the loss of plasmid direct repeats in the concatenate and translocation for the integration of the remaining repeats in another genomic location. However, these data establish a tight correlation between genetic and molecular analyses of the progeny of plants targeted at single-copy natural loci. Meiotic recombination events, leading to loss and/or rearrangements of integrated direct repeats have been observed in yeasts (Struhl, 1983). A similar mechanism may account for the meiotic recombination events observed in these analyses.

Discussion

Targeting frequencies

We have performed experiments to assess the efficiency of gene targeting in the moss *P. patens*, and our data provide phenotypic, genetic and molecular evidence demonstrating successful and efficient gene targeting of the three single-copy natural loci tested. Transformation frequencies in targeting conditions ranged from 0.3 to 2 integrative transformants per μg DNA, with a mean value at least one order of magnitude higher than the frequencies of illegitimate integration (0.05 transformants per μg DNA). The highest transformation frequencies achieved in these experiments were in the range of 10^{-3}, or about two clones per μg DNA. Such frequency corresponds to the rate of integrative transformation by homologous recombination in *S. cerevisiae* (Struhl, 1983), and to high frequencies of illegitimate integration following PEG-mediated DNA uptake in higher plant protoplasts (Negrutiu *et al.*, 1990).

The extent of sequence homology between the transforming DNA and the target has been shown to be essential for efficient gene targeting. Several hundred base pairs appear to be sufficient for gene targeting in *S. cerevisiae* (Rothstein, 1991), whereas several thousand base pairs are required to achieve gene targeting in mouse ES cells (Deng and Capecchi, 1992). In the experiments described here, sequence homology between the introduced plasmid and the target loci ranges from 2.3 to 3.6 kb, and the data indicate that such length is sufficient to promote efficient targeting to natural single-copy loci.

In yeast and ES cells, there is a correlation between targeting frequencies and the extent of sequence homology. In the experiments reported here, transformation frequencies may correlate with the extent of sequence homology present in the targeting vectors (Table 1). However, we consider that the different transformation frequencies observed with the targeting vectors instead reflect the natural variability of genomic regions, as already observed in *S. cerevisiae* (Rothstein, 1991), and further experiments are required to establish the relationship between the extent of sequence homology and targeting frequencies in *P. patens*. Interestingly, the difference observed between plasmid pGL-108 and pGL-420 may correlate with transcrip-

Figure 3. Southern Blot analysis of genomic DNA extracted from plants obtained with plamid pGL-420
(a) Predicted structure of targeted locus 420 following insertion by homologous recombination of two direct repeats of plasmid pGL-420. Organization of the Figure, symbols and legends are as described in Figures 1 and 2.
(b, c) Southern blot analysis of DNA extracted from plants obtained with pGL-420. Organization of the figure, symbols and legends are as described in Figure 2. DNA (2.5 μg) extracted from the wild-type strain (W), from a hygromycin-resistant transgenic plant obtained with pGL2 (H) and from four independent transgenic plants obtained with plasmid pGL-420 (1–4) was analysed. The 0.9 kb *Hin*dIII–*Bgl*I fragment was used as junction probe J, and the 2.7 kb *Hin*dIII fragment of λ420 was used as genomic probe G (see Figure 1).

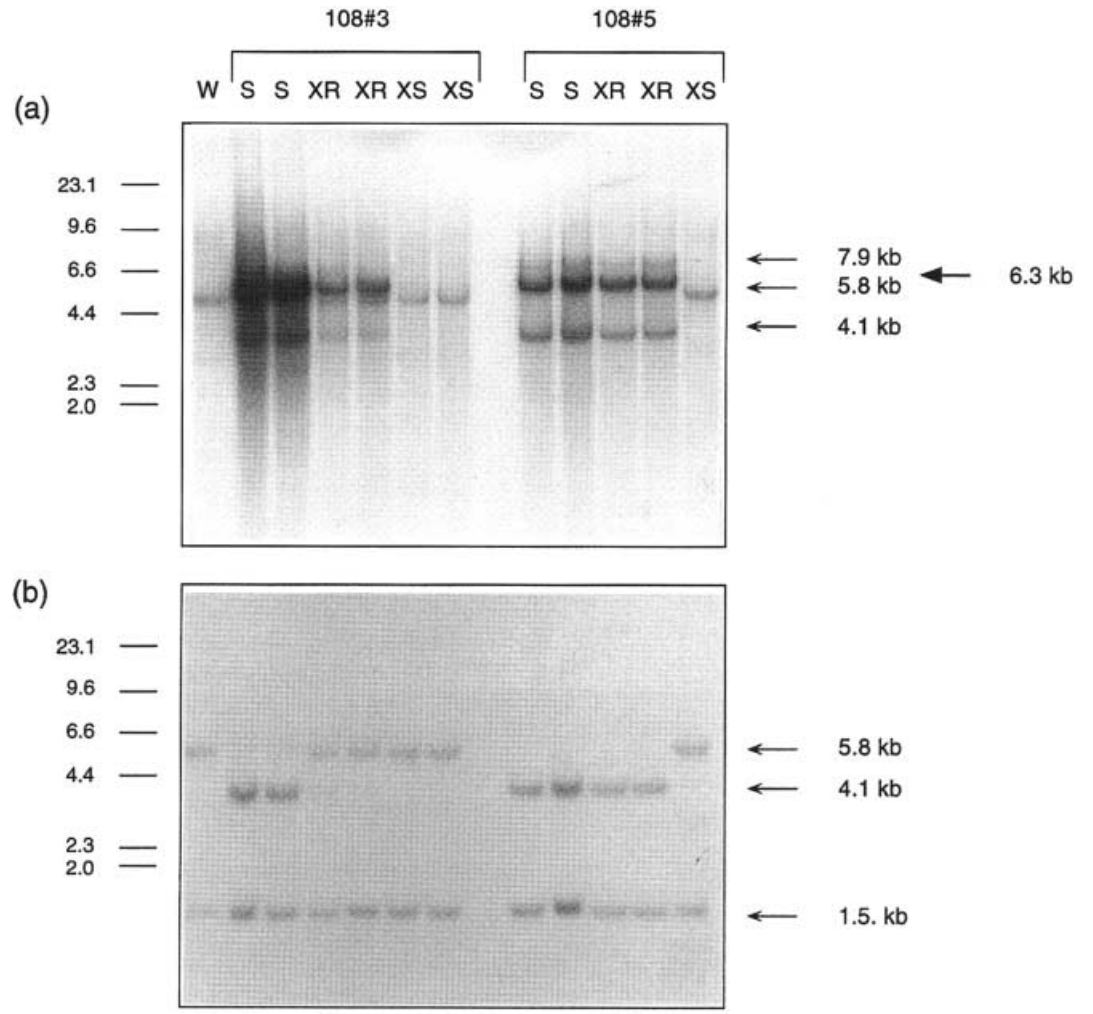


Figure 4. Southern blot analysis of genomic DNA extracted from the progeny of plants obtained with plamid pGL-108.
DNA (2.5 μg) from wild-type (W) and from selfed (S), hygromycin-resistant (XR) and hygromycin-sensitive (XS) F_1 siblings obtained from crosses of plants 108#3 and 108#5 with strain *nicB5ylo6* (Ashton and Cove, 1977) was digested with *Eco*RI and hybridized with the 3.6 kb *Hin*dIII 108 genomic probe G (a) or with the 1.7 kb *Hin*dIII junction probe J (b). Symbols are as described in Figure 2.

tional activity, since a transcript is detected with the 3.6 kb 108 genomic fragment, but not with the 2.7 kb 420 fragment upon Northern hybridization of poly(A)$^+$ mRNA extracted from 10-day-old protonemal tissue (data not shown).

In yeast and mouse ES cells, gene targeting frequencies have been shown to be increased by the presence of double-strand breaks in the region of homology (Capecchi, 1989a; Orr-Weaver *et al.*, 1981). The experiments reported here were performed with supercoiled DNA. However, we have observed that transformation frequencies with pHP-213 were approximately five times higher following linearization in the region of homology, without altering the ratio of targeted to random integration events (data not shown). This suggests that a similar mechanism exists in *P. patens*, and work is in progress to address this question further.

Gene targeting efficiency

Two independent estimations of the efficiency of gene targeting in *P. patens* can be made from these data. Mean frequencies of integrative transformation observed under targeting conditions are 10-fold higher than those observed in the absence of sequence homology, indicating that DNA integration occurs 10 times more frequently by homologous recombination than by illegitimate integration. Molecular evidence for the integration of the transforming plasmids by homologous recombination has been obtained for the three analysed loci, and for 93% (14/15) of the plants. These estimations are similar to those obtained in targeting experiments with artificial loci, where phenotypic, genetic and molecular evidence for successful targeting was obtained for the six loci tested and for 91% (10/11) of the plants (Schaefer, 1994; unpublished data). The data

presented here further demonstrate that efficient gene targeting in this moss is independent of the presence of artificial loci integrated in the genome. Thus, one can reliably conclude that the efficiency of gene targeting in *P. patens* is above 90%, and that illegitimate integration of exogenous DNA in the genome is a rare event. Such a high ratio of homologous to random integration is unique in plants, and is similar to that observed in *S. cerevisiae* (Struhl, 1983). It provides the methodological requirement to develop efficient gene knock-out and allele replacement approaches in the moss *P. patens*. When one realizes the importance of such methodology in the development of *S. cerevisiae* as an experimental model system, it is predicted that such a high ratio of homologous to random integration should enable the development of a new moss genetics similar to that developed in yeast (Struhl, 1983) and mouse (Capecchi, 1989b). Furthermore, the availability of both efficient gene targeting and cell lineage analysis in this haplobiontic moss will allow study of the basic mechanisms of plant development at a level which is not yet accessible in other plants.

Mitotic and meiotic stability of the targeted trans*-loci*

In all the transformants described here, we observed that transformation was achieved following the integration of several direct repeats of the targeting plasmids. Such insertion generates direct repeats of the transforming plasmid flanked by direct repeats of the targeted sequence, and this structure is prone to intra-chromosomal recombination events which may lead to mitotic and/or meiotic loss of the transforming sequences. In *S. cerevisiae*, mitotic loss of such structures is around 0.1%. In *P. patens*, mitotic loss of the resistance is only observed for transformants with a copy number above 50 per genome (D. Schaefer, unpublished data). Meiotic loss of such artificial loci in *S. cerevisiae* ranges from 1–10% (Struhl, 1983). In *P. patens*, meiotic stability of the integrated plasmid repeats is supported by the 100% transmission of the resistance to the progeny resulting from self-fertilization, since crosses are performed on non-selective medium. Yet, meiotic loss of the transforming sequence was observed in one out of 10 F_1 progeny, and recombination events leading to a reduction in the number of integrated copies was detected in the hygromycin-resistant F_1 progeny of plant 108#3 (Figure 4). These data indicate that the artificial loci are occasionally prone to meiotic recombination events when many plasmid copies are integrated into the genome and when the wild-type allele is present. We postulate that size difference between the artificial locus and its wild-type allele may be responsible for these meiotic recombinations which may be associated with duplication, deletion and translocation events.

Why is gene targeting so efficient in P. patens?

Such a high ratio of homologous to random integration in the moss *P. patens* is an unexpected result and raises the question of the factors controlling gene targeting in eukaryotes. Interestingly, a ratio of homologous to random integration above 10% has in most cases only been observed in haploid and/or lower eukaryotic cells (*Saccharomyces cerevisiae, Schizosaccharomyces pombe, Aspergillus nidulans, Neurospora crassa, Dictyostelium discoideum, Alternaria alternata, Ustilago maydis* and *Physcomitrella patens*). Such an observation suggests that efficient gene targeting could be correlated with the haplophase in eukaryotes, and we have made the following assumptions: (a) organisms with a dominant haplophase may have strategies to protect themselves against the integration of foreign DNA sequences in the genome, since such events are potentially immediately mutagenic, and (b) in contrast to diploid cells, a tight control of sequence homology to prevent deleterious recombination events between allelic sequences is not required in a haploid cell. In this situation, the presence of homologous sequences on the transforming DNA would increase the probability of integration through homologous recombination by the unrestricted formation of stable hybrid structures between the incoming DNA and the homologous genomic sequences, facilitating subsequent integration in the genome. Homologous sequences would therefore be required to allow integrative transformation to occur, resulting in a higher ratio of homologous to random integration events. Therefore, we propose that efficient gene targeting correlates with the predominance of the haplophase in the life cycle of *P. patens*, and that this correlation is associated with processes involved in the maintenance of genome integrity. The consequence of such a hypothesis is that efficient gene targeting may be a general phenomenon in other Bryophytes and haplobiontic eukaryotes. Furthermore, the general validity of this correlation would imply that efficient gene targeting could be achieved in the gametes of diplobiontic organisms.

Experimental procedures

Plant material and culture conditions

Targeting experiments to single-copy genomic sequences were performed with the Gransden wild-type (Ashton and Cove, 1977). *P. patens* was grown on the minimal medium described by Ashton *et al.* (1979) supplemented with 2.7 mM NH_4 tartrate, 25 mM glucose, 1.5 μM thiamine HCl, 1.8 μM paraaminobenzoic acid and 8 μM nicotinic acid unless otherwise stated. Cultures were grown in 9 cm Petri dishes on medium solidified with 0.7% Agar (Merck 1614) and overlaid with a 8 cm diameter cellophane disk (type 325P, Cannings, Avonmouth, Bristol, UK). Plates were grown in the culture room at 25°C with a light regime of 16 h light / 8 h darkness and a quantum irradiance of ~80 μE m^{-2} sec^{-1} (adapted

from Ashton and Cove, 1977; Knight and Cove, 1988). Protoplasts were isolated from 5- to 6-day-old protonemal cultures by incubation for 30 min in 1% Driselase (Fluka 44585), 0.48 M mannitol (4 ml per Petri dish). The suspension was filtered through a 100 μm stainless steel sieve, left for 15 min at room temperature to complete digestion of the cell walls and filtered again through a 50 μm sieve. Protoplasts were sedimented by low-speed centrifugation (500 rpm for 5 min) and washed twice in 0.48 M mannitol (adapted from Grimsley *et al.*, 1977; Knight *et al.*, 1988).

Transformation and selection of transgenic clones

PEG-mediated direct DNA transfer into protoplasts was performed as described by Schaefer *et al.* (1994) and samples were kept for 16 h in darkness after transformation. Protoplasts were then harvested by low-speed centrifugation, embedded in a top layer (0.7% agar, 0.48 M mannitol) and regenerated for 6 days in the same conditions as plant material on solid culture medium supplemented with 0.33 M mannitol and overlaid with a cellophane disk. Selection for antibiotic-resistant colonies was initiated 6 days after transformation by transferring the top layer onto plates without mannitol and supplemented with 40 mg l^{-1} geneticin sulphate (Gibco) or 25 mg l^{-1} hygromycin B (Calbiochem). Selection was maintained at a constant level by transferring the top layers onto fresh selective plates every 10 days. Integrative transgenic clones were identified by their unrestricted growth and isolated one month after the initiation of selection (see for details Knight, 1994; Schaefer, 1994; Schaefer *et al.*, 1991, 1994).

Genetic crosses

Strain *nicB5ylo6* is self-sterile, cross-fertile and carries a convenient phenotypic yellow marker (*ylo*) allowing easy identification of the colony in crossing experiments (Ashton and Cove, 1977). Protonemal inocula of a transgenic strain and of strain *nicB5ylo6* were transferred side by side on solid medium containing nitrate as the sole nitrogen source and regenerated at 25°C until some 50 gametophores were completely differentiated. Cultures were then irrigated with sterile water and transferred to 17°C for 3 weeks to induce gametogenesis. The cultures were brought back to 26°C and the maturation of sporophytes was followed visually. Mature spore capsules were harvested individually, crushed in 1 ml sterile water to disperse the spores and kept for three weeks at 4°C to ensure high spore germination rates. Spore capsules collected from the transgenic colony were generated by self-fertilization and the ones collected from the *nicB5ylo6* colony by cross-fertilization. Spores were germinated in light on solid medium and replicates of well-defined single spore-derived colonies were tested for the segregation of the antibiotic resistance and of the *ylo* marker (adapted from Ashton and Cove, 1977; Knight and Cove, 1988).

Plasmid construction

DNA manipulations were performed according to standard procedures (Sambrook *et al.*, 1989). A 3.6 kb *Hin*dIII fragment was isolated from the genomic clone λ108 (D. Schaefer, unpublished results) and cloned into the *Hin*dIII site of pGL2 to build plasmid pGL-108. A 3.6 kb *Bgl*II fragment isolated from the genomic clone λ420 (D. Schaefer, unpublished results) was subcloned in the *Bam*HI site of pBS-M13+ (Stratagene). A 2.7 kb *Hin*dIII fragment was recovered from this plasmid (one of the *Hin*dIII sites comes from the polylinker of pBS-M13+) and cloned into the corresponding site of pGL2 to construct pGL-420. A 2.3 kb *Eco*RI fragment was isolated from the genomic clone λ213 (D. Schaefer, unpublished results) and cloned into the *Eco*RI site located next to the 35S promoter of pHP23b to build plasmid pHP-213. *E. coli* HB101 was used to transform and amplify the plasmids which were PEG-purified according to standard procedures (Sambrook *et al.*, 1989).

Plant DNA analysis

Protonemal tissue was collected from 8- to 10-day-old culture, frozen in liquid nitrogen and ground in a coffee grinder with dry ice. DNA was extracted with cetyl-trimethyl-ammonium bromide according to Rogers and Bendich (1988), yielding 3–5 μg DNA per gram fresh weight. Southern blot analysis was performed according to standard methods (Sambrook *et al.*, 1989). DNA (2.5 μg) was digested with the appropriate restriction enzyme, fragments were separated by electrophoresis in 0.7% agarose and transferred to nylon membranes (Zeta probe, BioRad) under alkaline conditions (0.4 M NaOH). Probes were radiolabelled with [α-^{32}P]dCTP by random priming (Feinberg and Vogelstein, 1983). Hybridization was conducted in 3 × SSPE, 0.1% SDS, 2 × Denhardt's, 100 μg ml^{-1} yeast tRNA and 10^6 cpm ml^{-1} radiolabelled probes for 16 h at 60°C. The final wash was in 0.1 × SSPE at 65°C. Genomic reconstructions were based on a haploid genome size of 4.6×10^8 bp (Reski *et al.*, 1994).

Acknowledgements

We acknowledge Iris Schmidt and Luis Ruiz de Castaneda for technical assistance, Dr Barbara Hohn (FMI, Basel) and Dr Enzo Russo (MPI, Berlin) for helpful suggestions throughout the course of this study, and Dr Susan Reynolds (LPC, UNILausanne) for critical reading of the manuscript. This work was funded by the OFES (Office fédéral pour l'éducation et la science), on behalf on the European Communities BIOTECH Program, as part of the EUROMOSS project of the Project of Technological Priority 1993–1996.

References

ten Asbroek, A.L., Ouellette, M. and Borst, P. (1990) Targeted insertion of the neomycin phosphotransferase gene into the tubulin gene cluster of *Trypanosoma brucei*. *Nature*, **348**, 174–175.

Ashton, N.W. and Cove, D.J. (1977) The isolation and preliminary characterisation of auxotrophic and analogue resistant mutants in the moss *Physcomitrella patens*. *Mol. Gen. Genet.* **154**, 87–95.

Ashton, N.W., Grimsley, N.H. and Cove, D.J. (1979) Analysis of gametophytic development in the moss *Physcomitrella patens*, using auxin and cytokinin resistant mutants. *Planta*, **144**, 427–435.

Berg, P. (1991) Reverse genetics: its origins and prospects. *Bio/Technology*, **9**, 342–344.

Bilang, R., Iida, S., Peterhans, A. and Paszkowsi, J. (1991) The 3′-terminal region of the hygromycin-B-resistance gene is important for its activity in *Escherichia coli* and *Nicotiana tabacum*. *Gene*, **100**, 247–250.

Bollag, R.J., Waldmann, A.S. and Liskay, R.M. (1989) Homologous recombination in mammalian cells. *Annu. Rev. Genet.* **23**, 199–225.

Bopp, M. and Werner, O. (1993) Abscissic acid and dessication tolerance in mosses. *Bot. Acta*, **106**, 103–106.

Buerstedde, J.-M. and Takeda, S. (1991) Increased ratio of gene

targeted to random integration after transfection of chicken B cell lines. *Cell*, **67**, 179–188.

Capecchi, M.R. (1989a) Altering the genome by homologous recombination. *Science*, **244**, 1288–1292.

Capecchi, M.R. (1989b) The new mouse genetics: altering the genome by gene targeting. *Trends Genet.* **5**, 70–76.

Cove, D.J. (1983) Genetics of Bryophyta. In *New manual of Bryology* (Schuster, R.M., ed.). Tokyo, Japan: Hattori Botanical Laboratory, pp. 222–231.

Cove, D.J. (1992) Regulation of development in the moss *Physcomitrella patens*. In *Development. The Molecular Genetic Approach* (Brody, S., Cove, D.J., Ottolenghi, S. and Russo, V.E.A., eds). Heidelberg: Springer Verlag, pp. 179–193.

Cove, D.J. and Ashton, N.W. (1984) The hormonal regulation of gametophytic development in Bryophytes. In *The Experimental Biology of Bryophytes* (Dryer, A.F. and Duckett, J.G., eds). London: Academic Press, pp. 177–201.

Cove, D.J. and Knight, C.D. (1993) The moss *Physcomitrella patens*, a model system with potential for the study of plant reproduction. *Plant Cell*, **5**, 1483–1488.

Cruz, A. and Beverley, S.M. (1990) Gene replacement in parasitic protozoa. *Nature*, **348**, 171–173.

De Lozanne, A. and Spudich, J.A. (1987) Disruption of the *Dictyostelium* myosin heavy chain gene by homologous recombination. *Science*, **236**, 1086–1091.

Deng, C. and Capecchi, M.R. (1992) Reexamination of gene targeting frequency as a function of the extent of homology between the targeting vector and the target locus. *Mol. Cell. Biol.* **12**, 3365–3371.

Engel, P.P. (1968) The induction of biochemical and morphological mutants in the moss *Physcomitrella patens*. *Am. J. Bot.* **55**, 438–446.

Feinberg, A.P. and Vogelstein, B. (1983) A technique for labeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6–13.

Fotheringham, S. and Holloman, W.K. (1989) Cloning and disruption of *Ustilago maydis* genes. *Mol. Cell. Biol.* **9**, 4052–4055.

Grimm, C. and Kohli, J. (1988) Observations on integrative transformation in *Schizosaccharomyces pombe*. *Mol. Gen. Genet.* **215**, 87–93.

Grimsley, N.H., Ashton, N.W. and Cove, D.J. (1977) The production of somatic hybrids by protoplast fusion in the moss *Physcomitrella patens*. *Mol. Gen. Genet.* **154**, 97–100.

Halfter, U., Morris, P.-C. and Willmitzer, L. (1992) Gene targeting in *Arabidopsis thaliana*. *Mol. Gen. Genet.* **231**, 186–193.

Hartmann, E. and Jenkins, G.I. (1984) Photomorphogenesis of mosses and liverworts. In *The Experimental Biology of Bryophytes* (Dryer, A.F. and Duckett, J.G., eds). London: Academic Press, London, pp. 203–228.

Hinnen, A., Hicks, J.B. and Fink, G.R. (1978) Transformation of yeast. *Proc. Natl Acad. Sci. USA*, **75**, 1929–1933.

Hrouda, M. and Paszkowski, J. (1994) High fidelity extrachromosomal recombination and gene targeting in plants. *Mol. Gen. Genet.* **243**, 106–111.

Joyner, A.L. (1991) Gene targeting and gene trap screen using embryonic stem cells: new approaches to mammalian development. *Bio/Essays*, **13**, 649–656.

Kammerer, W. and Cove, D.J. (1996) Genetic analysis of the result of re-transformation of transgenic lines of the moss, *Physcomitrella patens*. *Mol. Gen. Genet.* **250**, 380–382.

Knight, C.D. (1994) Studying plant development in mosses: the transgenic route. *Plant Cell Environ.* **17**, 669–674.

Knight, C.D. and Cove, D.J. (1988) Time-lapse microscopy of gravitropism in the moss *Physcomitrella patens*. In *Methods in Bryology* (Glime, J.M., ed.). Tokyo, Japan: Hattori Botanical Laboratory, pp. 127–129.

Knight, C.D., Cove, D.J., Boyd, P.J. and Ashton, N.W. (1988) The isolation of biochemical and developmental mutants in the moss *Physcomitrella patens*. In *Methods in Bryology* (Glime, J.M., ed.). Tokyo, Japan: Hattori Botanical Laboratory, pp. 47–58.

Knight, C.D., Sehgal, A., Atwal, K. *et al.* (1995) Molecular responses to abscissic acid and stress are conserved between moss and cereals. *Plant Cell*, **7**, 499–506.

Kronstad, J.W., Wang, J., Covert, S.F., Holden, D.W., McKnight, G.L. and Leong, S.A. (1989) Isolation of metabolic genes and demonstration of gene disruption in the phytopathogenic filamentous fungus *Ustilago maydis*. *Gene*, **79**, 97–106.

Lee, K.Y., Lund, P., Lowe, K. and Dunsmuir, P. (1990) Homologous recombination in plant cells after *Agrobacterium*-mediated transformation. *Plant Cell*, **2**, 415–425.

Lichtenstein, C. and Barrena, E. (1993) Prospects for reverse genetics in plants using recombination. *Plant. Mol. Biol.* **21**, V–XII.

Miao, Z.-H. and Lam, E. (1995) Targeted disruption of the TGA3 locus in *Arabidopsis thaliana*. *Plant J.* **7**, 359–365.

Negrutiu, I., Dewulf, J., Pietrzak, M., Botterman, J., Rietveld, E., Wurzer-Figurelli, E.M., Ye, D. and Jacobs, M. (1990) Hybrid genes in the analysis of transformation conditions: II. Transient expression vs stable transformation – analysis of parameters influencing gene expression levels and transformation efficiency. *Physiol. Plant.* **79**, 197–205.

Offringa, R., de Groot, J.A., Haagsman, H.J., Does, M.P., van den Elzen, P.J.M. and Hooykaas, P.J.J. (1990) Extrachromosal homologous recombination and gene targeting in plant cells after *Agrobacterium*-mediated transformation. *EMBO J.* **9**, 3077–3084.

Ohl, S., Offringa, R., van den Elzen, P.J.M. and Hooykaas P.J.J., (1994) Gene replacement in plants. In *Homologous Recombination and Gene Silencing in Plants* (Paszkowski, J., ed.). Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 191–217.

Orr-Weaver, T.L., Szostak, J.W. and Rothstein, R.J. (1981) Yeast transformation: a model system for the study of recombination. *Proc. Natl Acad. Sci. USA*, **78**, 6354–6358.

Paietta, J.V. and Marzluf, G.A. (1985) Gene disruption by transformation in *Neurospora crassa*. *Mol. Cell. Biol.* **5**, 1554–1559.

Paszkowski, J., Baur, M., Bogucki, A. and Potrykus, I. (1988) Gene targeting in plants. *EMBO J.* **7**, 4021–4026.

Potrykus, I. (1991) Gene transfer to plants: assessment of published approaches and results. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42**, 205–225.

Puchta, H., Swoboda, P. and Hohn, B. (1994) Homologous recombination in plants. *Experientia*, **50**, 277–284.

Reski, R., Faust, M., Wang, X.-H., Wehe, M. and Abel, W.O. (1994) Genome analysis of a moss, *Physcomitrella patens* (Hedw.) B.S.G. *Mol. Gen. Genet.* **244**, 352–359.

Rogers, S.O. and Bendich, A.J. (1988) Extraction of DNA from plant tissues. In *Plant Molecular Biology Manual*, Volume A6 (Gelvin, S.B., Schilperoort, R.A. and Verma, D.P.S., eds). Dordrecht, The Netherlands: Kluwer Academic Publishers, pp. 1–10.

Rothstein, R. (1991) Targeting, disruption, replacement and allele rescue: integrative DNA transformation in yeast. *Methods Enzymol.* **194**, 281–301.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular*

Cloning: A Laboratory Manual. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schaefer, D.G. (1994) *Molecular Genetic Approaches to the Biology of the Moss* Physcomitrella patens. PhD Thesis, University of Lausanne, Switzerland. (www.unil.ch/lpc/docs/DSThesis.htm)

Schaefer, D.G., Zrÿd, J.-P., Knight, C.D. and Cove, D.J. (1991) Stable transformation of the moss *Physcomitrella patens. Mol. Gen. Genet.* **226**, 418–424.

Schaefer, D.G., Bisztray, G. and Zrÿd, J.-P. (1994) Genetic transformation of the moss *Physcomitrella patens.* In *Biotechnology in Agriculture and Forestry 29: Plant Protoplasts and Genetic Engineering V,* Volume 29 (Bajaj, Y.P.S., ed.) Berlin: Springer Verlag, pp. 349–364.

Shiotani, H. and Tsuge T. (1995) Efficient gene targeting in the filamentous fungus *Alternaria alternata. Mol. Gen. Genet.* **248**, 142–150.

Struhl, K. (1983) The new yeast genetics. *Nature,* **305**, 391 –397.

Thomas, K.R., Folger, K.R. and Capecchi, M.R. (1986) High frequency targeting of genes to specific sites in the mammalian genome. *Cell,* **4**, 419–428.

Timberlake, W.E. and Marshall, M.A. (1989) Genetic engineering in filamentous fungi. *Science,* **244**, 1313–1317.

Wang, T.L. and Cove, D.J. (1989) Mosses – lower plants with high potential. *Plants Today,* **2**, 44–50.

von Wettstein, F. (1932) Genetik. In *Manual of Bryology* (Verdoorn, F. ed.). The Hague, The Netherlands: Martinus Nijhoff, pp. 233–272.