II.11 Genetic Transformation of the Moss *Physcomitrella patens*

D.G. SCHAEFER, G. BISZTRAY, and J.P. ZRŸD

1. Introduction

1.1 Importance of *Physcomitrella patens*

Physcomitrella patens is a widely spread moss species which colonizes open habitat in cold temperate zones. For the developmental geneticist, haplobiontic plants, such as mosses, present distinct advantages over the diplobiontic angiosperms: the haploid gametophytic generation dominates over the diploid sporophytic generation during most of their life cycle. This facilitates mutant isolation and genetic analysis (Cove 1983; Ashton et al. 1988; Wang and Cove 1989). Since the first report of the successful isolation of biochemical and morphological mutants in *P. patens* (Engel 1968), this organism has been used as a model genetic system for physiological and developmental studies. In vitro propagation conditions have been defined which allow the complete life cycle to be achieved on a simple mineral medium in less than 3 months (Ashton and Cove 1977). Physiological studies have demonstrated that the main factors controlling development in *P. patens* were similar to those involved in angiosperms, i.e., light, gravity, and growth substances such as cytokinins and auxins (Cove et al. 1978; Cove and Ashton 1984, 1988). The simple morphology and the well-defined cell lineage in the development of the gametophyte (Cove 1992) offer the opportunity to study morphogenetic processes at the single cell level, permitting the efficient combination of genetic, cellular, and physiological approaches. An efficient protoplast system has been developed which allows regeneration of up to 80% of protoplasts into fertile plants (Grimsley, et al. 1977). The production of new chloronemata from these protoplasts is strictly light-dependent (Jenkins and Cove 1983), does not require an exogenous hormone supply, and does not involve the dedifferentiation stages that are typical in angiosperms. Somatic fusion of these protoplasts has been used for complementation analysis and dominance studies in fertile diploid plants (Grimsley et al. 1980; Ashton et al. 1988). Finally, chemical mutagenesis has been achieved using spores or somatic tissue to induce mutants of the following classes (review and references in Knight et al. 1988) :

[1] Laboratoire de Phytogénétique Cellulaire, Bâtiment de Biologie, Université de Lausanne, CH-1015 Lausanne, Switzerland

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(1) auxotrophy and analog resistance, (2) alteration of the gravitropic or phototropic responses (Knight et al. 1991), (3) alteration of the overall morphology, (4) cytokinin and auxin resistance.

1.2 Need for Transformation in *Physcomitrella patens*

The successful genetic transformation of *P. patens* should enable the following types of experiments to be performed:
1. The introduction and overexpression of genes involved in auxin and cytokinin biosynthesis or in light-mediated processes (phytochrome). Such an approach should provide insight into the role and the mode of action of these factors during plant development.
2. The introduction of defined DNA sequences (such as partial genomic or cDNA library) complementing the different mutants, thereby permitting the identification and molecular characterization of genes involved in the mutated phenotypes.
3. The generation of tagged mutants by the introduction of foreign DNA sequences (e.g., selectable markers or transposons) into specific genes (gene disruption experiments) and their subsequent isolation and characterization (Cove et al. 1991; Feldmann 1991; Koncz et al. 1992; Walbot 1992).
4. The targeting of genes by homologous recombination (Paszkowski et al. 1988; Capecchi 1989; Offringa et al. 1990; Halfter et al. 1992). This would imply the replacement or conversion of specific chromosomal genes by homologous DNA sequences carrying a point mutation or a deletion (reverse genetics through gain or loss of function experiments).
5. The study of transient expression of reporter genes driven by regulated promoters and the subsequent characterization of *cis* regulatory elements involved in the control of gene expression (for example, by 5' deletion analysis). This approach has already found several applications in studies of the regulation of gene expression in plant cells (for examples, see Callis et al. 1987; Lipphardt et al. 1988; Bonneville et al. 1989; Ainley and Key 1990; Harkins et al. 1990; Sheen 1990; Schaffner and Sheen 1991).

This chapter covers our current knowledge of the genetic transformation of *P. patens* and is illustrated using selected data from transient gene expression experiments, and phenotypic and genetic observations on resistant and transgenic plants.

2. Genetic Transformation of *Physcomitrella patens*

2.1 General Account of the Various Methods Tested for the Transformation of *P. patens*

Many techniques for the transformation of plant cells have been developed over the last 15 years (reviewed in Potrykus 1991). Four of these approaches have

been tested for the transformation of *P. patens:* (1) electroporation of protoplasts, (2) cocultivation of protoplasts or protonemata fragments with *Agrobacterium tumefaciens,* (3) bombardment of intact cells with DNA-coated microprojectiles, and (4) polyethylene glycol (PEG)-mediated direct gene transfer to protoplasts (Schaefer et al. 1991). The last approach is reviewed in Sections 2.2 and 2.3. The data obtained with the other approaches are briefly summarized here.

2.1.1 Electroporation-Mediated Direct Gene Transfer to Protoplasts

Electroporation of protoplasts was achieved according to Shillito et al. (1985) with a plasmid carrying a hygromycin resistance gene under the control of the 35S CaMV promoter. Two hygromycin-resistant clones were isolated out of 200 000 regenerating protoplasts and exhibited characteristics typical of unstable clones (see Sect. 2.3). Molecular analysis demonstrated that the introduced sequences were present in the resistant colonies (Schaefer 1994). Transient expression assays have also been performed with a 35S CAT reporter plasmid (Pietrzak et al. 1986), but no chloramphenicol acetyl transferase (CAT) activity could be detected in the extracts (D. Schaefer unpubl.). The low relative transformation frequency obtained with this method (ca. 10^{-5}) and the lack of evidence for stable integration and for transient expression of introduced sequences led us to conclude that this approach was unsuitable for the transformation of *P. patens.*

2.1.2 Agrobacterium-Mediated Gene Transfer to Protoplasts or Protonemal Fragments

Agrobacterium strains carrying disarmed vectors with a kanamycin resistance selectable marker were cocultivated with protoplasts or protonemal fragments of *P. patens.* No resistant colonies were recovered from 10^7 surviving cells (D. Schaefer and N. Grimsley unpubl.). With the same approach, transient expression assays were performed with a 35S GUS reporter gene but no β-glucuronidase (GUS) activity could be detected (C. Knight and B. Hohn unpubl.), although moss extracts did induce the expression of the *vir* genes of *Agrobacterium tumefaciens* (N. Grimsley pers. comm.). It seems therefore that the host range of *A. tumefaciens* for DNA transfer does not include the moss *P. patens.*

2.1.3 Bombardment of Intact Cells by DNA-Coated Microprojectiles

It has been recently reported that the biolistic approach can be used to transfer DNA into intact cells of P. *patens* (Sawahel et al. 1992). The data are supported by histochemical analysis of transient GUS expression in cells bombarded with particles coated with a 35S GUS construct. Regeneration of stable transgenic

plants has not yet been achieved and appears unlikely in view of the low frequency of stable integrative events when compared with other methods, such as chemical direct gene transfer (Potrykus 1991). Therefore, we believe that this method might find application in specific transient gene expression studies in differentiated tissues of *P. patens,* but is to date less efficient than the PEG procedure for routine transformation experiments.

2.2 Methodology and Protocols

The role of various steps involved in our published procedure (Schaefer et al. 1991) has been investigated using the transient expression of a 35S GUS expression cassette to monitor the efficiency of transformation. The GUS reporter gene (Jefferson et al. 1987) was chosen for transient expression assays in *P. patens* since preliminary studies have demonstrated that endogenous GUS activity in moss protein extracts was always below 0.05 nmol MU mg^{-1} min^{-1} and that a GUS assay run with purified β-glucuronidase or with extracts from a GUS transgenic tobacco plant was not affected by the presence of moss protein extracts. The following optimal protocol has been established (Schaefer 1994).

2.2.1 Protocol for the PEG-Mediated Transformation of Protoplasts

1. Isolate protoplasts from 5-6-day-old protonemata according to Grimsley et al. (1977).
2. Resuspend the protoplasts at a concentration of 1 to 1.5 x 10^6/ml in MMM solution (0.48 M mannitol, 15mM magnesium chloride, 0.1% MES, pH 5.6 with KOH) and proceed immediately with the transformation.
3. Dispense 30 μl (17 μg) of plasmid DNA (final concentration of 50 μg/ml) into 14-ml tubes.
4. Add 300 μl of protoplast suspension and mix gently.
5. Add 300 μl of PEG solution [to 100 ml of a mannitol 0.38 M calcium nitrate 0.1 M solution, add 40 g of PEG 4000 (Serva), and buffer to pH 8 with 10 mM Tris] and mix gently.
6. Heat shock 5 min at 45°C.
7. Bring back to room temperature and leave for an additional 10 min with occasional gentle mixing.
8. Progressively dilute the sample with 10 ml of protoplast liquid culture medium (Knight et al. 1988).

At this stage, protoplasts may be further cultured in liquid medium for the transient expression assay or embedded and regenerated in a top layer of 0.7% agar (Grimsley et al. 1977) for further selection on solid medium.

2.2.2 Additional Notes on the Protocol

1. The MMM solution is required to achieve a high transformation efficiency.
2. Carrier DNA has often been reported to increase the stable integration event

(Negrutiu et al. 1990). This approach was systematically omitted to avoid the generation of tagged mutants by the insertion of nonidentified carrier sequences.
3. A large precipitate containing DNA and cellular material will result upon addition of PEG if the final DNA concentration is higher than 70 µg/ml. A specific activity/DNA concentration dose response has been obtained with increasing concentration of plasmid DNA, provided the final concentration is maintained constant with sheared salmon sperm DNA (Fig. lc).
4. Heat shock is necessary, although some DNA uptake without heat shock has been occasionally observed. A specific activity/heat shock dose response has been obtained with increasing heat shock duration (Fig. la).

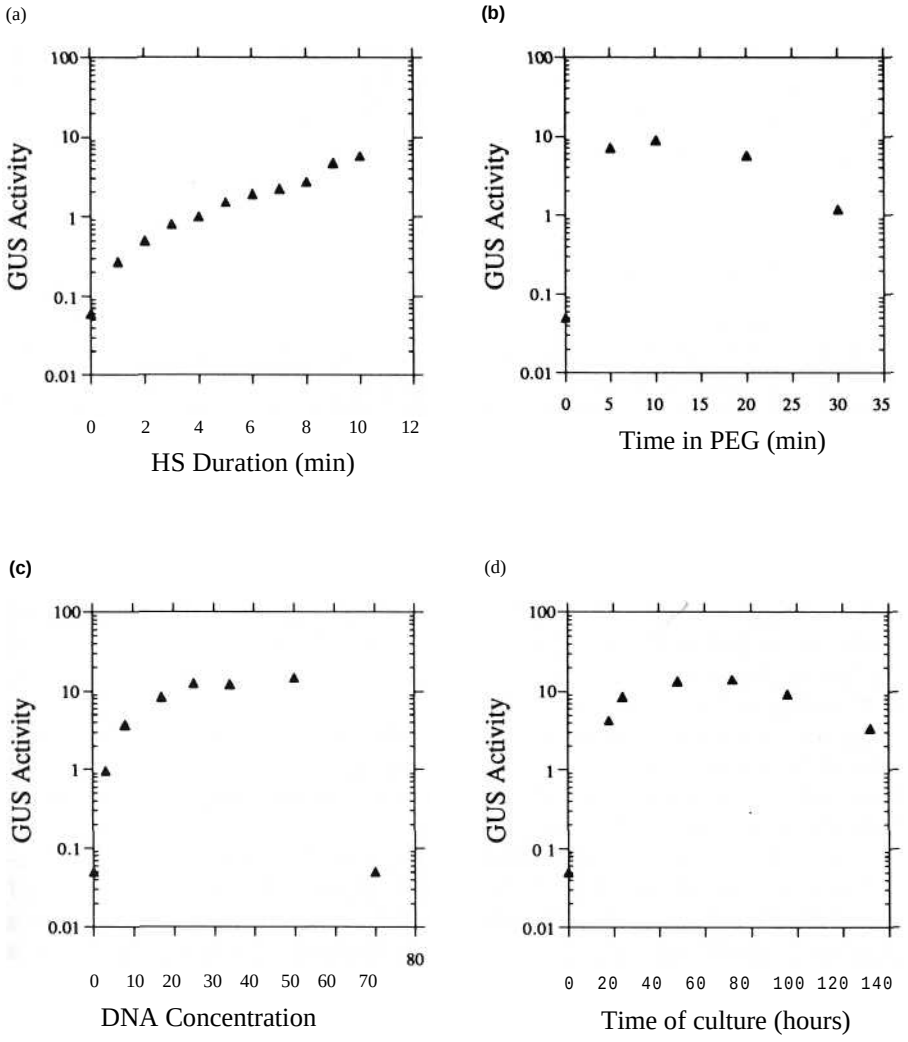


Fig. 1 a-d. Transient GUS expression in protoplasts *of Physcomitrella patens* as a function of **a** heat shock duration, **b** time in PEG, **c** DNA concentration, and **d** time after transformation. Specific activity is expressed on a log scale in nmol MU mg^{-1} min^{-1}

5. Longer incubation in PEG decreases cell survival and specific activity (Fig. lb).
6. High transient gene expression is readily measurable after 24 h of culture, reaching a maximum after ca. 40 h. A decrease in specific activity is observed after 4 days (Fig. 1d).
7. Selection for stable transformants should be undertaken using high antibiotic concentrations (ca. 5-10 x LD50). Under these conditions, the growth rate of
stable clones is similar to the untransformed wild type plated on nonselective medium, whereas the growth rate of unstable clones is seriously reduced.

With this procedure, an average 50% cell survival has been obtained in transient gene expression assays, while 30% of the initial number of cells will regenerate in transformation experiments. GUS-specific activities of 5-10 nmol MU mg^{-1} min^{-1} were routinely obtained using a 35S GUS construct and the sensitivity of the assay ranges over two orders of magnitude. A 30-fold increase in relative transformation frequency was obtained when antibiotic-resistant colonies were selected, but the number of stable clones remained the same (see Sect. 2.3).

2.2.3 Methods for the Analysis of Transient Gene Expression and the Characterization of Resistant Colonies

For transient gene expression assays, cells are usually collected 48 h after transfection and lysed by three cycles of freeze and thaw. Cellular debris are removed by centrifugation and GUS-specific activity of the soluble protein extract is determined according to Jefferson (1987) using methyl umbelliferyl glucuronide (MUG) at 2 mM as substrate.

Selection for antibiotic-resistant colonies is usually initiated 6 days after transformation by transferring the top layers on plates without mannitol and supplemented with 40 mg/l of Geneticin (G 418) or 25 mg/l of Hygromycin B. The initial relative transformation frequency is determined 10 days after transfer [RTF = (No. resistant colonies/No. regenerating colonies) * 100]. Additional counts are undertaken throughout the next month. Criteria for the definition of transgenic plants have been described (Potrykus 1991). These include mitotic and meiotic stability of the introduced sequences, correlation between phenotypic and molecular analysis of the original plant and of its progeny, and Mendelian segregation of the introduced character. Two additional types of experiments have been designed to characterize resistant colonies of *P. patens* (Schaefer and Zryd, in prep. b)

1. *Nonselective to selective test:* Protonemata from resistant colonies are fragmented for 30 s with a Polytron (position 4), plated on cellophane disks, and grown for 10 days on nonselective medium. The culture is then transferred to a selective medium and grown for an additional fortnight. The growth of a transgenic strain will not be affected by the transfer, whereas unstable strains will become moribund with only a few discrete regenerating foci remaining on selective plates. This test is used to assess the stability of the introduced sequences under nonselective growth conditions (mitotic stability index).

2. *Rescue experiments:* Taking advantage of the fact that every part of the plant is able to regenerate a protonema under standard culture conditions (Cove 1992), isolated gametophores from resistant colonies are plated on selective medium. The complete gametophore from transgenic plants will regenerate a protonema on selective plates, whereas this ability will be restricted to the resistant part of the gametophore from unstable strains (Fig. 3). With this test, mosaic structures within a resistant clone can easily be detected.

2.3 Results and Discussion

2.3.1 Light-Mediated Transient Gene Expression in Physcomitrella patens Protoplasts

P. patens protoplasts require light for their regeneration (Jenkins and Cove 1983) and may therefore be competent for the light-mediated transient expression of reporter genes driven by light-inducible promoters. The behavior of two different constructs has confirmed this assumption (Bisztray et al. unpubl.). The plasmid rbcS-GUS contains the GUS reporter gene under the control of a *Nicotiana tabacum* rubisco small subunit *(rbcS)* gene promoter and displays the characteristic light-inducible and tissue-specific expression pattern of *rbcS* genes in transgenic tobacco (Jefferson et al. 1987). The plasmid cab-GUS (kindly provided by F. Nagy, FMI, Basel) contains the GUS reporter gene under the control of the complete wheat *cab-1* gene promoter (Nagy et al. 1986). This promoter has been shown to contain the regulatory elements for phytochrome-mediated light induction and circadian control of gene expression in transgenic tobacco (Nagy et al. 1987, 1988; Fejes et al. 1990). *P. patens* protoplasts were transfected with these plasmids and cells were further grown under different light conditions. GUS-specific activity was determined at different times after

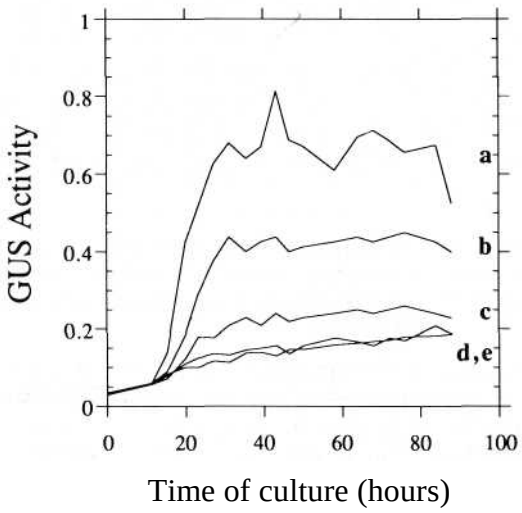


Fig. 2. The plasmid cab-GUS was introduced into *Physcomitrella patens* protoplasts and cells were grown under different light conditions: a white light, b red light, c red/far red light, d far-red light, e darkness. Red light was provided as 15 min pulses and far-red light as 5-min pulses every 2 h. Samples were taken at different times after transfection to determine GUS activity (in nmol MU mg^{-1} min^{-1})

transfection. The transient expression of both plasmids was light-regulated and the involvement of phytochrome in the regulation of cab-GUS was demonstrated by its red/far-red behavior (Fig. 2). These data suggest that some of the biochemical mechanisms involved in the light regulation of plant gene expression are conserved between angiosperms and bryophytes. Light-regulated transient gene expression in protoplasts has only been achieved in maize (Schaffner and Sheen 1991) and tobacco (Harkins et al. 1990) under well-defined conditions. It is believed that the regulatory elements required for light-mediated gene expression are different in monocots and dicots (Schaffner and Sheen 1991). Our data indicate that some of these elements may be shared, since both the tobacco *rbcS* and the wheat *cab-1* promoters are properly light-regulated in moss protoplasts.

2.3.2 Integrative and Replicative Transformation (Schaefer and Zryd, in prep. b)

The data presented in this section refer to transformation experiments performed with the supercoiled form of plasmids pHP 23 (Paszkowski et al. 1988) and pGL 2 (described in Schaefer et al. 1991) conferring resistance to kanamycin and hygromycin B, respectively. Negative controls containing no DNA, DNA without PEG, bacterial and promoterless expression cassettes never gave rise to resistant colonies. The PEG-mediated introduction of pHP 23 and pGL 2 into *P. patens* protoplasts generated a high number of resistant clones with an initial relative transformation frequency ranging from 5 to 30% (Table 1). However, 50 to 90% of these colonies will not grow beyond the 100-cell stage and it is likely that these clones represent plants in which antibiotic resistance is only expressed transiently. The remaining regenerants have been classified into three different categories according to the criteria summarized in Table 2.

Class I: Stable Transgenic Plants. These transformants have already been described (Schaefer et al. 1991). The relative transformation frequency is low, ranging from 10^{-5} to 10^{-4}. These clones display unrestricted growth and differentiation upon selective medium and maintenance of resistance after nonselective growth. The new character is always transmitted through meiosis. Molecular and genetic analyses have demonstrated that these plants carry multiple copies of the plasmid integrated at a single chromosomal locus.

Table 1. Protoplasts of *P. patens* were transformed with pHP 23 and subjected to increasing concentrations of G 418 at different times after transformation. The initial RTF was monitored 10 days after the initiation of selection. (Schaefer et al. in prep. b)

Selection	G 418-5	G 418-10	G 418-25	G 418-50
Day 2	18	15	10	7
Day 4	28	23	17	18
Day 6	33	34	26	23

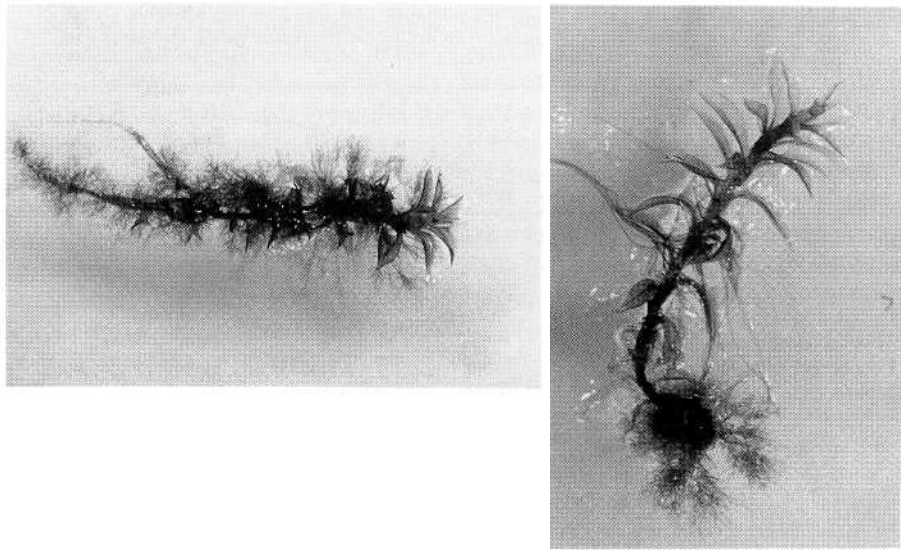
Table 2. Phenotypic characteristics of stable, unstable, and fast-growing unstable clones generated by the transformation of *P. patens* with antibiotic resistance genes. (Schaefer and Zryd, in prep. b)

Classes	I Stable transgenic plants	II Unstable replicative transformants	III Fast-growing unstable transformants
RTF	5×10^{-5}	10^{-1} to 10^{-2}	5×10^{-5}
Mitotic stability	100%	<5%	5 to 95%
Growth rate upon selective media	Normal	Reduced	+ Normal
Differentiation upon selective media	Normal	Altered	+ Normal
Meiotic transmission	100%	0%	Non mendelian
Phenotypic switches	None	To I and III	To I
Mosaic structures	None	Always	Frequent

Class II: Unstable Replicative Transformants. Unstable clones are characterized by their high frequency of occurrence and their high mitotic instability. These regenerants display a strong reduction in growth rate coupled with alteration in their patterns of differentiation upon selective medium. The resistance is rapidly lost in the absence of antibiotic and the presence of mosaic structures is common. Nevertheless, these clones can be propagated for many years if the selective pressure is maintained. Rescue experiments indicate that resistance to antibiotic is tissue-specific, being restricted to the protonema (Fig. 3) and accounting for the fact that no resistant spores have so far been recovered. Molecular analyses have shown that both the loss of resistance observed in the absence of selection and the non regeneration of gametophores in rescue experiments were correlated with a loss of the introduced sequences.

Class III:Fast-Growing Unstable Clones. This class is characterized by an improved mitotic stability, as compared with class II. These clones are observed with a frequency similar to that of the stable transgenic plants. They grow well on selective medium, but display a loss of resistance in the absence of selective pressure and often show the same tissue-specific regeneration pattern as unstable regenerants in rescue experiments. However, the presence of mosaic structures and the loss of resistance upon nonselective medium are reduced and occasionally difficult to demonstrate. Meiotic transmission of the character after selfing has occasionally been observed, but never accounted for 100% of the offspring. At the molecular level, a correlation between the phenotypic loss of resistance, in the absence of selective pressure, and a reduction in copy number has been established and preliminary data indicate that recombination events occurred within the introduced sequences.

Switches from class II to class III or class I and from class III to class I occurred spontaneously at rates similar to those observed for stable transformation events. However, this frequency increases following subculture, which involves the fragmentation of protonemata. Such switches would certainly require a recombination event and possibly chromosomal integration. The fact



a **b**

Fig. 3a,b. Rescue experiment using gametophores from a stable **a** and an unstable **b** clone on selective plates. Regeneration of protonemata occurs on the whole plant in the stable gametophore but is restricted to the filamentous tissue in the unstable (class II) and in the fast-growing unstable clones (class **III)**

that these switches are often associated with tissue disruption suggests that wound responses and DNA repair functions could be involved in this process. A model has been developed to account for these data, in which transformation of the moss *P. patens* is postulated as being essentially replicative with a low frequency of integration events by heterologous recombination. Bacterial plasmids would be maintained in moss cells as high molecular weight extrachromosomal arrays. The fast-growing unstable transformants would then correspond to replicative clones which have undergone an extrachromosomal recombination with genomic sequences conferring an improved mitotic stability to the introduced sequences. This type of transformation is similar to that observed in yeast (Struhl 1983) and has also been reported for *Caenorhabditis elegans* (Stinchcomb et al. 1985; Mello et al. 1991), but to our knowledge has never been reported in plants. If this model is confirmed, then transformation of the moss *P. patens* would represent a powerful model system for the identification of sequences involved in the replication and mitotic segregation of plant chromosomes.

2.3.3 Gene Targeting

The low frequency of stable transformation obtained with *Physcomitrella patens* led us to investigate the influence of homologous sequences on the

Table 3. Segregation analysis of clone GH 7 after selfing and crossing with the sensitive self-sterile *nicB5y!o6* strain. Both antibiotic resistance and the *ylo* phenotype were scored. The kanamycin and the hygromycin markers are linked and segregate independently from the *ylo* marker. (Schaefer et al. in prep. a)

	Total	Sensitive *ylo*	Sensitive wt	Kan^R	Hg^R	Kan^R + Hg^R *ylo*	Kan^R + Hg^R wt
GH 7x *nicB5ylo6*	134	29	33	0	0	35	37
GH 7 Self	104	0	0	0	0	0	104

transformation rate. Preliminary data have indicated that the addition of moss genomic DNA fragments on plasmids used for transformation increased the number of stable clones (W. Kammerer, pers. comm.). Since pHP 23 and pGL 2 are homologous, except for the coding sequence of the resistance gene, we have transformed six independent transgenic strains (initially transformed with pHP 23b or pGL 2) with the other plasmid and selected for double-resistant transgenic plants (Schaefer et al., in prep. a). A ten-fold increase in stable transformation was obtained with stable RTF ranging from 10^{-4} to 10^{-3}. Segregation analysis was conducted by crossing seven double-resistant transgenic plants with the sensitive self-sterile strain *nicB5ylo6* (Ashton and Cove 1977). The kanamycin and hygromycin markers cosegregated into six clones (Table 3), while the seventh clone did not display any linkage between the markers. These data strongly suggest that the second plasmid is integrated at the same locus as the first plasmid. Molecular analysis has to be undertaken to show whether or not this integration has occurred through homologous recombination. If such a mechanism of integration is confirmed, then transformation of the moss *P. patens* would represent the first haploid system suitable for the development of reverse genetics in plant research.

2.3.4 Overexpression of Morphogenetic Genes

P. patens has been developed as a model genetic system to study morphogenetic processes in plants. Among the major factors influencing moss development are cytokinin and phytochrome. The effects of the overexpression of these factors on the development of *P. patens* are currently being studied in our laboratory. Wild-type protoplasts were transformed with the potato phytochrome A gene *{phyA)* (Heyer and Gatz 1992) or with the isopentenyl transferase *(ipt)* gene from *A. tumefaciens* (Schmülling et al. 1989). Both genes were under the control of the 35S CaMV promoter *(35S-PhyA* from C. Gatz, unpubl., and *35S-ipt* from A. Spena, unpubl.). Stable clones have been obtained following transformation with the *35S-phy A* construct, whereas the *35S-ipt* has only been introduced into unstable clones. Both genes are biologically active in *P. patens* (Fig. 4). Phenotypically, regenerants overexpressing the *ipt* gene resembled wild-type plants

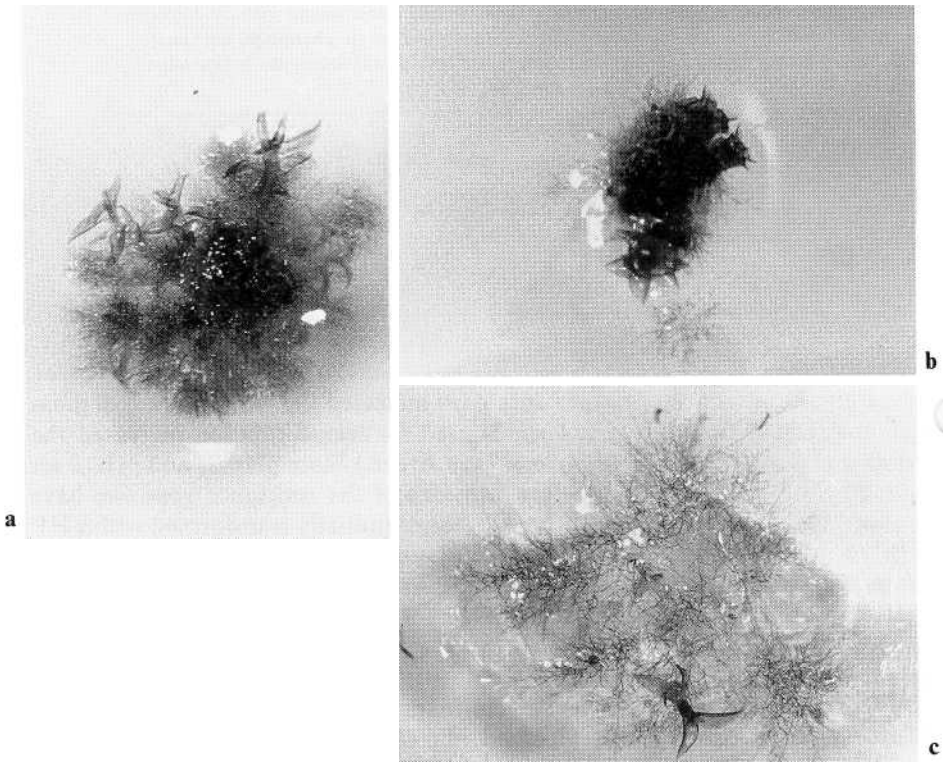


Fig. 4a-c Phenotype of regenerating colonies of wild-type stable kanamycin-resistant plants a, wild-type unstable kanamycin-resistant plant overexpressing the *ipt* gene b, and wild-type stable kanamycin-resistant plant overexpressing the potato *phyA* gene c. Colonies were grown on selective plates and photographs were taken one month after transformation.

maintained on medium supplemented with cytokinin (Ashton et al. 1979b) and the OVE mutants (Ashton et al. 1979a), i.e., strong inhibition of protonema development and excessive differentiation of buds (Schaefer 1994). The phenotype obtained following transformation with the potato *phyA* gene is more pleiotropic (Bisztray et al. in prep). It includes an overall reduction in the rate of branching of the protonema and a reduction in the number and size of the gametophores. This phenotype is reminiscent of the effect of monocot *phyA* genes expressed in transgenic dicotyledonous plants (Boylan and Quail 1989; Keller et al. 1989) and once again points towards conservation of morphological processes between angiosperms and bryophytes.

3 Summary and Conclusion

An efficient procedure based on the PEG-mediated direct gene transfer technique has been developed to genetically transform protoplasts of the moss

Physcomitrella patens. The introduction of plasmids carrying antibiotic resistance markers yields a very high number of resistant clones. However, stable transgenic plants represent less than 0.01% of regenerating colonies. The remaining resistant clones are probably replicative transformants carrying the resistance gene as an autonomously replicated extrachromosomal element. Replicative transformation is well documented in yeast and was the first step in the development of YAC (yeast artificial chromosome) vectors (Burke et al. 1987). A similar approach with *P. patens* might lead to the development of a moss artificial chromosome.

A ten-fold increase in the stable transformation frequency has been obtained when plasmids used for transformation were carrying sequences homologous to chromosomal DNA. Segregation analysis has demonstrated that the markers used in these experiments were linked. These data suggest that integration by homologous recombination occurs at a higher rate than random integration in the haploid *P. patens* and opens the way to the development of efficient reverse genetics.

The *ipt* gene of *Agrobacterium tumefaciens* and the *phyA* gene from potato have been shown to be biologically active in *P. patens,* suggesting that some of the mechanisms that control plant development are conserved between mosses and angiosperms.

An efficient transient gene expression assay has also been developed with this procedure. It was demonstrated that both the wheat *cab-1* and tobacco *rbcS* promoters were properly light-regulated in protoplasts of *P. patens.* This indicates a level of functional comparability in the regulation of gene expression between mosses and angiosperms.

Preliminary results indicate that the same type of transformation occurs in the moss *Ceratodon purpureas,* suggesting that replicative transformation could be a common mechanism in bryophytes (Schaefer 1994). The large size of the genome of P. *patens* (8×10^8 bp in 26 chromosomes, N. Grimsley, J.-P. Zryd, and M. Newton, unpubl.) and the lack of a genetic map constitute a disadvantage for the development of this organism as a model genetic system, and other mosses with a smaller genome could be more suitable for such an approach. However, the successful tranformation of *P. patens* is the first step towards the development of molecular genetics in bryophytes and the data presented in this review suggest that this approach could be extremely fruitful in the genetic dissection of plant developmental processes.

References

Ainley WM, Key JL (1990) Development of a heat shock inducible expression cassette for plants: characterisation of parameters for its use in transient expression assays. Plant Mol Biol 14:949-967

Ashton NW, Cove DJ (1977) The isolation and preliminary characterisation of auxotrophic and analogue resistant mutants in the moss *Physcomitrella patens.* Mol Gen Genet 154:87-95

Ashton NW, Cove DJ, Featherstone DR (1979a) The isolation and physiological analysis of mutants of the moss *Physcomitrella patens,* which over-produce gametophores. Planta 144:437-442

Ashton NW, Grimsley NH, Cove DJ, (1979b) Analysis of gametophytic development in the moss*Physcomitrella patens,* using auxin and cytokinin resistant mutants. Planta 144:427-435
Ashton NW, Boyd PJ, Cove DJ, Knight CD (1988) Genetic analysis in *Physcomitrella patens.* In:Glime JM (ed) Methods in bryology. Proc Bryol Meth Worksh, Mainz. Hattori Botanical Laboratory, Tokyo, pp 59-72
Bisztray G, Lamparter T, Gatz C, Heyer A, Schaefer DG, Zryd J-P, Hartmann E, Hughes J Preliminary studies of the moss *Physcomitrella patens* transformed with potato *PhyA.* Physiol Plant (in prep)
Bonneville J-M, Sanfaçon H, Fütterer J, Hohn T (1989) Posttranscriptional (rans-activation in cauliflower mosaic virus. Cell 59:1135-1143
Boylan MT, Quail PH (1989) Oat phytochrome is biologically active in transgenic tomatoes. Plant Cell 1:765-773
Burke DT, Carle GF, Olson MV (1987) Cloning of large segments of exogenous DNA into yeast by means of artificial chromosome vectors. Science 236:806-812
Callis J, Fromm M, Walbot V (1987) Introns increase gene expression in cultured maize cells. Genes Dev 1: 1183-1200
Capecchi MR (1989) Altering the genome by homologous recombination. Science 244:1288-1292
Cove DJ (1983) Genetics of Bryophyta. In: Schuster RM (ed) New manual of bryology. Hattori Botanical Laboratory, Tokyo pp 222-231
Cove DJ (1992) Regulation of development in the moss, *Physcomitrella patens.* In: Brody S, Cove D J, Ottolenghi S, Russo VEA (eds) Developmental biology. A molecular genetic approach. Springer Berlin, Heidelberg New York, pp 179-193
Cove DJ, Ashton NW (1984) The hormonal regulation of gametophytic development in bryophytes. In: Dryer AF, Duckett JG (eds) The experimental biology of bryophytes. Academic Press, London, pp 177-201
Cove DJ, Ashton NW (1988) Growth regulation and development in *Physcomitrella patens:* an insight into growth regulation and development of bryophytes. Bot J Linn Soc 98:247-252
Cove DJ, Schild A, Ashton NW, Hartmann E (1978) Genetic and physiological studies of the effect of light on the development of the moss *Physcomitrella patens.* Photochem Photobiol 27:249-254
Cove DJ, Kammerer W, Knight CD, Leach MJ, Martin CR, Wang TL (1991) Developmental genetic studies of the moss *Physcomitrella patens.* In: Jenkins GI, Schuch W (eds) Molecular biology of plant development. Society for Experimental Biology, Cambridge, pp 31-43
Engel PP (1968) The induction of biochemical and morphological mutants in the moss *Physcomitrella patens.* Am J Bot 55 :438-446
Fejes E, Pay A, Kanevsky I, Szell M, Adam E, Kay S, Nagy F (1990) A 268 by upstream sequence mediates the circadian clock-regulated transcription of the wheat Cab-1 gene in transgenic plants. Plant Mol Biol 15:921-932
Feldmann KA (1991) T-DNA insertion mutagenesis in *Arabidopsis:* mutational spectrum. Plant J 1: 71-82
Grimsley NH, Ashton NW, Cove DJ (1977) The production of somatic hybrids by protoplast fusion in the moss *Physcomitrella patens.* Mol Gen Genet 154:97-100
Grimsley NH, Featherstone DR, Courtice GRM, Ashton NH, Cove DJ (1980) Somatic hybridization following protoplast fusion as a tool for the genetic analysis of the development in the moss *Physcomitrella patens.* In: Ferenczy L, Farkas GL (eds) Advances in protoplast research. Proc 5th Intl Protoplast Symp, July 9-14, 1979, Szeged, Hungary. Pergamon Press, Oxford, pp 363-376
Halfter U, Morris P-C, Willmitzer L (1992) Gene targeting in *Arabidopsis thaliana.* Mol Gen Genet 231:186-193
Harkins KR, Jefferson RA, Kavanagh TA, Bevan MW Galbraith DW (1990) Expression of photosynthesis-related gene fusions is restricted by cell type in transgenic plants and in transfected protoplasts. Proc Natl Acad Sci USA 87:816-820
Heyer A, Gatz C (1992) Isolation and characterisation of a cDNA-clone coding for potato type A phytochrome. Mol Gen Genet 18:535-544
Jefferson RA (1987) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep 5:387-405
Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucoronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO 6:3901-3907

Jenkins GI, Cove DJ (1983) Light requirement for the regeneration of protoplasts of the moss *Physcomitrella patens.* Planta 157:39-45

Keller JM, Shanklin J, Vierstra RD, Hershey HP (1989) Expression of a functional monocotyledon nous phytochrome in transgenic tobacco. EMBO J 8:1005-1012

Knight CD, Cove DJ, Boyd PJ, Ashton NW (1988) The isolation of biochemical and developmental mutants in the moss *Physcomitrella patens.* In: Glime JM (ed) Methods in bryology. Proc Bryol Meth Worksh, Mainz. Hattori Botanical Laboratory, Tokyo, pp 47-58

Knight CD, Futers TS, Cove DJ (1991) Genetic analysis of a mutant class of *Physcomitrella patens* in which the polarity of gravitropism is reversed. Mol Gen Genet 230:12-16

Koncz C, Nemeth K, Redei GP, Schell J (1992) T-DNA insertional mutagenesis in *Arabidopsis.* Plant Mol Biol 20:963-976

Lipphardt S, Brettschneider R, Kreuzhaler F, Schell J, Dangl JL (1988) UV-inducible transient expression in parsley protoplasts identifies regulatory cis-elements of a chimeric *Antirrhinum majus* chalcone synthase gene. EMBO J 7:4027-4033

Mello CC, Kramer JM, Stinchcomb D, Ambros V (1991) Efficient gene transfer in *Caenorhabditis elegans:* extrachromosomal maintenance and integration of transforming sequences. EMBO J 10:3959-3970

Nagy F, Kay S, Boutry M, Hsu M-Y, Chua N-H (1986) Phytochrome-controlled expression of a wheat *Cab* gene in transgenic tobacco seedlings. EMBO J 5:1119-1124

Nagy F, Boutry M, Hsu M-Y, Wong M, Chua N-H (1987) The 5'-proximal region of the wheat *Cab-1* gene contains a 268-bp enhancer-like sequence for phytochrome response. EMBO J 6:2537-2542

Nagy F, Kay SA, Chua N-H (1988) A circadian clock regulates transcription of the what *Ca*b-1 gene. Genes Dev 2: 376-382

Negrutiu I, Dewulf J, Pietrzak M, Botterman J, Rietveld E, Wurzer-Figurelli EM, Ye D, 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 JA, Haagsman HJ, Does MP, van den Elzen PJM, Hooykaas PJJ (1990) Extrachromosal homologous recombination and gene targeting in plant cells after *Agrobacterium* mediated transformation. EMBO J 9: 3077-3084

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

Pietrzak M, Shillito RD, Hohn T, Potrykus I (1986) Expression in plants of two bacterial antibiotic resistance genes after protoplast transformation with a new plant expression vector. Nucleic Acids Res 14:5857-5868

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

Sawahel W, Onde S, Knight C, Cove D (1992) Transfer of foreign DNA into *Physcomitrella patens* protonemal tissue by using the gene gun. Plant Mol Biol Rep 10:314-315

Schaefer DG (1994) Molecular genetic approaches to the biology of the moss *Physcomitrella patens.* PhD Thesis, University of Lausanne

Schaefer DG, Zryd J-P, Knight CD, Cove DJ (1991) Stable transformation of the moss *Physcomitrella patens.* Mol Gen Genet 226:418-424

Schaefer DG, Nodin M-H, Zryd J-P Gene targeting in the moss *Physcomitrella patens,* (in prep a)

Schaefer DG, Zryd J-P Replicative transformation in the moss *Physcomitrella patens.* (in prep b)

Schäffner A, Sheen J (1991) Maize *rbcS* promoter activity depends on sequence elements not found in dicot *rbcS* promoters. Plant Cell 3:997-1012

Schmülling T, Beinsberger S, De Greef J, Schell J, Van Onckelen H, Spena A (1989) Construction of a heat-inducible chimeric gene to increase the cytokinin content in transgenic plant tissue. FEBS Lett 249:401-406

Sheen J (1990) Metabolic repression of transcription in higher plants. Plant Cell 2:1027-1038

Shillito RD, Saul M, Paszkowski J, Müller M, Potrykus I (1985) High efficiency direct gene transfer to plants. Bio/Technology 3:1099-1103

Stinchcomb DT, Shaw JE, Carr SH, Hirsh D (1985) Extrachromosomal DNA transformation of *Caenorhabditis elegans.* Mol Cell Biol 5:3484-3496

Struhl K (1983) The new yeast genetics. Nature 305:391-397
Walbot V (1992) Strategies for mutagenesis and gene cloning using transposon tagging and T-DNA insertional mutagenesis. Annu Rev Plant Physiol Plant Mol Biol 43:49-82
Wang TL, Cove DJ (1989) Mosses - lower plants with high potential. Plants Today 2:44-50