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MOLECULAR GENETIC APPROACHES TO THE BIOLOGY OF THE MOSS PHYSCOMITRELLA PATENS

THESE DE DOCTORAT

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To Life,

A fascinating Mystery

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RESUME DE LA THESE

La mousse haplobiontique *Physcomitrella patens* est utilisée comme système génétique modèle pour l'étude du développement des plantes. Cependant, l'absence d'un protocole efficace de transformation a constitué jusqu'à présent un gros désavantage méthodologique pour le développement futur de ce système expérimental.

Les résultats présentés dans le premier chapitre relatent la mise au point d'un protocole de transformation basé sur la technique de transfert direct de gènes dans des protoplastes par précipitation au PEG. Un essai d'expression transitoire de gènes a été mis au point. Ce protocole a été adapté afin de permettre l'introduction *in vivo* d'anticorps dans des protoplastes. Le protocole modifié permet d'introduire simultanément du DNA et des IgG dans les cellules, et nous avons démontré que ces anticorps peuvent inactiver spécifiquement le produit d'un gène co-introduit (GUS), ainsi que certaines protéines impliquées dans des processus cellulaires (tubuline). Cet essai, baptisé "essai transitoire d'immuno-inactivation *in vivo*", devrait être directement applicable à d'autres protoplastes végétaux, et permettre l'élaboration de nouvelles stratégies dans l'étude de processus cellulaires.

Le second chapitre est consacré aux expériences de transformation de la mousse avec des gènes conférant une résistance à des antibiotiques. Nos résultats démontrent que l'intégration de gènes de résistance dans le génome de *P. patens* est possible, mais que cet événement est rare. Il s'agit là néanmoins de la première démonstration d'une transformation génétique réussie de cet organisme. L'introduction de gènes de résistance aux antibiotiques dans les protoplastes de *P. patens* génère à haute fréquence des clones résistants instables. Deux classes de clones instables ont été identifiés. La caractérisation phénotypique, génétique et moléculaire de ces clones suggère fortement que les séquences transformantes sont concaténées pour former des structures de haut poids moléculaire, et que ces structures sont efficacement répliquées et maintenues dans les cellules résistantes en tant qu'éléments génétiques extrachromosomaux. Ce type de transformation nous permet d'envisager des expériences permettant l'identification des séquences génomiques impliquées dans la replication de l'ADN de mousse.

Plusieurs lignées transgéniques ont été retransformées avec des plasmides portant des séquences homologues aux séquences intégrées dans le génome, mais conférant une résistance à un autre antibiotique. Les résultats présentés dans le troisième chapitre montrent que les fréquences de transformation intégrative dans les lignées transgéniques sont 10 fois plus élevées que dans la lignée sauvage, et que cette augmentation est associée à une coségrégation des gènes de résistance dans la plupart des clones testés. Ces résultats génétiques indiquent que l'intégration de séquences d'ADN étranger dans le génome de *P. patens* a lieu en moyenne 10 fois plus fréquemment par recombinaison homologue que par intégration aléatoire. Ce rapport homologue/aléatoire est 10000 fois supérieur aux rapports obtenus avec d'autres plantes, et fournit l'outil indispensable à la réalisation d'expériences de génétique inverse dans cet organisme à haplophase dominante.

THESIS SUMMARY

The moss *Physcomitrella patens* is used as a model genetic system to study plant development, taking advantage of the fact that the haploid gametophyte dominates in its life cycle. But further development of this model system was hampered by the lack of a protocol allowing the genetic transformation of this plant.

We have developed a transformation protocol based on PEG-mediated direct gene transfer to protoplasts. Our data demonstrate that this procedure leads to the establishment of an efficient transient gene expression assay. A slightly modified protocol has been developed allowing the *in vivo* introduction of antibodies in moss protoplasts. Both DNA and IgGs can be loaded simultaneously, and specific antibodies can immunodeplete the product of an expression cassette (GUS) as well as proteins involved in cellular processes (tubulins). This assay, named transient *in vivo* immunodepletion assay, should be applicable to other plant protoplasts, and offers new approaches to study cellular processes.

Transformations have been performed with bacterial plasmids carrying antibiotic resistance expression cassette. Our data demonstrate that integrative transformation occurs, but at low frequencies. This is the first demonstration of a successful genetic transformation of mosses. Resistant unstable colonies are recovered at high frequencies following transformation, and two different classes of unstable clones have been identified. Phenotypical, genetic and molecular characterisation of these clones strongly suggests that bacterial plasmids are concatenated to form high molecular arrays which are efficiently replicated and maintained as extrachromosomal elements in the resistant cells. Replicative transformation in *P. patens* should allow the design of experiments aimed at the identification of genomic sequences involved in moss DNA replication.

Transgenic strains have been retransformed with bacterial plasmids carrying sequences homologous to the integrated transloci, but conferring resistance to another antibiotic. Our results demonstrate an order of magnitude increase of integrative transformation frequencies in transgenic strains as compared to wild-type, associated with cosegregation of the resistance genes in most of these double resistant transgenic strains. These observations provide strong genetic evidence that gene targeting occurs about ten times more often than random integration in the genome of *P. patens*. Such ratio of targeted to random integration is about 10 000 times higher than previous reports of gene targeting in plants, and provides the essential requirement for the development of efficient reverse genetics in the haplodiplobiontic *P. patens*.

1. GENERAL INTRODUCTION

The suitability of mosses as model systems to study plant biology

Mosses (division Bryophyta) are a very successful group of small non vascular green plants widely distributed around the world and able to endure extreme environmental conditions. Their suitability as model systems to study plant cellular and developmental processes was already recognised in the 20s and 30s, and is associated with the following characteristics (for ref., see (Dyer and Duckett 1984; Wang and Cove 1989)).

(1) Mosses were the first plants to be successfully propagated under axenic conditions and for some species, several generations can be completed in one year on simple mineral media.

(2) Mosses anatomical structures are simple, with relatively few cell types and organs composed of single cell sheets. However, despite this apparent simplicity, mosses display sophisticated morphological structures associated with complex morphogenetic processes.

(3) The haploid gametophyte dominates in the life cycle of Bryophytes, which facilitates mutagenesis and genetic analysis. Yet, sexually fertile diploid gametophyte can be generated by apospory, a process by which sporophytic tissue is induced to regenerate a diploid protonema, and haploidisation of a diploid gametophyte can be obtained in some species following differentiation of spore capsules from unfertilized gametophytic tissue (apogamy).

(4) The gametophyte development can be subdivided in two phases: the protonema stage, which is characterised by a branched network of filamentous cells displaying unidimensional apical growth, and the gametophore stage, which displays three-dimensional apical and intercalary growth. The former allow physiological and developmental processes to be monitored *in vivo* at the single cell level, whereas the latter displays developmental processes analogous to that of higher plants.

(5) Mosses development is regulated by similar growth substances (such as auxins, cytokinins, abscisic acid, gibberelins) and environmental factors (such as light and gravity) to those regulating higher plants morphogenesis.

(6) Mosses display an incredible regeneration capacity and in some species any part of the plant can regenerate a protonema under appropriate growth conditions. This regeneration is associated with direct differentiation of protonematal cells from differentiated tissues, and usually does not involve callus formation and dedifferentiation. One can therefore consider that in these species, every single moss cell is developmentally totipotent.

These features fulfil some of the major experimental requirements for the establishment of a model system, and no other plants reach such an ideal situation. Yet the recent development of mosses as model systems was hampered by the lack of an efficient procedure allowing genetic transformation of these plants. The purpose of the work presented in this dissertation was to develop procedures allowing to use the powerful molecular genetic approach in studies of the moss *Physcomitrella patens*.

Physcomitrella patens, a small moss with high potential

P. patens is a monoecious widely spread moss which colonises open habitat in cold temperate zones. Recent work with this moss was initiated in the middle of the 70s in the group of Pr. David Cove, who is now in Leeds. Two reasons have led to this choice: the short life cycle of the plant *in vitro* and the successful chemical induction and genetic characterisation of biochemical and developmental mutants achieved by Paulinus Engel (Engel 1968). Since then, important progresses have been achieved in the development of *P. patens* as an experimental genetic model system, and the main features of this system will be briefly described here, emphasizing the developmental processes which can be advantageously studied in this plant (for a complete and recent review, see (Cove 1992)).

Culture conditions.

In vitro propagation conditions have been standardised and the complete life cycle can be achieved in about 10 to 12 weeks in both continuous or discontinuous (16h / day) white light at 25^{o}C on a simple mineral medium (Ashton and Cove 1977). Large scale production of material in an air lift fermenter has been developed (Boyd, et al. 1988b), as well as conditions for continuous feeding of colonies with defined media (Ashton, et al. 1979). Time-lapse video microscopy has been established to monitor responses *in vivo* at the level of individual cells (Knight and Cove 1988). Fragmented protonema can be conveniently stored at 4^{o}C in sterile water for up to one year, dry sporophytes can be stored for several years, and a method for cryopreservation of self-sterile strains has been devised (Grimsley and Withers 1983).

P. patens life cycle

The life cycle of *P. patens* can be completed in 10 to 12 weeks in axenic culture and under well defined conditions (reviewed and ref. in Cove 1992). The haploid spore germinates within 48 hours after inoculation and gives rise to the protonema, a branched network of tubular cells composed of three different cell types. The first cells to differentiate, primary chloronemata, contain many chloroplasts, have a cell wall perpendicular to the axis of the filaments and constitute the photosynthetic assimilatory part of the colony. Caulonemata differentiate from some apical chloronemata around three days after germination. These cells are more vacuolated, contain fewer chloroplasts and have an oblique cell wall. Their cell cycle is 3 times shorter than that of chloronemata and they constitute the adventitious part of the protonema. Caulonemal side branch initials differentiate along caulonemal filaments. These cells can adopt four

different developmental pathways at different frequencies: quiescence (< 5%), development of a secondary chloronemal cell (90%), of a new caulonemal cell (5%) or of a leafy shoot primordia, the bud (3%). The relative frequencies of each developmental fates accounts for the general morphology of the colony. The differentiation of tetrahedral buds starts around ten days after spore germination. This developmental step corresponds to the transition from unidimensional apical growth to three-dimensional caulinary growth, and can be considered as being analogous to the establishment of a primitive meristem in higher plants. Filamentous rhizoids, resembling caulonemal filaments, differentiate from the bottom part of the bud, whereas the bud itself develops the gametophore. This latter is composed of a non vascular stem and of leaves formed by a single layer of cells. About a month after spore germination, colonies with well differentiated gametophores are transferred at 17^{o}C to induce gametogenesis and irrigated with sterile water to favour fertilisation events. Archegonia and antheridia differentiate on the gametophore, and the fusion of ciliated antherozoids with the archegonia forms the diploid sporophyte. Each sporophyte is the product of a single fertilisation event, and is structurally simple, with a very short stalk and a spore capsule containing around 4000 haploid spores generated in tetrads by spore mother cells. Mature sporophytes are collected about a month following the induction of gametogenesis and spores have to be vernalized (10 days at 4^{o}C) to achieved high germination frequencies.

Beside this normal life cycle, an efficient protoplast system has been developed which allows regeneration of up to 80 % of protoplasts into fertile plants (Grimsley, et al. 1977b). The production of new chloronemata from these protoplasts is strictly light-dependent (Jenkins and Cove 1983a), does not require an exogenous hormone supply and does not involve the dedifferentiation stages that are typical in angiosperms. One can therefore consider that protoplast regeneration is physiologically similar to spore germination.

Genetic analysis in *P.patens* .

Genetic analysis is facilitated in *P. patens*, since the haplophase dominate in the life cycle (reviewed and ref. in Ashton, et al. 1988). The size of the haploid genome of *P. patens* has been estimated around $8 \cdot 10^8$ base pairs (0.8 pg) in 26 chromosomes (M. Newton, N. Grimsley and J.-P. Zrÿd, unpubl. data, and Newton 1984). *P. patens* is self-fertile, since it is a monoecious species and does not display any mating type. To avoid the tedious identification of selfed versus crossed sporophytes in crosses between two self fertile strains, genetic crosses are usually conducted with at least one strain being a self-sterile but cross-fertile auxotrophic mutant. In these conditions, differentiated sporophytes collected on the mutant colony are usually generated by cross fertilisation. As an alternative method to sexual crossing, somatic hybridization of protoplasts has been established and used for complementation analysis and dominance studies in self-fertile diploid plants (Grimsley, et al. 1977a; Watts, et al. 1985). All segregation analysis performed so far, following sexual or parasexual crossing approaches, have demonstrated true haploidy of the gametophyte, despite the fact that the presence of 26 chromosomes could be indicative of an ancestral allopolyploidy (see (Cove 1983) and

(Featherstone, et al. 1990) for a discussion of the expected and monitored segregation ratio in haploid and diploid strains).

Chemical mutagenesis of *P. patens* has been achieved using spores (Ashton and Cove 1977) or somatic tissue (Boyd, et al. 1988a), and a large collection of mutants of the following classes have been isolated (reviewed and ref. in (Knight, et al. 1988): (1) auxotrophs, (2) analogue-resistant mutants, (3) mutants altered in phototropic or gravitropic responses (Knight, et al. 1991), (4) morphological mutants altered at different stages of protonema and gametophore development, and (5) hormone-resistant mutants (for auxins or cytokinins).

No genetic map of the genome of *P. patens* has yet been established, and consequently none of the isolated mutants have been mapped. Genetic linkage between different mutants has not yet been observed either. This constitute a serious disadvantage for further development of *P. patens* as a model genetic system. However, with the random amplified polymorphic DNA approach recently developed (RAPD) (Williams, et al. 1990), this methodological gap could be quite rapidly filled, since this technique is specially efficient for the mapping of haploid genomes.

Phytohormonal regulation of *P. patens* development

The role of auxins and cytokinins in the development of *P. patens* gametophyte has been investigated (reviewed and ref. in (Cove and Ashton 1984; Ashton, et al. 1988; Cove and Ashton 1988). These studies have indicated that normal development of the gametophyte was modulated by a fine tuning in the auxin/cytokinin ratio. Wild-type tissue grown on auxin supplemented medium displays an increased production of caulonemal filaments, whereas the presence of cytokinin induces a massive overproduction of gametophores, with up to 90% of caulonemal side branch initials developing a bud. Several classes of mutants resistant to these growth factors have been isolated, some of them being blocked in the chloronema-caulonema transition (*cal* -), others being unable to produce buds (*gam* -). Genetic analysis of these mutants identified several complementation groups and recessive mutations, indicating that the mutated phenotype was the result of loss of gene function. Physiological analysis showed that some of them are impaired in their ability to synthesize these substances, since exogenously supplied phytohormones restored the wild-type phenotype, while others may be impaired in the uptake and/or transport of these substances, or in the transduction pathway, since the mutated phenotype persisted even in the presence of an exogenous hormone supply. Different categories of *ove* mutants (gametophore overproducing) have also been isolated (Ashton, et al. 1979), which resemble the wild-type grown on cytokinin supplemented medium. Biochemical analysis demonstrated that these mutants have a higher cytokinin content as compared to the wild-type, and at least three complementation groups have been identified (Featherstone, et al. 1990). All these mutants provide very interesting model systems to study at the level of individual cells the phytohormonal regulation of plant elementary developmental processes, with the efficient combination of cellular, physiological and genetic approaches.

Light regulation of *P. patens* development.

Light plays an essential role in the normal development of *P. patens* and acts synergisticaly with phytohormones (Cove, et al. 1978; Jenkins and Cove 1983a; Ashton, et al. 1988). Spore germination, chloronema and protoplast division, and bud formation are light dependent processes that are blocked in darkness and far-red light. Under these conditions, caulonemal cells divide and etiolate, as illustrated by a dramatic increase in cell length associated with disappearance of chloroplasts and of side branch initials. New gametophores do not develop but differentiated gametophores etiolate too, forming very elongated stems and reduced leaves. The normal light growth pattern can be progressively restored with white or red light irradiations of increasing fluence or duration, but each developmental process will be restored at different light levels. For example, chloroplast development or caulonemal side branch initial differentiation are induced at very low fluences of red light, whereas spores and protoplasts germinate only after several hours of white or red light. Phytochrome is considered as the main photoreceptor involved in these processes, and red / far-red reversibility has been demonstrated for the establishment of the dark growth pattern.

The availability of a protoplast system displaying light-dependent physiological regeneration led us to investigate the involvement of phytochrome in this response. We have been able to show that the red light induced cell division could be partially reverted by a subsequent far-red irradiation (Y. Fracheboud and D. Schaefer, unpubl.), that most protoplasts divide and regenerate along the same axis under polarized red light (J.-P. Rey and S. Rometsch, unpubl.), and that a preliminary far-red treatment synchronises a population of protoplasts for at least the next two or three cell cycles (K. Bremer, A. Kakulya and D. Schaefer, unpubl.). These data provide physiological evidence for the involvement of phytochrome in the control of the cell cycle as well as in the establishment of cell polarity prior to mitosis. To our knowledge, these observations provide the first evidence for the implication of phytochrome in such processes in plant protoplasts, and there are no equivalent protoplast systems in higher plants where these responses could be studied. It is therefore believed that protoplasts of *P. patens* provide a physiological and genetic homogenous material to study the transduction pathway of phytochrome-mediated responses, a fundamental question in plant developmental biology which lack a competent unicellular experimental system to be efficiently investigated (for a recent discussion of the importance of single cell experimental systems in this field, see (Harter, et al. 1993; Neuhaus, et al. 1993)).

Photomorphogenetic mutants, analogous to the *hy*, *cop* or *det* mutants isolated in *Arabidopsis*, have not yet been isolated in *P. patens* (for a discussion of photomorphogenetic mutants in higher plants, see (Chasan 1993; Pepper, et al. 1993)). Further mutagenesis and screenings designed to isolate photomorphogenetic mutants should be performed, since the isolation of such mutants would complete the tools available to study photomorphogenesis in *P. patens*.

Tropic responses in *P. patens*

Protonemata and gametophores display both photo- and gravitropic responses (reviewed and ref. in (Cove and Knight 1987; Knight and Cove 1989)). Yet

phototropism appears to be epistatic to gravitropism in *P. patens* since gravitropic responses are only shown in darkness or in very low levels of light. Tropic responses of protonematal filaments offers the opportunity to follow these processes *in vivo* at the level of individual cells and this approach has been advantageously used to study gravitropic and phototropic responses in the moss *Ceratodon purpureus* (see for examples (Hartmann and Weber 1988; Walker and Stack 1990)). Caulonemal apical cells and gametophores display negative gravitropism in darkness. Several mutants belonging to three different complementation groups have been isolated, among which the very interesting *gtr* C mutants, in which the polarity of gravitropism is reversed (Jenkins, et al. 1986; Knight, et al. 1991). All these mutants display normal phototropic behaviour, indicating that the mutated phenotype is specific to the response to gravity.

Primary and secondary chloronemata, caulonemata and gametophores growth is influenced by light direction. Primary chloronemata and caulonemata display positive phototropism at low light intensities and perpendicular phototropism at high light intensities, whereas secondary chloronemata and gametophores display positive phototropism at all light intensities. Similar responses were monitored in primary chloronemata grown under red polarized light, with growth perpendicular to the electrical vector (E) at low light intensities and parallel to E at high light intensities (Jenkins and Cove 1983c). Several mutants, identified by the absence of phototropic responses in the gametophores and corresponding to three complementation groups, have been isolated (Jenkins and Cove 1983b). Interestingly, the phototropic responses of primary chloronemata is not lost in these mutants, but the switch from low light to high light tropic response occured at lower intensities than in wild-type. Physiological analysis supported the hypothesis that these responses were controlled by the photoequilibrium in the phytochrome pool.

Light and gravity are two essential environmental factors involved in plant morphogenesis. The possibility to investigate the role of these factors *in vivo* at the level of individual cells in wild-type and mutant strains offers the opportunity to combine efficient cellular, physiological and genetic approaches. Polarotropic responses are likely of no biological signification, but provide a valuable experimental model to study the mechanism by which a dichroic photoreceptor (in this case phytochrome) can determine cell polarity, and to investigate the cellular localisation of this receptor.

Molecular biology in *P. patens*.

Molecular biology in *P. patens* is still at its infancy. So far, only one gene encoding chlorophyll a/b binding protein and a *myb*-related gene have been cloned with heterologous probes and sequenced (Long, et al. 1989; Leech, et al. 1993). We have demonstrated that integrative transformation following PEG-mediated direct gene transfer to protoplasts was possible (Schaefer, et al. 1991). The data presented in this thesis are entirely devoted to the development of efficient molecular genetic approaches in *P. patens*. Alternative transformation methods, including *Agrobacterium* mediated transformation (D. Schaefer and N. Grimsley, unpubl.; C. Knight and B. Hohn, unpubl.), electroporation (D. Schaefer, unpubl.) and DNA transfer mediated by high velocity DNA-coated micro-projectiles (Sawahel, et al. 1992), have so far been

unsuccessful. The isolation of developmental genes by substractive hybridization of cDNA libraries as well as insertional mutagenesis by transposon are currently being investigated. However there is no evidence so far that transposons can be efficiently integrated in *P. patens* genome following their excision from a transforming plasmid (Cove, et al. 1991).

The general features of *P. patens* development described in this introduction illustrate the potential of this moss as a model system to investigate plant development. Fundamental questions, such as the role of auxins and cytokinins on plant cell growth and differentiation, the mechanisms underlying phytochrome-mediated responses, the mechanisms underlying tropical responses, or the transition from unidimensional growth to the establishment of a three-dimensional primitive meristem can be advantageously investigated in this plant with the efficient combination of physiological, cellular and genetic approaches at the level of individual cells. The data presented in this dissertation will demonstrate that it is now possible to add the powerful molecular genetic approach to investigate these questions. Further development of *P. patens* as a model system for the genetic analysis of plant development would be facilitated by the generation of tagged mutants, and perspectives in that view will be discussed at the end of the dissertation.

2. RESULTS

2.1. DEVELOPMENT OF A SENSITIVE TRANSIENT GENE EXPRESSION ASSAY AND AN EFFICIENT TRANSIENT *IN VIVO* IMMUNODEPLETION ASSAY FOLLOWING PEG-MEDIATED UPTAKE OF PLASMID DNA AND ANTIBODIES IN PROTOPLASTS OF THE MOSS PHYSCOMITRELLA PATENS

Abstract.
The conditions for PEG-mediated direct DNA transfer in protoplasts of *Physcomitrella patens* were optimized, using transient expression of a 35S GUS construct to monitor transfection efficiency. Osmolarity of the PEG solution, position and duration of heat shock, plasmid DNA concentration and incubation time in PEG solution were identified as the main factors affecting transfection efficiency. High levels of transient gene expression were achieved with this procedure and the sensitivity of the assay ranged over two orders of magnitude. Experiments performed with GUS constructs driven by heterologous light-inducible promoters indicated that phytochrome-mediated transient gene expression can be monitored in protoplasts of *P. patens*.
A slightly modified protocol was developed to introduce simultaneously plasmid DNA and antibodies in protoplasts. It was demonstrated that FITC-labelled antibodies can be efficiently introduced into protoplasts following PEG-mediated uptake, and that the expression of a 35S GUS construct introduced simultaneously was not altered by the presence of antibodies. Moreover, transient GUS expression could be blocked by antibodies raised against ß-glucuronidase, while anti-ß-tubulin monoclonal antibodies were shown to interfere with protoplast survival, regeneration and ploidy. Both responses were shown to be proportional to the concentration of antibodies in the samples, indicating that this approach can be used to immunotitrate and immunodeplete *in vivo* active factors in cellular processes.

Abbreviations: PEG, polyethylene glycol; GUS, ß-glucuronidase from E Coli; CaMV, Cauliflower mosaic virus; IgG, immunoglobulin G; FITC, fluorescein isothiocyanate; MUG, methylumbelliferyl ß-D-glucuronide; MU, 4-methylumbelliferone; BSA, bovine serum albumin; MES, morpholino ethanesulfonic acid; ppl, protoplast; wt, wild-type; rbcS, rubisco small subunit; cab, chlorophyll a/b binding protein.

2.1.1 INTRODUCTION

PEG-mediated direct gene transfer to protoplasts has been developed as an alternative method to transform plant species which were not responsive to *Agrobacterium tumefaciens* (Paszkowski, et al. 1984; Negrutiu, et al. 1987). Both transient gene expression and integrative transformation have been achieved and the

role of different physical and chemical parameters on the efficiency of the procedure has been carefully investigated (Maas and Werr 1989; Gharti-Chhetri, et al. 1990; Negrutiu, et al. 1990; Gharti-Chhetri, et al. 1992). These studies have demonstrated that the mechanism of DNA uptake is based on the synergistic interaction of protoplasts and DNA with PEG, divalent cations such as Ca^{2+} and / or Mg^{2+} and heat shock. Plasmid DNA and protoplasts are mixed to allow adsorbtion of the DNA on the cell surface and the subsequent addition of PEG permeabilises the plasmalemma and simultaneously precipitates extracellular DNA within the cells. The process is almost instantaneous and DNA uptake occurs in most of the cells. The efficiency of the method relies on the high survival rate of PEG-treated protoplasts and the procedure is widely applicable since high levels of transient gene expression have been obtained with all plant species tested so far.

In our published procedure (Schaefer, et al. 1991), the survival rate of *P. patens* protoplasts following PEG-mediated DNA uptake was low. This led to an investigation of the role of various steps involved in the protocol, using transient expression of a 35S GUS cassette to monitor transfection efficiency.

Transient gene expression assay is a powerful analytical tool to study the regulation of gene expression. The assay relies on the fact that expression cassettes are readily transcribed and translated following uptake, allowing the expression of a reporter gene to be assayed within 24 to 48 hours after transfection. With this procedure and in competent cells, fine dissection of promoters can be achieved by 5' deletion analysis and *cis*- and *trans*- acting factors can be rapidly identified and characterised. Several features of the regulation of gene expression in plants have been successfully addressed this way, including:

(1) the role of introns for efficient gene expression (Callis, et al. 1987; Maas, et al. 1992),

(2) the identification of regulatory sequences in promoters responsive to growth factors (Marcotte, et al. 1988), heat shock (Ainley and Key 1990), anaerobic stress (Walker, et al. 1987), UV light and elicitors (Lipphardt, et al. 1988) and white light (Harkins, et al. 1990; Schäffner and Sheen 1991),

(3) interactions between energy metabolism and gene expression (Sheen 1990),

(4) more complex processes such as posttranscriptionnal transactivation of dicistronic mRNA mediated by viral genes (Bonneville, et al. 1989; Fütterer and Hohn 1991) or interactions between nuclear oncogenes such as *ras2, fos* and *jun*, signal transduction and gene expression (Hilson, et al. 1990a; Hilson, et al. 1990b).

However this approach has not yet been successful in the study of several other mechanisms of regulation, and it is believed that this limitation is associated with the competence of protoplasts for the investigated response. The physiological regeneration of *P. patens* protoplasts led to the postulate that some of these limitations might be circumvented in these cells, and a sensitive transient gene expression assay was therefore established.

Promoters of the genes encoding chlorophyll a/b binding protein (*cab*) and rubisco small subunit (*rbcS*) have been used as models to study light- and phytochrome-mediated gene expression in higher plants (Thompson and White 1991). *Cis*- and *trans*- acting factors have been identified (reviewed in (Gilmartin, et al. 1990; Quail 1991)), but progresses in the analysis of the transduction pathway was slowed down by the lack of a competent unicellular system allowing transient gene expression experiments to be performed (discussed in (Neuhaus, et al. 1993)). The involvement of phytochrome in the early stages of *P. patens* protoplasts regeneration led us to investigate the possibility of developing a phytochrome-mediated transient gene expression assay in these cells. Plasmid rbcSGUS contains the GUS reporter gene under the control of a tobacco *rbcS* promoter and displays the characteristic light-inducible and tissue-specific expression pattern of *rbcS* gene in transgenic tobacco plants (Jefferson, et al. 1987). Plasmid cabGUS (F. Nagy, unpubl.) contains the GUS reporter gene under the control of the complete wheat *cab-1* gene promoter (Nagy, et al. 1986). This promoter was shown to contain the regulatory elements for phytochrome-mediated light induction and circadian control of gene expression in transgenic tobacco plants (Nagy, et al. 1987; Nagy, et al. 1988; Fejes, et al. 1990). Transient expression of these two constructs was investigated in protoplasts grown in different light conditions. Both constructs were expressed in moss protoplasts and the involvement of phytochrome in the regulation of cab-GUS expression was strongly suggested by its responses to red/far red irradiations.

Professor J.-P. Zrÿd's original idea of uploading monoclonal antibodies in *P. patens* protoplasts was directed towards the development of an *in vivo* immunolabeling assay. Initial experiments were performed with the sonication loading method (Fechheimer, et al. 1986; Fechheimer, et al. 1987) with FITC-labelled antibodies and uptake efficiency was monitored by fluorescence microscopy. Although labelled cells were obtained, this approach was hampered by severe problems of reproducibility and cell survival (J.-P. Zrÿd, unpubl.). The high efficiency of the PEG-mediated DNA uptake procedure and the fact that PEG is known to precipitate, beside DNA, several other biological macrostructures and macromolecules including proteins (for ref. see (Lis 1980)) led me to postulate that this method could be used to introduce antibodies in protoplasts. The main idea in this approach was to develop a new type of assay designed not only to label, but also to specifically inactivate cellular proteins with antibodies *in vivo* in a way similar to that achieved with antisense mRNA, and to be able to associate this approach with transient gene expression. To test the feasibility of such an assay, experiments were performed to answer the following questions.

(1) Is it possible to introduce antibodies in protoplasts following PEG-mediated uptake and what are the main parameters affecting uptake efficiency ?

(2) Is it possible to introduce simultaneously antibodies and plasmid DNA in protoplasts in order to achieve both efficient IgG uptake and transient gene expression ?

(3) Is it possible to inactivate *in vivo* the expression of a reporter gene with antibodies raised against the product of this gene or against trans-acting proteins involved in its expression ?

(4) Is it possible to specifically block cellular processes following the introduction of antibodies directed against one of the protein components of this process ?

(5) Is the inactivating process proportional to the concentration of IgGs in the samples, to determine whether this approach can be used to immunotitrate active proteins involved in cellular processes ?

(6) Is immunodepletion reversible which would indicate that the process is transient, being limited by the rate of IgGs proteolysis in the cells ?

The data presented in this chapter will demonstrate that antibodies and DNA can be efficiently uploaded in *P. patens* protoplasts following PEG-mediated uptake and that specific antibodies are able to inactivate products of transient gene expression and proteins involved in cellular processes. The level of inactivation is proportional to the concentration of antibodies in the samples and the process is transient as demonstrated by its reversibility. This new type of assay has been named "Transient *in vivo* immunodepletion" and it is believed that this approach could find applications in biological research.

2.1.2. RESULTS

2.1.2.1. Development of a sensitive transient gene expression assay

Transfections were performed in duplicates with supercoiled plasmid NcoGUS and each experiment was repeated with 2 or 3 different batches of protoplasts. The variation between duplicates never exceeded 15 % and similar qualitative results were obtained from different batches. Variability between separate experiments was more important with GUS specific activities ranging from 5 to 15 nmol MU·mg prot^{-1}·min^{-1} under optimum conditions. Negative controls, including untreated protoplasts, protoplasts and DNA without PEG, and protoplasts and PEG without DNA, never displayed specific activities higher than 0.05 nmol MU·mg prot^{-1}·min^{-1}. Data presented in figure 1 summarize the role of the main parameters affecting transient gene expression in *P. patens*. For reasons of clarity, data from one typical experiment only are reported.

The MMM solution was required to achieve high transformation efficiency. A 4 fold increase in specific activity was recorded as compared to protoplasts resuspended in mannitol 0.48M (data not shown).

Osmolarity of the PEG solution was found to be important for efficient DNA uptake (Figure 1-I). High levels of GUS expression were correlated with high survival rate of protoplasts and optimum mannitol concentration was established at 0.38 M. A 4 fold increase in specific activity was achieved as compared with our initial protocol (Schaefer, et al. 1991).

Duration of incubation in PEG was critical for efficient transient gene expression (Figure 1-II). Maximum expression was achieved within 5 to 15 minutes following addition of PEG. Longer incubation of up to 30 minutes resulted in a 10 fold decrease in specific activity which was correlated with a strong reduction in cell viability. Optimum incubation time was thus established at 10 minutes following heat shock.

Heat shock was essential to achieve high levels of transient gene expression. Both its position in the procedure and its duration were investigated. Heat shock was effective when performed on the whole batch of protoplasts in MMM (Figure 1-III A), and after (Figure 1-III C) but not prior (Figure 1-III B) to the addition of PEG to the samples. Though detectable GUS activities were routinely obtained without heat shock, a 10 fold increase in specific activity was monitored following 5 to 10 minutes of heat shock. This response to heat shock was not associated with variation in cell viability, and was apparently not saturated within 10 minutes (Figure 1-IV), suggesting that heat shock was affecting both the uptake process and the physiology of the cells. A 5 minute heat shock following the addition of PEG was established as a standard condition.

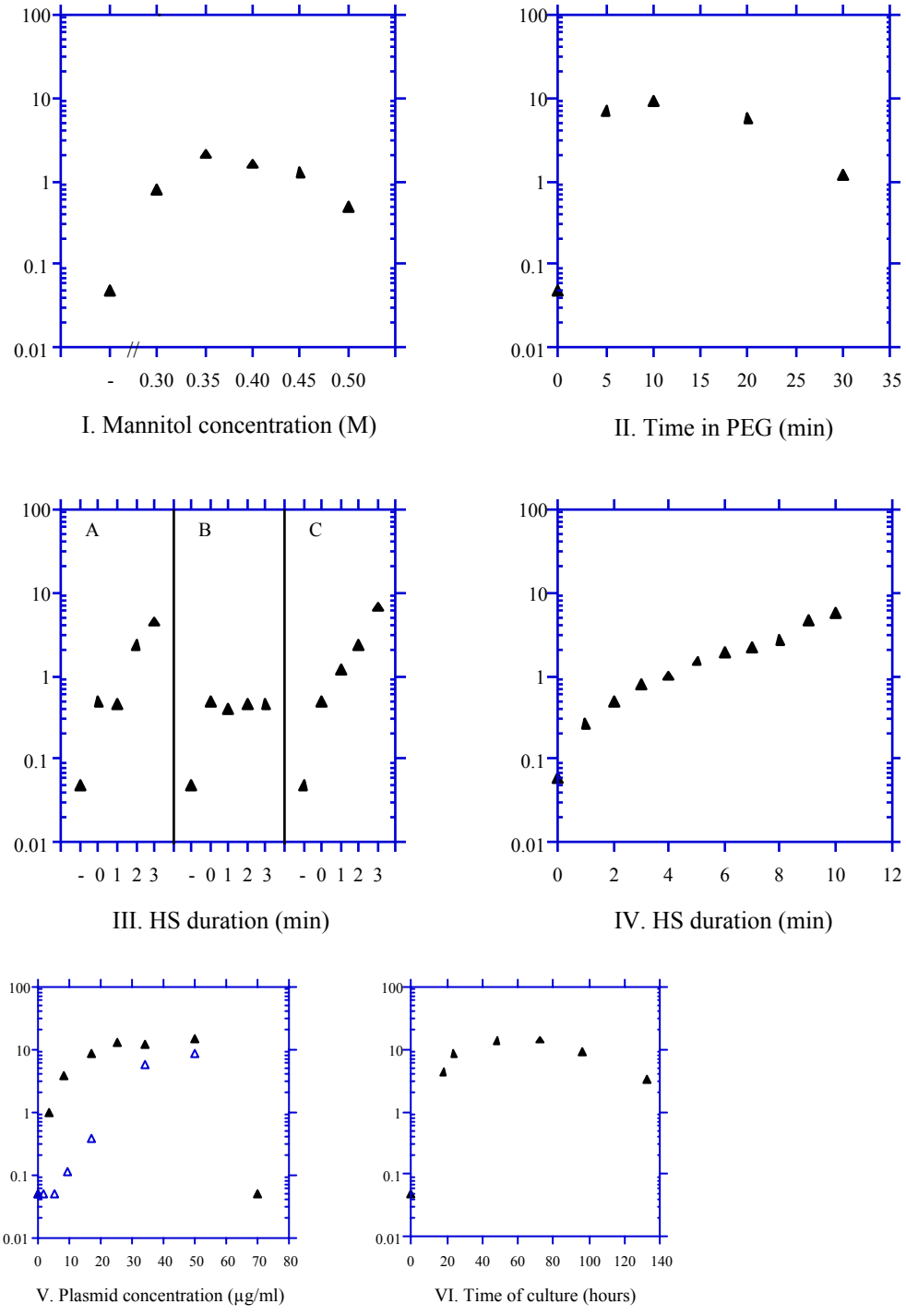

Figure 1. Effect of several transfection parameters on transient GUS expression in P. patens protoplasts. GUS specific activity in nmol MU·mg prot^{-1}·min^{-1} is expressed on a log scale as a function of (I) osmotic strength of PEG solution; (II) time in PEG; (III) heat shock position (A) ppl in MMM, (B) ppl + DNA, (C) ppl + DNA + PEG; (IV) heat shock duration; (V) plasmid DNA concentration with (Δ) and without (Δ) carrier DNA and (VI) time of culture.

DNA concentration was a critical factor for efficient DNA uptake (Figure 1-V). When the final DNA concentration was maintained constant at 50 µg/ml with sheared salmon sperm DNA, detectable GUS activity was monitored with as few as 3 µg/ml plasmid DNA and increased proportionally with DNA concentration up to 20 µg/ml

where saturation was reached. When DNA concentrations higher than 70 µg/ml were tested, a large precipitate containing DNA and cellular material was observed and GUS activity fell to undetectable levels. When increasing concentrations of plasmid DNA alone were tested, detectable GUS activity was monitored at 10 µg/ml and increased proportionally with DNA concentration until 40 µg/ml where saturation was reached. These data clearly showed that efficient DNA uptake occurred within a narrow interval of DNA concentrations, ranging from 35 to 50 µg/ml total DNA, and that carrier DNA did neither improve uptake efficiency nor transient gene expression levels, since similar GUS activities were obtained under saturating conditions. Optimum DNA concentration was thus established at 50 µg/ml plasmid DNA alone.

Transient gene expression as a function of time of culture was also investigated (Figure 1-VI). High levels of GUS activity were achieved within 18 hours, reaching a maximum between 30 and 70 hours following transfection. Specific activity after 5 days was about one fifth of maximum. GUS assays were therefore performed 48 or 72 hours following uptake.

In the original protocol a washing step with MMM was performed at the end of the procedure to eliminate PEG. This step was omitted since it resulted in a 3 fold decrease in specific activity which was associated with decreased cell viability, suggesting that permeabilised cells were preferentially lysed upon low speed centrifugation in MMM. Moreover, survival in liquid culture medium was about 2 fold higher in PEG-treated protoplasts as compared to untreated controls and cell regeneration was not altered (data not shown). Protoplast concentrations ranging from 0.5 to 2.5 x 10^6 / ml and sample volumes ranging from 50 to 1500 µl protoplast suspension were also tested and found to be not significant for efficient gene expression, indicating that the assay could easily be scaled up or down (data not shown). Standard assays were performed with 300 µl of protoplasts at 1.5 x 10^6 / ml and the soluble protein content of such samples ranged from 100 to 200 µg, corresponding to ca. 20 enzymatic assays. PEG concentrations, ranging from 30 to 40 % were also tested and found to be equally effective for transient gene expression (data not shown). Finally, it was reported that pH of the PEG solution was optimum between 6 and 7 for efficient DNA uptake (Maas and Werr 1989). This parameter was not investigated since the pH of the samples following the addition of PEG was measured and found to be around 6.7.

Optimum transfection conditions were thus established as described in the Material and Methods. With this procedure, an average of 50% cell survival was obtained in transient gene expression assays while about 30% of the cells regenerated in transformation experiments. GUS specific activities ranging from 5 to 15 nmol MU·mg^{-1}·min^{-1} were routinely obtained with NcoGUS and the sensitivity of the assay ranged over two orders of magnitude above background. These specific activities were in the same range than those measured in protein extracts from a transgenic tobacco plant transformed with a similar construct (data not shown).

Phytochrome-mediated transient gene expression

Several experiments were performed with plasmids NcoGUS, rbcSGUS and cabGUS to investigate the possibility of monitoring phytochrome-mediated transient gene expression. Data from these experiments will be only briefly presented here, since, although preliminary experiments were performed by myself, further experiments were done by Dr. G. Bisztray (Bisztray, et al. in preparation). Both plasmids rbcSGUS and cabGUS were expressed in light, indicating that these promoters were functional in *P. patens*. However, GUS specific activities monitored with these constructs were at least ten fold lower than those with plasmid NcoGUS, while cab and rbcS promoters are considered as strong plant promoters (for example, similar GUS specific activities were monitored with similar 35S-GUS and rbcSGUS constructs in transgenic tobacco plants (Jefferson, et al. 1987)). These low specific activities could indicate either that these promoters were not fully active in *P. patens*, or that the presence of glucose in protoplast liquid culture medium inhibited the activity of these promoters (for a discussion of the effects of a carbon source on the activity of light-inducible promoters, see (Sheen 1990; Harter, et al. 1993)). GUS activities were systematically two to three fold higher in white light as compared to that in darkness for both light-inducible constructs, but similar data were monitored for NcoGUS (data not shown). These results could indicate that the overall transcriptional activity was restricted in dark grown protoplasts and did not allow to conclude unambiguously that the expression of rbcSGUS and cabGUS was properly light regulated.

Therefore, transient expression of NcoGUS and cabGUS was investigated in protoplasts irradiated with red and far red light, since the reversion of a red light induced response by a subsequent far red irradiation was considered as a criterion establishing phytochrome involvement, and since plasmid NcoGUS was not expected to respond to such light treatments. Protoplasts were irradiated with short pulses of red and far red light, since the wheat *cab* promoter is known to display low fluence phytochrome-mediated responses (Nagy, et al. 1987). The red light-induced expression of NcoGUS was not reversed by a subsequent far red irradiation, whereas the expression of cabGUS was two fold higher in red light as compared to that in red / far red or in far red light alone (Table 1). Similar results were reproducibly monitored in several independent experiments and the kinetic of expression of cabGUS in different light conditions was investigated to confirm these point measurements (Figure 2).

Table 1. Protoplasts were transfected with plasmid NcoGUS or cabGUS and regenerated for 72 hours in different light conditions. GUS specific activities are expressed in nmol MU·mg^{-1}·min^{-1}.

Plasmid	GUS Activity Red	GUS Activity Red / far red	GUS Activity Far red
Nco GUS	1.60	2.20	1.70
Cab GUS	0.51	0.31	0.25

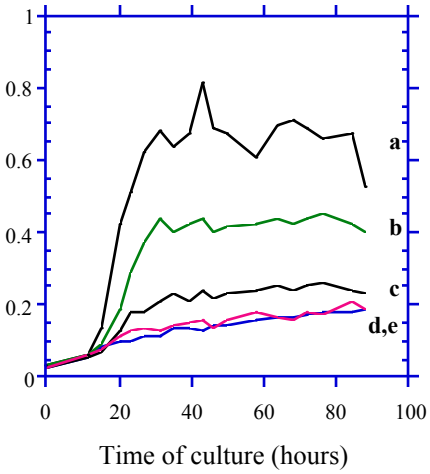

Figure 2. Protoplasts were transfected with plasmid cabGUS and regenerated under different light conditions: (a) white light, (b) red light, (c) red/far red light, (d) far red light, (e) darkness. Samples were taken at different times after transfection to determine GUS activity (in nmol MU·mg^{-1}·min^{-1})

These results provide physiological evidence for the involvement of moss phytochrome in the regulation of the cabGUS construct transiently expressed in *P. patens* protoplasts. It is therefore concluded that these cells are competent for phytochrome-mediated transient gene expression experiments. These data also show that heterologous higher plants promoters are functional in moss cells and suggest that some of the mechanisms involved in the regulation of gene expression by phytochrome are conserved between wheat and *P. patens*.

2.1.2.2. PEG-mediated IgG uptake in P. patens protoplasts

Since FITC-labelled IgGs could be introduced in *P. patens* protoplasts by sonication (J.-P. Zrÿd, unpubl.), the first step in this study was to compare this method with PEG-mediated uptake. FITC-labelled IgG working concentration (1 mg/ml) and the requirement for BSA as carrier (5 mg/ml) were established in a set of preliminary experiments (data not shown). FITC labelling of protoplasts was recognised by the presence of many fluorescent particles in the cells, sometimes associated with a weak diffused fluorescence (Figure 4). The intensity of the fluorescence decreased during the next days of culture, but fluorescent particles could still be detected 48 hours after uptake (data not shown). The experiment described in figure 3 was designed to define the ability of heat shock, sonication and PEG treatments, applied independently or in combination, to promote efficient IgG uptake in moss protoplasts. The following parameters were recorded: cell survival, % of FITC-labelled cells and intensity of labelling.

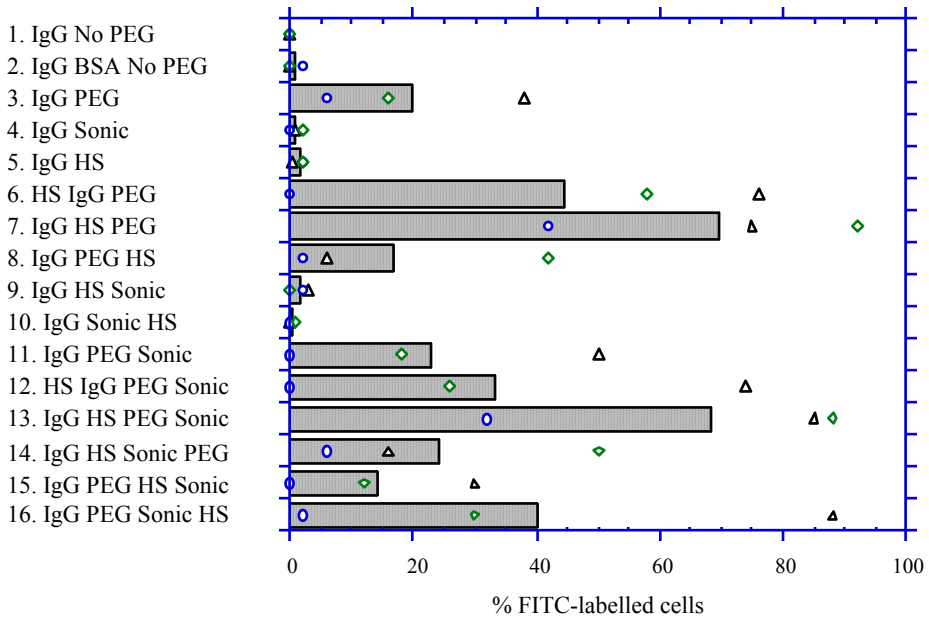

Figure 3. Determination of the efficiency of heat shock, sonication and PEG treatments in promoting IgG uptake in P. patens protoplasts. The experiment was repeated 3 times (Δ, ◊, o)*, and bars represent the mean percentage of FITC-labelled cells as determined from these values. Samples treated with the sequence IgG HS PEG ± sonication were also the more heavily labelled samples (lanes 7 and 13)*

The following conclusions were drawn from this experiment.

(1) Passive uptake of FITC-labelled IgGs did not occur when antibodies were simply mixed with protoplasts (lanes 1 and 2).

(2) Reproducible uptake resulting in 20 to 40% labelled cells were monitored when PEG alone was added, but not when heat shock or sonication alone were performed (lanes 3, 4, 5).

(3) Combination of PEG and heat shock induced efficient and reproducible IgG uptake with up to 90% of the cells being labelled (lanes 6, 7, 8). The position of heat shock in the procedure was critical and needed to be investigated further.

(4) Combination of heat shock and sonication did not promote IgG uptake (lanes 9, 10).

(5) Combination of heat shock, PEG and sonication induced IgG uptake to similar levels as for combination of PEG and heat shock alone (lanes 11 to 16 and compare 3 with 11, 6 with 12, 7 with 13 and 8 with 15). The position of heat shock in the protocol was once again critical. Sonication did not improve uptake efficiency but rather seemed to increase variability between samples and was occasionally associated with high cell mortality. It was therefore concluded that sonication was detrimental to efficient IgG uptake in moss protoplasts.

(6) Maximum uptake occurred when protoplasts, IgG and BSA were mixed together, heat shocked and subsequently treated with PEG (lanes 7 and 13). Under these conditions, survival rate was about 30% and an average of 80% of the living cells were

strongly labelled with many (< 20) fluorescent particles, sometimes accompanied by a weak diffuse fluorescence of the cytoplasm (Figure 4). These observations suggested that FITC-labelled IgGs were precipitated in cells upon the addition of PEG by a similar mechanism than DNA. These conditions were therefore chosen to further characterise the uptake process and to test the possibility of introducing simultaneously IgG and plasmid DNA in protoplasts.

Figure 4 link

Figure 4. FITC-labelled protoplasts of P. patens as observed 6 hours after PEG-mediated IgG uptake. From top left to bottom right: untreated protoplasts, protoplasts incubated with FITC-IgGs but without PEG, treated sample in UV light (excitation 390 - 490 nm, emission cut off 515 nm), treated sample in UV light (excitation 450 - 490 nm, emission cut off 515 nm). Samples were heat shocked prior to the addition of PEG and illustrate heavily labelled protoplasts.

A complete characterisation of each step of the protocol was not carried out but some additional experiments were performed to further define the role of heat shock, PEG and BSA on PEG-mediated IgG uptake. Heat shocks of increasing duration were performed before (Figure 5.I A) or after (Figure 5.I B) the addition of PEG. More than 80% of the cells were labelled following a 3 minute heat shock performed before the addition of PEG whereas only half of the cells were labelled when a 5 minute heat shock was performed after the addition of PEG. These data confirmed the results presented in figure 3 and clearly established the optimum position of heat shock in the protocol (mean value and standard deviation out of 4 independent experiments for a 3 minute heat shock: before PEG, 75 ± 20 % FITC-labelled cells; after PEG, 20 ± 16 %). A 3 minute heat shock performed before the addition of PEG was established as a standard condition.

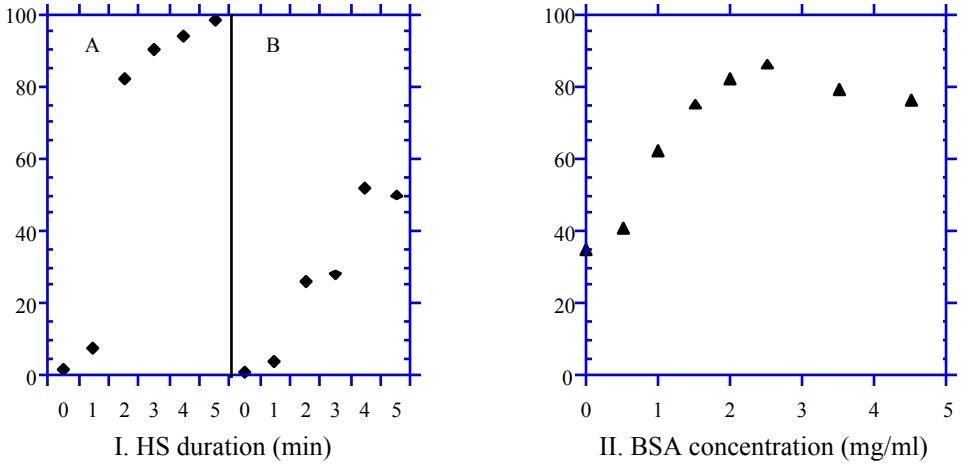

Figure 5. Dependence of PEG-mediated IgG uptake on (I) heat shock position and duration (A before PEG, B after PEG), and (II) BSA concentration.

PEG-mediated IgG uptake was performed in the presence of increasing concentration of BSA (Figure 5.II). Percentage of labelled cells increased proportionally with BSA concentration and reached a plateau at 2.5 mg/ml where over 80% of cells were labelled. Altering the ratio between BSA and IgGs (from 5+1 to 1+5) did not improve uptake efficiency as monitored by the percentage of labelled cells or by the intensity of labelling (data not shown). Standard IgG and BSA concentrations were thus established at 1 mg/ml and 5 mg/ml respectively.

Kinetics of IgG uptake as a function of time of incubation in PEG were also determined (data not shown). The percentage of labelled cells increased proportionally with the duration of incubation and reached a plateau within 15 minutes after the addition of PEG. At this stage more than 80% of the cells were labelled. Longer incubations in PEG, up to 35 minutes, did not induce a reduction in the percentage of labelled cells nor a decrease in cell survival, as for PEG-mediated DNA uptake, suggesting that BSA could play a role in stabilising protoplasts during incubation in PEG. Fifteen minutes in PEG following heat shock was established as a standard condition. These data confirmed results from previous experiments and permitted establishment of the protocol for PEG-mediated IgG uptake in protoplasts as described in the Material and Methods.

A preliminary analysis by Western blot was performed to confirm at the molecular level that IgGs were effectively introduced into protoplasts and to investigate their fate during the following days of culture (figure 6). Duplicate samples were treated with a mixture of FITC-labelled goat IgGs (1 mg/ml) and biotin-labelled rabbit IgGs (100 µg/ml) and cells were collected after 0, 3, 9, 19, 27, 43, 51 and 67 hours of culture. Negative controls included untreated protoplasts, and protoplasts incubated with IgGs in the absence of PEG. Survival was about 30%, with over 90% of treated cells being labelled. Cellular proteins were extracted, separated by SDS PAGE and transferred to nitrocellulose. 9 and 0.9 ng of biotin-labelled rabbit IgG in 10 µg BSA were loaded in parallel as positive controls. When the membrane was exposed to UV light (365 nm), two fluorescent bands of ca 55 and 27 kD were detected in all treated samples but not in the negative controls. When the membrane was stained with Ponceau red, the 55 and 27 kD bands were detected in all treated samples, but not in the negative controls, and the intensity of the signal decreased with increasing time of culture. Detection of biotin-labelled IgG was achieved with alkaline phosphatase-labelled streptavidin and phosphatase activity was detected using naphthyl-phosphate as substrate. Two positive bands with a molecular weight of ca 55 and 27 kD were monitored in all treated samples but not in the negative controls. These molecular weight were consistent with the 25 and 50 kD expected following the separation of IgGs by SDS PAGE. The intensity of the signals decreased proportionally with culture duration and was equivalent to the 9 ng positive control after 43 hours, indicating that about 0.1% of the initial amount of biotin-labelled IgGs was still present in the protein

extracts. These data, though preliminary, strongly suggested that IgGs were actually introduced into the cells following PEG-mediated uptake.

2.1.2.3. PEG-mediated DNA and IgG transfer in P. patens protoplasts

The next step of this set of experiments was to test the possibility of introducing simultaneously antibodies and plasmid DNA in protoplasts, in order to achieve both

figure 6 link

Figure 6. Western blot analysis of protein extracts from protoplasts transfected with FITC- and biotin-labelled IgGs. Duplicated samples were collected at different times following uptake and the whole protein extract (30 - 50 µg) was loaded in each lane, electrophoresed on SDS-PAGE and transferred to nitrocellulose. Top panel: membrane was exposed to 365 nm UV light, middle: Ponceau Red staining, bottom: Western Blot analysis. Lanes: (1) untreated protoplasts, (2) protoplast and PEG without FITC-IgG, (3) protoplasts and FITC-IgG without PEG, (4 and 5) 0 hour, (6 and 7) 3 hours, (8 and 9) 9 hours, (10) 2 µg FITC-IgG in 20 µg BSA, (11 and 12) 19 hours and (13) molecular weight markers: 97.4, 66.2, 45 and 31 kD.

efficient IgG uptake and transient gene expression. Since BSA played a crucial role in IgG uptake, this parameter was the first to be investigated. Both FITC labelling and transient gene expression were monitored in a preliminary experiment (data not shown). The experiment described in table 2 was designed to test the effect of BSA (5 mg/ml) on DNA uptake, IgG uptake and co-uptake of DNA and IgGs. Transfections were performed as described in Material and Methods and the data clearly demonstrated that PEG-mediated DNA transfer and IgG uptake in moss protoplasts could be performed simultaneously. GUS specific activity of ca 3 nmol $MU \cdot mg^{-1} \cdot min^{-1}$ were recorded when both DNA and IgGs were present in the samples, corresponding to one third of the mean specific activity monitored following PEG-mediated DNA transfer. As expected BSA was required for efficient IgG uptake, but its role in efficient transient gene expression could not be established from this experiment.

Table 2. GUS specific activity (in nmol $MU \cdot mg^{-1} \cdot min^{-1}$) and percentage of FITC-labelled cells following PEG-mediated uptake of IgGs and / or DNA in the presence or in the absence of BSA (5 mg/ml).

Treatment	FITC-labelled cells (%)	GUS specific activity
DNA	0	4.7
DNA + BSA	0	2.9
IgG	12	0.05
IgG + BSA	90	0.03
DNA + IgG	8	2.8
DNA + IgG + BSA	98	3.3

To further define the role of BSA in the process, DNA uptake was performed in the presence of increasing concentration of BSA. GUS specific activity increased with increasing concentration of BSA up to 2.5 mg/ml BSA where a specific activity of 2.4 nmol MU·mg^{-1}·min^{-1} was recorded. Higher concentrations of BSA, up to 5 mg/ml, induced a 4 fold reduction of GUS activity, indicating that high concentration of BSA could be detrimental to efficient transient gene expression (data not shown).

2.1.2.4. Functional applications of PEG-mediated IgG uptake: *in vivo* immunodepletion

The final purpose of the development of an IgG uptake assay was to test the possibility of immunodepleting *in vivo* cellular proteins with specific antibodies. Two different approaches were followed: in the first one, co-uptake of NcoGUS and anti-GUS IgGs were performed in order to test the possibility of inactivating the product of transient gene expression. In the second, anti-tubulin antibodies were introduced in moss protoplasts in order to test whether they interfere with the normal regeneration process of these cells. Predictions for these experiments were as follows. Anti-GUS antibodies should inactivate the GUS protein, resulting in a reduction or a loss of activity in the extracts. This inactivation should be proportional to the concentration of IgG in the samples, and its reversibility, if it occurred, would probably not be detectable due to the short culture time employed in the transient gene expression assay. In the second test, anti-tubulin antibodies should deplete free cellular tubulin and/or interact with the extremity of the microtubule, thereby disturbing the turn-over of microtubules with the following putative consequences: (a) cell death due to complete disorganisation of the microtubular cytoskeleton, (b) delay in protoplast division and regeneration due to perturbation of microtubules involved in cell polarisation and growth, and (c) polyploidisation and/or mutagenesis due to disorganisation of the mitotic spindle and of the microtubules involved in cell division (colchicine-like response). The level of inactivation should be proportional to the concentration of antibodies in the samples and the transient nature of the process could be detected by testing the reversibility of the inhibition of cell division.

Immunodepletion of transient gene expression

Despite the fact that co-uptake of IgG and DNA was not completely optimized, inhibition of transient GUS expression with anti-GUS polyclonal antibodies was achieved in 2 independent experiments (table 3).

Table 3. Inhibition of transient GUS expression following co-uptake of pNcoGUS and anti-GUS IgGs of increasing concentration. Experiments were performed in duplicates and GUS specific activity is expressed in nmol MU·mg^{-1}·min^{-1} (mean value ± standard deviation). Percent of control was calculated on the mean GUS activity determined from the 2 positive controls. Heat shock was performed before the addition of PEG in exp.1 and after in exp. 2.

Treatment	IgG conc. (mg/ml)	Exp. 1 GUS	% control	Exp. 2 GUS	% control
No DNA	-	0.02 ± 0.01	-	0.03 ± 0.02	-
DNA	-	0.85 ± 0.03	-	1.45 ± 0.17	-
DNA + goat IgG	0.2	0.79 ± 0.08	-	-	-
DNA + FITC IgG	1	-	-	5.81 ± 0.66	-
DNA + GUS IgG	25	-	-	0.03 ± 0.02	0.8
DNA + GUS IgG	2.5	-	-	0.21 ± 0.02	5.8
DNA + GUS IgG	1	0.24 ± 0.02	29	-	-
DNA + GUS IgG	0.25	-	-	0.80 ± 0.37	22
DNA + GUS IgG	0.1	0.72 ± 0.08	88	-	-
DNA + GUS IgG	0.01	0.83 ± 0.09	100	-	-

In both experiments, inactivation was proportional to the concentration of IgGs in the samples and 50% inhibition was recorded at similar antibody concentration (ca 0.1 to 0.5 mg/ml). When higher concentrations of IgG were tested, complete inhibition of GUS activity was monitored. These data strongly indicated that the product of transient gene expression could be inactivated by specific antibodies co-introduced in cells upon transfection.

Tubulin immunoinactivation

Delays in protoplast regeneration, cell disorganisation and a decrease in cell survival were monitored following PEG-mediated uptake of anti-tubulin monoclonal antibodies in two independent experiments, and responses were shown to be proportional to IgG concentration in one of them. Negative controls containing pre-immune serum, goat IgG and FITC-labelled IgGs didn't display such responses. Surprisingly, a delay in protoplast regeneration was also monitored when anti-tubulin IgGs and protoplasts were simply mixed, diluted and cultivated without PEG treatment (IgG concentration in culture medium, 2 to 6 µg/ml) (data not shown). These observations already indicated that anti-tubulin IgGs could interfere with protoplast regeneration and the following experiment was designed to further characterise this interaction. PEG-mediated IgG uptake was performed with different amounts of anti β-tubulin IgGs and cells were directly diluted in protoplast liquid culture medium. After 40 hours, half of each sample was washed and resuspended in fresh medium. The percentage of dividing protoplasts was monitored daily and cell survival was determined 14 days after uptake. At this stage, one aliquot of each culture was transferred to standard solid culture medium and grown for an additional 10 days. The exact number of colonies was determined, and those displaying abnormal protonematal development were isolated and regenerated on nitrate solid medium for further phenotypical characterisation. Alteration of protonematal development such as reduced

level of ramification and thigmotropic-like growth are typical features of diploid clones generated by somatic fusion, and were used as criteria to identify putative diploids.

The following conclusions were drawn from this experiment. Anti-tubulin IgGs concentration-dependent (a) inhibition of protoplast division and regeneration and (b) decrease in cell survival were clearly monitored in unwashed samples, and were already observed with IgG concentrations in the culture medium in the nanomolar range (initial conc. 7.5 µg/ml, final conc. 0.2 µg/ml) (Figure 7 and table 4). Similar qualitative results were monitored in washed samples treated with 100 times more anti-tubulin IgGs, and the delay in protoplast regeneration was clearly monitored up to 9 days following uptake, indicating that intracellular antibodies were sufficient to induce these responses *in vivo* (Figure 7 and table 4). Extracellular anti-tubulin IgGs was considered as being responsible for the difference between the treatments, thereby confirming previous results which indicated that anti-tubulin IgGs diluted in the culture medium could interfere with protoplast regeneration. It was concluded that these antibodies were extremely efficient in altering protoplast regeneration, but that the mechanism by which extracellular IgGs were interfering with this process could not be established from these data.

The transient nature of the inactivation process was strongly suggested by the fact that all surviving cells divided and regenerated a colony, and it was postulated that the delays in protoplast regeneration reflected the rate of IgG degradation.

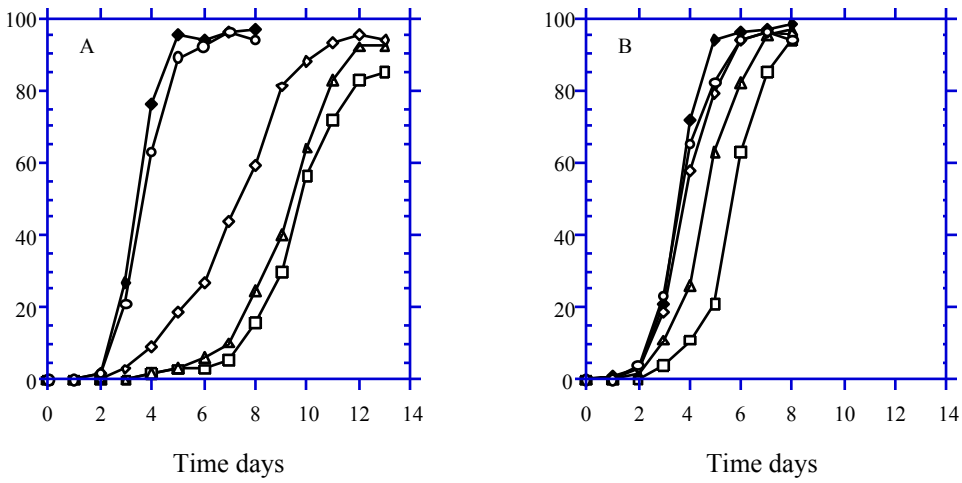

Figure 7. Kinetics of protoplast division following PEG-mediated uptake of anti ß-tubulin monoclonal antibodies. Samples were diluted 40 times with liquid culture medium and half of each culture was washed 40 hours after uptake and resuspended in fresh medium (panel B). Final IgG concentration prior to PEG addition was 0.75 (□), 0.075 (△), 0.0075 (◇) and 0.00075 (○) mg/ml. Goat IgG (_) was at 0.2 mg/ml.

Diploid clones were occasionally recovered (1 to 3%) following PEG-mediated DNA uptake and were considered to be generated by PEG-induced somatic fusion (data

not shown). Diploid-like colonies were recovered at significantly increased frequencies in the presence of a high concentration of anti-tubulin IgGs both in washed and unwashed samples (table 4). Interestingly, these clones were not all diploids and could be classified in 3 different phenotypical categories: true diploid clones, compact dark green colonies and putative mutants (Figure 8), and each category was more frequent in treated samples.

Diploid clones were easily identified, since they resembled colonies regenerated in somatic fusion experiments, and were characterised by a reduced number of differentiated gametophores and a brown protonema. Compact dark green colonies were characterised by the presence of compact gametophores with dark-green unexpanded leaves and by a strong increase of rhizoid production. Two putative mutants have been isolated: one of them displayed wild-type morphology but yellow, resembling the *nicB5ylo6* mutant, whereas the other one display a sparse developmental phenotype characterised by a strong reduction in the number of differentiated gametophores and an extremely reduced protonema. Mutants have never been recovered at such frequencies in other experiments with protoplasts and the genetic status of these clones will be investigated further. Nevertheless, these data suggested that anti-tubulin IgGs was acting in the cells, since it was difficult to imagine an extracellular mechanism capable of inducing polyploidy or mutagenesis.

Table 4. Survival rate and frequencies of putative diploids and mutants following PEG-mediated anti ß-tubulin antibodies uptake in P. patens protoplasts. Survival (a) corresponds to the % of the initial number of protoplasts regenerating 14 days after uptake. Colonies (b) corresponds to the total number of regenerated colonies from which polyploid or mutated clones were isolated. Diploid, mutant (c) corresponds to the number of the different phenotypes monitored: first position are diploids, second correspond to dark green compact colonies and third to developmental mutants.

Treatment - IgG concentration (µg/ml)	Survival (%) (a)	50% division (days)	Total colonies (b)	Diploid, mutant (c)	%
Unwashed					
Goat IgG - 200	24	3.5	635	13, 2, 0	2.4
Tub IgG - 750	13	9.5	247	16, 6, 1	9.3
Tub IgG - 75	13	9.0	317	13, 6, 1	6.3
Tub IgG - 7.5	14	7.0	467	12, 2, 0	3.0
Tub IgG - 0.75	22	3.5	585	15, 5, 0	3.4
Washed					
Goat IgG - 200	26	3.5	503	16, 1, 0	3.4
Tub IgG - 750	10	5.5	306	17, 5, 0	7.2
Tub IgG - 75	23	4.8	498	6, 1, 0	1.4
Tub IgG - 7.5	26	3.5	544	17, 2, 0	3.5
Tub IgG - 0.75	24	3.5	517	14, 0, 0	2.7

Though preliminary, the data presented in this section clearly show the possibility of inactivating the product of transient gene expression and proteins involved in cellular processes following PEG-mediated IgG uptake. Possible mechanisms, further development and potential applications of this "transient *in vivo* immunodepletion assay" will be discussed in the next section.

Figure 8 link

Figure 8. Illustration of the dark-green and the sparse phenotype of colonies regenerated following PEG-mediated IgG uptake. Top: wild-type-, middle: sparse-, and bottom: dark green colonies.

2.1.3 DISCUSSION

PEG-mediated DNA uptake

Optimum conditions for PEG-mediated direct gene transfer to *P. patens* protoplasts were established and the main factors affecting transfection efficiency identified. These factors were identical to the main factors affecting transient gene expression in monocots (Maas and Werr 1989) and dicots (Negrutiu, et al. 1987; Negrutiu, et al. 1990), thereby confirming the wide field of application of the PEG procedure. The main difference between these studies and our data lies in the role of heat shock in the uptake process. In these reports, heat shock was either not performed (Maas and Werr 1989), or found to be detrimental for efficient transient gene expression (Negrutiu, et al. 1990), whereas it was essential for high level transient gene expression and for efficient IgG uptake in moss protoplasts. Moreover, our data indicated that heat shock could play a role both in the uptake process itself, possibly by increasing cell membrane fluidity, and on the physiology of the cells, by increasing gene expression. In a recent report (Zakai, et al. 1993), a constant increase in transient gene expression was monitored in preheated petunia protoplasts and the authors postulated that the assembly of transcriptionally active nucleosomes was enhanced under heat shock conditions, thereby stimulating gene expression. A similar heat shock response could account for the effect of heat shock on transient gene expression in *P. patens* protoplasts.

GUS specific activities in the range of 10 nmol $MU \cdot mg^{-1} \cdot min^{-1}$ corresponded well to specific activities monitored in other plant protoplasts transfected with similar constructs, demonstrating that the 35S promoter is efficiently recognised by the transcription machinery of *P. patens*. With a sensitivity extending over two orders of magnitude above background, this assay should allow the analysis of different strength promoters under different growth conditions. The establishment of a transient gene expression assay in *P. patens* provides a valuable new tool to be used in studies with this organism.

Phytochrome-mediated transient gene expression

The data presented here provide physiological evidence that *P. patens* protoplasts are competent for phytochrome-mediated transient gene expression experiments. This offers the opportunity of studying the transduction pathway of this regulatory mechanism in these cells. However, a difference of a factor 2 between induced (red) and uninduced (red/far red or far red) gene expression, as monitored for the cabGUS construct, is too low to provide a reliable system for further investigations of this regulation, and this small difference might be due to the fact that heterologous promoters were used in these experiments. The successful isolation of genomic clones carrying *cab* (Long, et al. 1989) and *rbcS* (D.Schaefer, unpubl.) genes from *P. patens* should allow rapid isolation of their promoters. Northern blot analysis should be performed to investigate their transcription level in response to different light treatments, but it is predicted that their expression will probably be phytochrome-

regulated, as suggested by the fact that chloroplast differentiation is induced with low fluences of red light. Expression vectors should be constructed with these promoters and the experiments presented here should be repeated with these constructs, in order to test the possibility of improving the ratio of induced to uninduced gene expression. Nevertheless, the data presented here demonstrate that *P. patens* protoplasts are competent for phytochrome-mediated transient gene expression experiments, and indicate that some of the mechanisms involved in the regulation of gene expression are conserved between higher plants and mosses. The possibility of monitoring several phytochrome-mediated processes in *P. patens* protoplasts, such as the establishment of cell polarity prior to mitosis, the control of the cell cycle and the regulation of gene expression offers a unique opportunity to investigate the mechanisms underlying phytochrome actions in single cells.

PEG-mediated IgG uptake

Conditions for efficient and reproducible PEG-mediated IgG uptake in *P. patens* protoplasts were established. The data presented here strongly suggested that the mechanism mediating antibody uptake was similar to that of DNA uptake, i.e. the simultaneous PEG-mediated precipitation of IgG and permeabilisation of the cell membrane. The following arguments led to this conclusion: (1) cells were mainly labelled with fluorescent particles rather than by a diffuse fluorescence of the cytoplasm, suggesting that FITC-labelled IgGs were precipitated in the cells, (2) IgG uptake was dependent on protein concentration in the samples, indicating that a minimum concentration (above 2 mg/ml) was required to achieve precipitation, (3) heat shock was required for efficient IgG uptake, as for efficient DNA uptake, (4) uptake occurred within 15 minutes and maximum uptake was occasionally monitored after 5 minutes of incubation in PEG, indicating that the process was extremely rapid, (5) preliminary Western blot analysis showed that IgGs were actually introduced into the cells, (6) reproducible co-uptake of IgG and DNA was achieved. The protocol established here should be considered as almost optimum and its efficiency and reproducibility has been clearly demonstrated. It is predicted that this procedure could be adapted to most plant protoplast systems, and possibly to yeast and bacteria, since PEG-mediated DNA uptake is a classical technique to transfect these cells.

PEG-mediated IgG and DNA uptake

The possibility of simultaneously introducing DNA and IgG into moss protoplasts was clearly demonstrated. Both FITC labelling and transient gene expression have been simultaneously recorded in 3 independent experiments and inactivation of the GUS protein with anti-GUS antibodies in 2 others. However, optimization of the protocol has not been performed yet and additional experiments are required to fully characterise this procedure. Experiments should focus on the definition of the type (IgG or BSA) and of the optimum concentration of carrier protein to achieve both efficient IgG uptake and gene expression, on the optimum pH, as well as on the duration and the position of heat shock in the protocol. This last point is specially important since the position of

heat shock, as established in the protocol for IgG uptake, has been found to be unfavourable for efficient transient gene expression.

Transient *In vivo* immunodepletion assay

Data presented here strongly suggested that the GUS protein was specifically inactivated by anti-GUS antibodies co-introduced into moss protoplasts upon transfection. Several hypotheses can be formulated to account for this immunodepletion. (1) Anti-GUS antibodies interact *in vivo* with the GUS protein upon translation, thereby preventing the formation of a mature functional protein. GUS activity will never be present in the extract and this hypothesis may be difficult to demonstrate. (2) Antibodies interact *in vivo* with the mature protein and the complex remains in solution. If this complex is stable GUS activity could be recovered from the supernatant of the extract following dissociation of the GUS-IgG complex. Alternatively, if this complex is recognised as an abnormal protein, it will be degraded by cellular proteases, thereby preventing the recovery of GUS activity. (3) Antibodies interact *in vivo* and immunoprecipitate the mature protein. In this case, GUS activity could be recovered from the pellet of the extract following dissociation of the GUS-IgG complex. (4) Immunodepletion occurs *in vitro* during protein extraction. This last possibility seems unlikely since attempts to immunoprecipitate *in vitro*, under similar experimental conditions, GUS activity from transgenic tobacco plant extracts were unsuccessful (data not shown). Beside the fact that complete inhibition of GUS activity, as achieved in one experiment, points towards a highly specific mechanism, which most probably occurred in the cell, our data preclude any conclusions to be made about these hypotheses.

The role of microtubules in *P. patens* morphogenesis is well documented (reviewed in (Doonan and Duckett 1988) and our results fit with the predicted biological effects of anti-tubulin IgG on protoplast regeneration. These data strongly suggested that anti-tubulin antibodies were specifically interfering *in vivo* with the different cellular microtubule arrays, thereby inducing alterations in protoplast survival, regeneration and ploidy. However, the fact that IgGs diluted in culture medium could mediate similar responses came as a complete surprise and the following hypotheses were formulated to account for this result. Antibodies could be actively or passively taken up by protoplasts during culture and subsequently interfered with tubulin and/or microtubules inside the cell. Alternatively, microtubules may be linked to the plasmamembrane by microtubule-associated proteins (discussed in (Cyr 1991)), and these sites could be recognised by extracellular antibodies, thereby blocking the normal turn-over of microtubules and consequently the regeneration of moss protoplasts. These two hypotheses were not mutually exclusive and our data could not discriminate between them.

Taken together, these data clearly demonstrate the feasibility of a "Transient *in vivo* immunodepletion assay" following PEG-mediated transfer of DNA and IgGs in moss protoplasts. Both the expression of a reporter gene and proteins involved in cellular processes can be effectively and reversibly inactivated by specific antibodies.

The mechanism of immunodepletion can not be established from the data, but that it occurs *in vivo* is sustained by the following arguments: (1) microscopic observations and Western blot analysis showed that IgG were in the cells following PEG-mediated uptake, (2) GUS is a soluble cytoplasmic protein, and inactivation most probably occured in the cells, (3) alteration of protoplast regeneration following PEG-mediated anti-tubulin IgG uptake was clearly monitored in washed samples, and (4) if one can imagine a mechanism by which plant cell polarity and growth could involve membrane-associated microtubules (discussed in (Cyr 1991)), an alteration in cell ploidy strongly suggests that part of the immunodepletion process occured in the cells. One objective has not been reached in this work, that is the possibility to block the expression of a reporter gene with antibodies raised against *trans*-acting factors involved in its expression. It is believed that a steroid-inducible gene expression system, as described by Schena (Schena, et al. 1991), would be appropriate to test the feasibility of such an assay for the following reasons: (1) it has been shown to be functional in plant cells, (2) it is well characterised at the molecular level, and several antibodies against different epitopes of the receptor are available, (3) transduction of the signal is solely mediated by the cytoplasmic hormone receptor which, once activated and translocated to the nucleus, directly interacts with the upstream regulating sequences of the gene, thereby avoiding any interference with endogenous transduction pathways.

Introduction of antibodies in mammalian cells (Chakrabarti, et al. 1989), yeast and plant protoplasts (Cyr 1991) has been successfully achieved by electroporation. Surprisingly, this approach has not been extensively used to perform *in vivo* immunodepletion, despite the fact that the feasibility of such an assay has been clearly demonstrated (Chakrabarti, et al. 1989). A rapid computer assisted search in the literature indicated that PEG-mediated uptake of antibodies has never been performed in plant protoplasts. It is demonstrated here that this approach can be performed in *P. patens* protoplasts and that specific antibodies can inactivate *in vivo* the expression of a reporter gene and proteins of the cytoskeleton. This assay presents several interesting features to perform loss of function experiments: (1) large numbers of cells can be treated at the same time, (2) it is rapid, reliable and experiments can be performed within a few days, (3) responses are proportional to the concentration of IgGs, thus allowing modulation of the level of inactivation, (4) the target to be inactivated is not a gene, which will subsequently be translated in a protein, but the protein itself, (5) the specificity of antibodies for their antigene permit complete immunodepletion of the targeted protein, which is not achieved with antisense messengers, as well as a fine dissection of the functional domains of the targeted protein with different monoclonal antibodies, (6) co-uptake of plasmid DNA and IgG can be performed, thus allowing the study of the regulation of gene expression, (7) this approach can be used to perform loss of function experiments on most cellular processes, providing one has an appropriate antibody.

This assay provides an alternative approach to study cellular processes in plant protoplasts. Experiments should be performed to test the possibility to inactivate

viruses in infected cells, as well as to test the feasibility of such an assay in yeast, bacteria and animal cells.

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2.1.4. MATERIAL AND METHODS

Plant material, plasmid DNA, antibodies and chemicals

P. patens wild-type strain was used in this study. The techniques for tissue propagation, protoplasts isolation and culture are described in Annex A. Plasmid pNcoGUS (gift from J.-M. Bonneville, unpubl.) consists of the Sal 1 - EcoR1 fragment of pRAJ 275 (Jefferson 1987) carrying the GUS reporter gene cloned under the control of the constitutive 35S CaMV promoter and terminator in vector pDH51 (Pietrzak, et al. 1986). Plasmid rbcSGUS was constructed by cloning the HindIII - EcoR1 expression cassette of pBI 131.1 (gift from R.A. Jefferson, described in (Jefferson, et al. 1987)) in the corresponding sites of pUC 19. Plasmid cabGUS was a gift from F. Nagy, FMI, Basel (unpubl.). Plasmids were amplified and PEG-purified according to standard techniques (Sambrook, et al. 1989).

The following antibodies were used in this study: goat anti-mouse IgG-FITC conjugate (Sigma F-9006), rabbit anti-goat IgG biotin-labelled (Sigma B-7014), rabbit IgG-FITC conjugate (Sigma F-7256), purified rabbit IgG (Sigma I-5006), rabbit anti-ß-glucuronidase antibody (Clontech 1511-1) and mouse anti-ß-tubulin monoclonal antibody (Sigma T-4026). BSA, MU and salmon sperm DNA were purchased from Sigma, MUG from Sigma or Fluka and PEG 4000 from Serva.

Light treatments

Transfections were performed with plasmids NcoGUS, rbcSGUS or cabGUS and protoplasts were further cultivated at 25^{o} C in white light (16 h / day, 60 $\mu mol \cdot m^{-2} \cdot s^{-1}$), in darkness, in red light (15 min every 2 h, fluence at 660 nm: 4 $\mu mol \cdot m^{-2} \cdot s^{-1}$), in far-red light (5 min every 2 h, fluence at 730 nm: 2 $\mu mol \cdot m^{-2} \cdot s^{-1}$), or in red/far red light (15 min red + 5 min far red every 2 h.). Red light was provided by red fluorescent tubes Philips TL 20W/15 L4. White light was filtered through a Plexiglas FRF-700 nm cut off filter (Westlake Plastics, Lenni, Pa., USA) to provide far red light. *P. patens* growth under this far red light was similar to that in darkness.

PEG-mediated DNA uptake

The following protocol has been established (modified from (Schaefer, et al. 1991)).

- Isolate protoplasts from 5-6 days old protonemata (Annex A).

- Resuspend the protoplasts at a concentration of 1.5 x 10^6 / ml in MMM solution (mannitol: 0.48 M, $MgCl_2$: 15 mM, MES: 0.1%, pH 5.6 with KOH) and proceed immediately with the transformation.

- Dispense 30 µl of plasmid DNA (500 µg/ml in Tris 10 mM, EDTA 1 mM, pH 8.0) into 14 ml polypropylene tubes.

- Add 300 µl of protoplast suspension and mix gently.

- Add 300 µl of PEG solution and mix immediately (PEG 4000, 33 % w/v in 0.38M mannitol, 0.1M $CaNO_3$, buffered to pH 8.0 with 10 mM Tris).

- Heat shock 5 minutes at 45º C and then leave for an additional 10 minutes at room temperature with occasional mixing.

- Progressively dilute the sample with 10 ml of protoplast liquid culture medium and transfer to 5 cm Petri dish.

Protoplasts were further cultivated in protoplast liquid culture medium for transient expression assays.

PEG-mediated IgG uptake

The following protocol has been established:

- Isolate protoplasts from 5-6 days old protonemata (Annex A).

- Resuspend the protoplasts at a concentration of 2 x 10^6 / ml in MMM solution (same as for DNA uptake) and proceed immediately with the uptake.

- Dispense 50 µl of protoplast suspension in 5 ml polypropylene tubes.

- Add 50 µl of BSA 10 mg/ml in mannitol 0.48M (final concentration 5 mg/ml).

- Add 100 µg IgG in 10 µl or less and mix gently (final concentration 1 mg/ml).

- Add 10 µl of plasmid DNA (500 µg/ml in TE) to the tubes if co-uptake of IgG and DNA is desired (final concentration 50 µg/ml).

- Heat shock 3 minutes at 45º C.

- Add 110 µl of PEG solution (PEG 4000, 40 % w/v in 0.38M mannitol, 0.1M $CaNO_3$, buffered to pH 8.0 with 10 mM Tris), mix immediately and leave for an additional 15 minutes at room temperature with occasional mixing.

- Progressively dilute the sample with 5 ml of mannitol 0.48M and harvest the cells by low speed centrifugation (600 g for 5 min.). Resuspend the protoplasts in 5 ml mannitol 0.48M and repeat washing in mannitol.

- Resuspend the cells in 5 ml protoplast liquid culture medium and transfer to a 5 cm Petri dish.

- Alternatively samples can be directly diluted with 5 ml liquid culture medium and transferred to a 5 cm Petri dish.

When sonication was tested for IgG uptake, samples were immersed for 15 seconds in a Branson 2200 sonicbath, by holding the tubes in the zones of turbulence. A detailed description of the role of each step of the procedure will be presented in the results section.

GUS assay

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 ß-glucuronidase or with extracts from a GUS transgenic tobacco plant was not affected by the presence of moss protein extracts (data not shown). GUS assays were performed essentially according to (Jefferson 1987) using methyl umbelliferyl glucuronide (MUG) as fluorogenic substrate. Cells were collected 48 hours after transfection unless otherwise stated, resuspended in 100 µl SSE (50 mM $NaPO_4$ pH 7.0, 1 mM Na_2EDTA, 0.1% Sarkosyl, 0.1% Triton X-100, 10 mM ß-mercaptoethanol) and lysed by 3 cycles of freeze and thaw. Cellular debris were removed by centrifugation (10000 rpm for 10 min) and protein content of the supernatant was determined according to (Bradford 1976). Enzymatic assays were performed in duplicates in microtiter plates and methylumbelliferone (MU) concentration determined with a Titertek Fluoroskan II (® Flow Lab) by comparison with a serial dilution of a 1 mM MU standard solution. 5 to 10 µg of protein extract were mixed with 100 µl of reaction buffer (SSE containing 2 mM MUG) and incubated at 37^{o}C. Fluorescence of the extracts was measured at regular time intervals and a minimum of 4 time points were taken. GUS specific activity of the extracts was then calculated and expressed in nmol MU·mg prot^{-1}·min^{-1}. Protein extracts from a 35S-GUS transgenic tobacco plant were used as an internal positive control in all enzymatic assays. Specific GUS activities in tobacco extracts ranged from 30 to 40 nmol MU · mg^{-1} · min^{-1}.

Monitoring IgG uptake

Survival rate, percentage of FITC-labelled cells and of dividing protoplasts were established by counting 200 to 300 cells under the microscope (Leitz Diavert).

Western blot analysis

Protein extracts from samples following PEG-mediated IgG uptake were prepared as for the GUS assay. Proteins were separated by SDS PAGE and transferred to nitrocellulose according to standard procedure (Harlow and Lane 1988). Biotin-labelled IgG was detected with alkaline phosphatase-labelled streptavidin using naphthyl-phosphate as substrate.

2.2. INTEGRATIVE AND REPLICATIVE GENETIC TRANSFORMATION OF THE MOSS *PHYSCOMITRELLA PATENS*

Abstract

The PEG-mediated introduction of bacterial plasmids carrying antibiotic resistance genes in protoplasts of *Physcomitrella patens* generates resistant clones at frequencies in the range of 10^{-1} / surviving cell under optimum conditions, and three classes of resistant clones were identified.

Class I integrative transformants were recovered at frequencies in the range of 10^{-5} / surviving cell. These clones displayed unrestricted growth upon selective medium, full mitotic stability of the character on non-selective medium and at all developmental stages, 100% meiotic transmission of the character after selfing and mendelian segregation of the character after crossing with a sensitive strain. Molecular analysis demonstrated the presence of hybrid fragments between host DNA and foreign genes and electrophoretic comigration of the transforming sequences with high molecular weight DNA. These criteria demonstrated integrative transformation of the moss *P. patens* .

Class II unstable resistant clones were recovered at frequencies in the range of 10^{-1} / surviving cell. These clones displayed strong mitotic instability of the transforming sequences, associated with restricted growth upon selective medium, strong mosaicism in the resistant colonies and rapid loss of the resistance following a period of non-selective growth. Maintenance of the transforming sequences was shown to be restricted to the protonema of the resistant colonies, accounting for the absence of meiotic transmission of the character after selfing. Preliminary molecular analysis established a correlation between phenotypical and physical loss of the resistance gene and indicated that these sequences were maintained as high molecular weight structures. These data suggested that the transforming sequences were concatenated possibly by recombination to form high molecular weight extrachromosomal arrays which were most likely replicated in *P. patens* protonema.

Class III resistant clones were recovered at frequencies similar to class I integrative transformants. These clones were characterised by an improved mitotic stability of the transforming sequences, associated with improved growth rate on selective medium and reduced loss of the resistance following a period of non-selective growth as compared to class II resistant clones. Resistant gametophores and non-mendelian transmission of the unstable character after selfing have been occasionally but reproducibly monitored. Molecular analysis showed (1) that the transforming sequences were present in high copy number and were arranged as tandem repeats in high molecular weight concatenates, (2) that the phenotypical loss of resistance was correlated with physical loss of the resistance gene, and (3) provided evidence for the occurrence of recombination events within the transforming plasmids. These data strongly suggested that the transforming plasmids were concatenated to form high molecular weight extrachromosomal arrays, which were efficiently replicated in *P. patens*, and suggested that the improved mitotic stability of these arrays could be associated with recombination of the transforming sequences with moss genomic elements involved in the replication and/or the mitotic segregation of replicated DNA. Delayed integration events were monitored in both class II and III resistant clones, yet at higher frequencies in class III, and molecular analysis provided preliminary evidence for the occurrence of such event between extrachromosomal recombined sequences and the moss genome.

Preliminary experiments performed with YAC derivatives indicated that class III resistant clones were recovered at frequencies 100 fold higher than with non YAC vectors, suggesting that some functional elements of these vectors were improving mitotic stability of the transforming sequences in *P. patens*. Preliminary transformations performed on another Bryophyte, *Ceratodon purpureus*, indicated

that extrachromosomal replicative transformation could be a general feature of moss transformation. Finally, preliminary transformations performed with heterologous morphogenetic genes provided phenotypical evidence that the *ipt* gene of *Agrobacterium tumefaciens* and the *rolA* gene of *Agrobacterium rhizogenes* were biologically active in *P. patens*, and that the induced phenotype could be observed in class II unstable resistant clones.

Abbreviations: PEG: Polyethylene glycol, YAC: Yeast Artificial Chromosome, ARS: autonomously replicating sequence, CEN: centromere, TEL: telomere, SAR: scaffold attachment region, NOS: nopaline synthase, Ocs: octopine synthase, NptII: neomycin phosphotransferase, Aph IV: hygromycin phosphotransferase, Ipt:isopentenyltransferase, CTAB: cethyl trimethyl ammonium bromide, RTF, relative transformation frequencies, CaMV: cauliflower mosaic virus, pA: polyadenylation sequences, 19S: promoter of the 19S CaMV transcript, 35S: promoter of the 35S CaMV transcript, GUS: glucuronidase, bp: base pair, kb, kilobase pair, G-418: geneticin, Hg: hygromycin B.

2.2.1. INTRODUCTION

In theory, the genetic transformation of eukaryotes with bacterial vectors carrying selectable markers can be achieved by integrative or replicative transformation. Integrative transformation refers to the genetic modification of an organism mediated by the integration of foreign DNA sequences in the host genome. Criteria demonstrating integrative transformation include mitotic stability, mendelian segregation of the new character, and molecular evidence for the presence of hybrid fragments between host and foreign DNA. Replicative transformation refers to the genetic transformation of an organism associated with extrachromosomal maintenance of efficiently replicated DNA sequences. Replicative transformation in eukaryotes should however be subdivided into two different classes: (1) sequence specific replicative transformation, which requires the presence of host origin of DNA replication sequences within the transforming plasmid, and (2) autonomous replicative transformation, where DNA replication is initiated at specific bacterial sequences, or at non specific sequences within the transforming plasmid. Criteria establishing replicative transformation include high transformation frequencies, often high copy number of the transforming sequences, mitotic instability of the character associated with mosaicism in the transformant, non-mendelian transmission of the new character, a tight correlation between phenotypical and physical loss of the genetic marker, possible occurrence of phenotypical changes associated with delayed integration of the extrachromosomal DNA in the host genome, and evidence for extrachromosomal location and for the presence of a DNA replication origin within the transforming sequences.

In reality, the genetic transformation of eukaryotes is almost always achieved by integrative transformation, since bacterial plasmid sequences are usually not recognised as functional origins of DNA replication in eukaryotic cells. In plants, neither sequence specific, nor autonomous replicative transformation has yet been reported. In protozoa and metazoa, efficient autonomous replication of bacterial plasmids has been reported in *Paramecium tetraurelia* (Gilley, et al. 1988), *Dictyostelium discoideum* (De Lozanne and Spudich 1987), *Caenorhabditis elegans* (Stinchcomb, et al. 1985; Mello, et al.

1991), Zebra fish (Stuart, et al. 1988), and during the embryonic development of sea urchin (McMahon, et al. 1985) and *Xenopus laevis* (Marini, et al. 1988). On the other hand, sequence specific replicative transformation has been reported in *Leishmania major* (Kapler, et al. 1990) and in *Tetrahymena thermophila* (Yu and Blackburn 1990). In fungi, sequence specific replicative transformation is well documented in *Saccharomyces cerevisiae* (reviewed in (Struhl 1983; Newlon 1988)) and *Schizosaccharomyces pombe* (for further references, see (Hayles and Nurse 1992)), and is associated with the presence of autonomous replicative sequences (ARS) providing a functional DNA replication origin within the transforming plasmids. Yeast transformation with ARS plasmids was the first step in the identification of structural and functional elements involved in DNA replication in yeast (for a recent review of the structure of eukaryotic origins of DNA replication, see (DePamphilis 1993)), and in the development of the first engineered artificial chromosome, the YAC vector (Yeast Artificial Chromosome) (Burke, et al. 1987). Yet, sequence independent autonomous replicative transformation has also been reported in *Schizosaccharomyces pombe* (Heyer, et al. 1986), in *Neurospora crassa* (Hughes, et al. 1983; Stohl and Lambovitz 1983), and possibly in *Aspergillus nidulans* (see (Timberlake and Marshall 1989)), indicating that replication of bacterial plasmids in fungi could occasionally be observed.

Sequence independent replicative transformation reports demonstrate that, in some organisms, bacterial plasmids can be efficiently replicated in eukaryotic cells. However, these plasmids were in most cases not replicated as single monomeres, but were shown to be initially concatenated to form high molecular weight arrays composed of many copies of the transforming plasmid, and only these high molecular weight structures were efficiently replicated (see (McMahon, et al. 1985; Stinchcomb, et al. 1985; Heyer, et al. 1986; Marini, et al. 1988; Stuart, et al. 1988)). This indicates that the size of the extrachromosomal element and/or reiteration of functional elements is important to achieve efficient autonomous replication.

The main characteristics of these extrachromosomal replicative transformants will be illustrated by the characterisation of *C. elegans* transformants (Stinchcomb, et al. 1985; Mello, et al. 1991). These studies indicated that the formation of high molecular weight arrays involves a large pool of plasmid molecules, and is mediated by processes such as homologous pairing, homologous recombination and ligation. These processes are not always conservative and lead to the formation of complex and heritable structures which are different among independent transformants. Such rearranged structures are evidenced by a complex hybridization pattern in Southern blot analysis, which is often associated with a smeared hybridization signal (see figure 2 in (Stinchcomb, et al. 1985), compare figure 2 and 7 in (Mello, et al. 1991), and note that smeared hybridization signals have also been obtained in sea urchin (McMahon, et al. 1985), Zebra fish (Stuart, et al. 1988), *S. pombe* (Heyer, et al. 1986) and *X. laevis* (Etkin, et al. 1984)). However, extensive rearrangements of the transforming plasmids during concatenation might be specific to *C. elegans*, since plasmid monomeres have been shown to be arranged in tandem repeats in the extrachromosomal arrays of *X. laevis* (Marini, et al. 1988), sea urchin (McMahon, et al. 1985), and *S. pombe* (see (Heyer, et al. 1986)). Transformed worms were shown to display mosaicism for the extrachromosomal array, and this was also true for *X. laevis* and Zebra fish. Two major

groups of extrachromosomal transformants were identified in *C. elegans* according to their ability to transmit the transformed phenotype to the progeny. Both non-mendelian meiotic transmission of the unstable phenotype and delayed integration events were monitored in *C. elegans*, and the latter was also monitored in *X. laevis* (Etkin and Pearman 1987), in Zebra fish (Stuart, et al. 1988) and in sea urchin (Flytzanis, et al. 1985). Finally, occurrence of recombination events between the transforming sequences and host genomic DNA was neither demonstrated, nor eliminated.

The data presented in this chapter will provide phenotypical, genetic and molecular evidence demonstrating both integrative and most probably replicative transformation of *P. patens* following PEG-mediated uptake of bacterial plasmids in protoplasts. Integrative transformants have already been described (Schaefer, et al. 1991), and the data presented here will summarise all the data obtained with transgenic plants during this work. Two major classes of most likely replicative transformants were identified according to the mitotic stability of the transforming sequences, and to the ability to transmit the unstable phenotype to the offspring. Though no direct evidence demonstrating the effective replication of bacterial sequences in moss cells will be presented, these clones will be referred as replicative transformants in the following part of this chapter. Several indirect evidences are presented in this report, which sustains the hypothesis of replicative transformation; high frequencies of transformation, strong mosaicism of the transformants, non-mendelian meiotic transmission of the new character, and a tight correlation between phenotypic and physical loss of the resistance. Beside this, for these clones, it has been possible to grow much more resistant cells than the initial number of plasmid molecules present in the transformation samples. Delayed integration events were monitored in both classes. Preliminary phenotypical evidence indicating that YAC vectors could display an improved mitotic stability in extrachromosomal transformants as compared to bacterial plasmids will be presented, as well as data suggesting that extrachromosomal replicative transformation could be a general feature of Bryophyte transformation.

2.2.2. RESULTS

2.2.2.1. Methodological considerations

The data presented in this chapter have been monitored in experiments performed with 7 different plasmids (Table 1). These plasmids share at least the 2.3 kb Pvu II - Eco R1 fragment of pBR 322, carrying the Col E1 ori site and the ampicilin resistance gene (Balbas, et al. 1986). Two experiments were performed with the initial protocol described in (Schaefer, et al. 1991). All other experiments were performed according to the optimized procedure described in the previous chapter. In these latter experiments, carrier DNA was systematically omitted to avoid the generation of tagged mutants by the insertion of non identified DNA sequences. In each transformation, negative controls included untreated protoplasts, protoplasts incubated with plasmid DNA in the absence of PEG and protoplasts incubated with PEG in the absence of plasmid DNA. Resistant colonies have never been recovered from these samples, nor when transformation was performed with pKC7 (Rao and Rogers 1979), a plasmid carrying a bacterial expression cassette encoding *npt II* (data not shown). Two different assays were developed to investigate the phenotypical characteristics of the resistant clones recovered in these experiments.

1). The non-selective to selective growth assay was designed to investigate the mitotic stability of the transforming sequences following a period of non selective growth. In this assay, integrative transformants did not display loss of resistance on non-selective medium, and both survival and growth rates were not affected by the subsequent transfer to selective medium, demonstrating mitotic stability of the character. On the other hand, replicative transformants, carrying mitotically unstable sequences, were loosing their resistant phenotype during the non-selective growth period. Such a loss was detected by the apparition of dead sectors in the regenerated protonema following transfer of the culture to selective medium. With this assay, the level of mitotic instability of the transforming sequences could be estimated by the amount of dead protonema recovered on selective medium. The same assay was used to investigate the meiotic stability of the resistance. When a resistant clone was selfed, the observation that 100% of its spores, germinated on non-selective medium, maintained their resistance upon transfer to selective medium established stable meiotic transmission of the character.

2). Rescue experiments were designed to investigate the mitotic stability of the transforming sequences during development. In integrative transformants, all differentiated gametophores were able to regenerate a protonema on selective medium, independently from the initial growth conditions. Yet in replicative transformants, regeneration was restricted to protonematal filaments associated with some gametophores, when the transforming sequences were not transmitted to the leafy shoots, and to some differentiated gametophores isolated from selective medium when the transforming sequences were occasionally transmitted to this developmental stage.

With this assay, the question of the tissue specificity of extrachromosomal replicative transformation was successfully addressed.

Table 1. List of the different plasmids tested for the transformation of P. patens.

Plasmid name (size in bp)	Selectable cassette	pBR 322 sequences (Balbas, et al. 1986)	References
pLGV neo 2103 (7564 bp)	NOS-npt II-pA Ocs	Nucl. 665 - 4361 Col E1 ori - bom	(Hain, et al. 1985)
pABD 1 (5265 bp)	19S-npt II-pA CaMV	Nucl. 2066 - 4361 Col E1 ori	(Paszkowski, et al. 1984)
pABD 2 (5265 bp)	19S-inverted npt II-pA CaMV	Nucl. 2066 - 4361 Col E1 ori	(Paszkowski, et al. 1984)
pHP 23b (4374 bp)	35S-npt II-pA CaMV	Nucl. 2066 - 4361 Col E1 ori	(Paszkowski, et al. 1988)
pGL 2 (4458 bp)	35S-aph-pA CaMV	Nucl. 2066 - 4361 Col E1 ori	(Karesh, et al. 1991)
pPCV 002 (8500 bp)	NOS-npt II-pA Ocs	Nucl. 1666 - 4361 Col E1 ori - bom	(Koncz and Schell 1986)
YAC RC AB1 YAC RC AB2 (12500 bp)	35S-npt II-pA CaMV	Nucl. 2066 - 4361 Col E1 ori	(Marchuk and Collins 1988), modified by G.Bonnema, unpubl.

2.2.2.2. Initial selection

In all experiments performed, and independently of the protocol used or of the plasmids tested, two different phenotypes which were subsequently shown to correspond to 3 different classes of resistant clones, were recovered. Initial RTF monitored under optimum conditions were in the range of 10^{-1}, but most of these resistant clones displayed strong reduction of growth rate upon selective medium, which was systematically associated with the presence of resistant and sensitive sectors within the colony (Figure 1). The number of these clones decreased during further selective growth and these resistant colonies were shown to lose their resistant phenotype following a period of non-selective growth. Yet, since these clones could be subcultured and maintained over years on selective medium, they have been classified as unstable transformants (class II). Resistant clones displaying unrestricted growth upon selective medium were recovered at frequencies ranging from 10^{-4} to 10^{-5} per surviving protoplasts. However, when these clones were tested in the non-selective to selective assay, only some of them displayed clear maintenance of the resistance after non-selective growth (see Figure 3). These stable clones were further characterised and shown to be true transgenic plants (class I) with multiple copies of the plasmid

integrated at a single genomic locus (Schaefer, et al. 1991). Despite their ability to display unrestricted growth upon selective medium, the other fast growing resistant clones were shown to display loss of the resistant phenotype in the absence of selective pressure (see Figure 8). These clones were therefore classified as fast growing unstable (class III).

figure 1 link

Figure 1. Five weeks old kanamycin resistant clones selected on G-418 50 µg/ml. A fragment of a stable clone (Kan R 7.01, arrow) displaying unrestricted growth can be identified among numerous dead or mosaic class II resistant clones.

2.2.2.3. Initial transformation frequencies

Transformation frequencies monitored in the two experiments performed with the initial protocol are presented in Table 2. All other transformations were performed with the improved procedure established in the development of the transient gene expression assay. Transformation frequencies as a function of time of selection and of increasing concentrations of antibiotics are presented in Table 3. In this experiment, the lowest concentrations were chosen as corresponding to the minimal concentration of antibiotic killing 100% wild-type untransformed protoplasts (data not shown). The role of heat shock on transformation efficiency was also investigated, as well as RTF monitored following the introduction of different plasmids (Table 2). Plasmids pHP 23b and pGL 2 were used as references in these experiments, and the data were compared with the results monitored with these constructs.

figure 2 link

Figure 2. Initial transformation frequencies. Protoplasts were transformed with supercoiled pHP 23b and selected 4 days after DNA uptake on increasing concentrations of G-418. Each plate represent 1/4 of a sample. Pictures were taken 18 days after the initiation of selection. Top plates, untreated protoplasts regenerating on non-selective medium TE + or selective TE - medium.

The following conclusions were drawn from these experiments.

(1) When transformation was performed with the optimum protocol, survival rate of protoplasts was increased about 3 to 5 times as compared to the initial protocol, with a mean survival rate of 28.5 ± 13.1 %. This increased survival was associated with at least a 10 fold increase in initial RTF. The importance of heat shock for efficient transformation was also clearly demonstrated and appeared similar to that for efficient transient gene expression.

(2) Initial RTF monitored with the optimum protocol were very high as compared to other plant systems, ranging from 5 to 35% with both plasmid pHP 23b and pGL 2 (Figure 2). These frequencies were higher when selection was applied later in the regeneration process, indicating that the transforming plasmids have to undergo some modifications to be maintained in the resistant colonies, and when the selective pressure was low in the media. Under strong selective conditions (G-418 at 50 µg/ml or Hg at 25 µg/ml), mean RTF determined from 4 independent experiments were 14.9 ± 3.5 %,

corresponding to 860 ± 280 resistant clones per µg DNA for plasmid pHP 23b, and 7.8 ± 3.8 %, corresponding to 500 ± 260 resistant clones per µg DNA for plasmid pGL 2. Similar RTF were monitored with plasmid pABD 1 and pABD 2, but when plasmid pLGV neo 2103 and pPCV 002 were tested, initial RTF were about hundred fold lower, indicating that the size of the plasmid could influence transformation efficiency.

(3) Fast growing resistant colonies, corresponding to transgenic (class I) or fast growing unstable clones (class III), were readily identified during the first month following the initiation of selection. However, frequencies of such events remained in the range of 10^{-4} to 10^{-5}, despite the increase in initial RTF monitored with the optimum protocol.

(4) In experiment 2, five out of seven stable clones and seven out of ten class III clones were recovered following transformation with linear plasmid. These data indicated that such plasmid structure might favour integration events and the generation of class III clones.

(5) Every tested plasmid conferred resistance to hygromycin B or G-418, indicating that the NOS, 19S CaMV and 35S CaMV promoters were functional in *P. patens*. Surprisingly, resistant clones were also obtained with plasmid pABD 2, which carries the *nptII* gene in reverse orientation with respect to the 19S CaMV promoter, and which was therefore considered as a negative control. Sequence analysis of this plasmid has shown that there were two ATG codons located 5' of and in frame with the coding sequence of *nptII* gene (data not shown). These start codons could possibly correspond to the initiation sites of a fusion protein conferring resistance to G-418. This assumption was confirmed by the fact that kanamycin resistant transgenic tobacco plants were occasionally recovered with pABD 2, probably following the integration of this plasmid in the vicinity of a endogenous promoter (M. Saul, personal communication). Since " a couple of As and Ts could be sufficient to promote gene expression in plants " (C. Koncz, quoted personal communication), it was considered that the CaMV polyadenylation sequences of this plasmid were possibly recognised as a promoter by the transcription machinery of *P. patens*, and that this plasmid was actually functional.

Table 2. Comparison of the initial RTF monitored following transformation with the initial (protocol 1) or optimised (protocol 2) procedures. Different plasmids in supercoiled (1,2) or linear (2) form were tested with protocol 1. Two class III resistant clones were recovered in exp. 1 (Kan^R ABD2 S6 and ABD2 L1), and 10 in exp. 2 (Kan^R 2.01, 3.03, 4.01, 6L1, 6L2, 6.03 and 12.02 and Hg^R 10.02, 14.01 and 15.04). RTF as a function of heat shock duration (4) was tested with supercoiled plasmid p HP 23b and protocol 2, as well as RTF following transformation with different plasmids (5).

Protocol 1	Survival (%)	Initial RTF (%)	Initial RTF per µg DNA	Stable (name)	Stable RTF ($\cdot 10^{-5}$)
1. Plasmid					
LGV 2103	2.8	0.60	11	0	-
ABD 1	4.5	1.30	40	0	-
ABD 2	4.8	0.33	11	0	-
HP 23b	5.5	0.70	25	1 (HP 23 L2)	4.5
2. Plasmid structure					
LGV 2103 sc	11.1	0.17	25	0	-
ABD 1 sc	8.2	0.24	32	0	-
ABD 1 lin	10.0	0.01	2	0	-
ABD 2 sc	9.0	0.98	118	0	-
ABD 2 lin	10.1	0.12	16	0	-
HP 23b sc	10.6	0.79	103	2 (7.01, 11.01)	2.4
HP 23b lin	8.3	0.41	45	2 (8.01, 12.01)	3.0
GL 2 sc	10.5	0.13	18	0	-
GL 2 lin	10.5	0.09	13	3 (15.01, 15.02, 15.03)	2.4
Protocol 2.					
4. Heat shock (min)					
0	24.7	2.0	135	0	
2	21.8	3.7	215	0	
4	17.8	5.6	265	1	1.4
6	24.9	5.9	392	2	2.0
8	19.8	9.1	480	2	2.5
5. Plasmid					
ABD 1	39.1	13.4	1400	1	0.6
ABD 2	47.2	11.0	1400	1	0.5
HP 23b	42.5	11.5	1300	3	1.8
GL 2	51.1	5.4	733	3	1.5
LGV neo 2103	50.2	0.1	14	0	
PCV 002	67.7	0.1	23	1	0.4

Table 3. RTF as a function of antibiotic concentration and of time of initiation of selection. Transformation samples were pooled before being distributed to the appropriate treatment to standardize uptake efficiency. The initial number of surviving protoplasts was 60 000 per treatment, corresponding to 15% survival. The total number of resistant clones was determined 15 days following the initiation of selection. In this experiment, half of the samples were selected for the identification of stable clones. Eight stable transgenic HgR plants and three table transgenic Kan^R plants were isolated (clones Hg 1, Hg 2, Hg 3, Hg 4, Hg 5, Hg 6, Hg 28 NS/S, Hg 23/25, and G-418 1, G-418 15 NS/S and G-418 Is13/25). This corresponded to a mean integrative RTF of $2.2 \cdot 10^{-5}$ for pGL 2 and $0.8 \cdot 10^{-5}$ for pHP 23b. Four class III replicative transformants were isolated, and one of them was further characterised (Hg Is 31).

Plasmid and antibiotic conc.	Day 2 RTF (%)	Per µg DNA	Day 4 RTF (%)	Per µg DNA	Day 6 RTF (%)	Per µg DNA
HP 23b						
G-418 5	17.8	710	29.2	1170	35.1	1400
G-418 10	14.2	570	21.4	860	26.6	1060
G-418 25	9.4	380	18.5	740	23.3	930
G-418 50	6.9	280	14.8	590	20.7	830
GL 2						
Hg 2.5	20.0	800	23.3	930	33.7	1350
Hg 5	13.5	540	13.2	530	15.5	620
Hg 12.5	6.2	250	6.2	250	11.8	470
Hg 25	4.0	160	6.3	253	9.5	380

The following working hypothesis was developed to account for these initial data. Bacterial plasmids have two possible fates once introduced in *P. patens* protoplasts. They can be integrated in the genome and thus generate transgenic plants (class I), but this event is rare, occurring at a mean RTF of 10^{-5}. Alternatively, bacterial plasmids can be efficiently replicated and maintained in the resistant cells as extrachromosomal elements, as suggested by the high transformation frequencies monitored during the initial selection stages. Yet, these extrachromosomal elements display strong mitotic instability, as illustrated by the strong reduction of growth upon selective medium associated with mosaicism of the colonies, and by the rapid loss of the resistant phenotype in the absence of selection, and continuous selection is required to maintain resistance in these unstable clones (class II). However, these plasmids or episomal elements can recombine upon transformation, or later during somatic growth, with moss genomic sequences simultaneously precipitated with plasmid DNA during PEG uptake, and functionally involved in replication and/or mitotic segregation of chromosomal DNA. Such recombination would confer an improved mitotic stability to the extrachromosomal element, accounting for the origin of class III clones. The data presented in the following part of this section will characterise the three classes of resistant clones and provide phenotypical, genetic and molecular evidences for the validity of this working hypothesis.

2.2.2.4. Characterisation of class I integrative transgenic clones

Transformation frequencies.

As already mentionned, these clones were initially identified by their unrestricted growth upon selective medium and by their ability to maintain the resistant phenotype following a period of non selective growth. Mean RTF determined from 7 independent experiments were $1.4 \cdot 10^{-5}$ (16 out of 1123000) for supercoiled plasmid pHP 23b and $1.7 \cdot 10^{-5}$ (15 out of 880000) for supercoiled plasmid pGL 2. These frequencies appeared to be independent of the uptake protocol and of the initial RTF, and were similar when other plasmids were tested (Tables 2 and 3).

Phenotypical analysis.

The mitotic stability of the resistant phenotype was clearly demonstrated by the following experiments. All these clones were submitted to the non-selective to selective assay, and it was shown that protonemata regenerated on non-selective medium perfectly continued growth upon subsequent transfer to selective medium, and did not display any dead sectors (Figure 3). Rescue experiments were performed on most of these clones, and demonstrated in each case that all gametophores were able to regenerate a protonema upon selective medium, independently from the initial culture conditions (Figure 3). Finally, protoplasts were isolated from eight independent strains and all but one (strain Hg^R 15.02) regenerated on selective medium independently from the initial growth conditions. Protoplasts of strain 15.02 were not able to germinate on selective medium. However, when regeneration was started on non-selective medium and the culture further transferred to selective medium, these protoplasts regenerated normally (data not shown), suggesting a position effect rather than mitotic instability. All these clones displayed normal growth and regeneration on increasing concentration of antibiotics (up to 50 µg/ml G-418 or 25 µg/ml Hygromycin B), allowing the complete life cycle to be normally achieved under such conditions.

Genetic analysis.

The meiotic stability of the introduced sequences was demonstrated by the following data. Eighteen transgenic clones were successfully selfed on both selective and non-selective media. In 17 of them, 100% of the offspring germinated on selective medium independently from the initial culture conditions (Figure 3). When these spores were further regenerated and selfed, 100% meiotic transmission of the character was demonstrated up to the fourth generation (data not shown). In one case, clone Hg^R 15.02, spores, like protoplasts, did not germinate on Hygromycin. However, when these spores were germinated on non-selective medium and further transferred to selective medium, 100% of them regenerated normally. These data demonstrated that the introduced sequences were meiotically stable, and in the case of strain 15.02, it was postulated that the inability of spores and protoplasts to germinate on Hygromycin could be associated with a position effect.

figure 3 link

Figure 3. Phenotypical and genetic characteristics of transgenic clones. (a) Non selective to selective growth assay on clones Kan^R 7.01, 8.01, 11.01 and 12.01 (from left to right). Top row: non-selective to selective growth, bottom row: selective growth. (b)Rescue experiment: the whole gametophore regenerate a protonema. (c) 100 % meiotic transmission of the character. Spores from the fourth generation of HP 23 L2 were germinated under selective (left) and non-selective to selective conditions (right). (d) Independent 1:1 segregation of the Kan^R and ylo marker in spores of HP 23 L2 x nicB5ylo6 germinated under non-selective to selective conditions.

In order to investigate the number of integration sites in the genome of transgenic strains, nine independent transgenic strains were successfully crossed with the self-sterile strain *nicB5ylo6* . A 1:1 segregation of the resistant character was monitored in every tested offspring, demonstrating that integration of the transforming sequences occured in a single genomic locus in each tested strains (see Table 2 in chapter 2.3). In these crosses, independent segregation of the yellow marker among sensitive and resistant spores was also monitored, demonstrating that these trans-loci were unfortunately not linked to this marker (Figure 3). Finally, in order to test a possible linkage between independent trans-loci, protoplasts from Kan^R strain HP23 L2 and Hg^R strain 15.01 were somatically fused, and diploid double resistant colonies were regenerated and selfed. Segregation data are presented on Table 4. Independent segregation of the kanamycin and the hygromycin resistant markers was monitored, demonstrating that these genes were integrated at different genomic loci. These genetic data demonstrated that in each strain tested, integration of the transforming sequences occured in a single genomic locus, and that these loci were independent in two different transgenic strains.

Table 4. Segregation of the kanamycin and hygromycin markers in the progeny of the diploid double resistant clone HP23 L2 + 15.01 produced by somatic fusion. Spores were initially germinated on non-selective PP NH_4 medium and further transferred to selective media. Expected frequencies assumes that in (a) bivalents and in (b) tetravalents are formed during meiosis (Cove 1983).

Selection	No. spores tested	Antibiotic resistant	Observed frequency	Expected frequency (a)	Expected frequency (b)
G-418	89	76	85.4	83.3	78.6
Hg	85	66	77.6	83.3	78.6
G-418 + Hg	80	43	53.7	44.0	61.9

Molecular analysis.

Southern Blot analysis was performed to determine the number of copies integrated in the genome and to confirm at the molecular level the phenotypical and genetic data. DNA from three independent Kan^R transgenic strains were digested with the restriction enzymes Eco R1 or Pst 1, fragments were separated by agarose gel

electrophoresis, transferred to nitrocellulose, and hybridized with the ^{32}P-labelled 800 bp Hind III fragment of pHP 23b carrying the *npt II* coding sequence (Figure 4). Eco R1 cuts twice in the plasmid, giving rise to a 1733 bp fragment containing the complete plant expression cassette and to a 2641 bp fragment of bacterial sequences. Pst 1 cuts twice in the plasmid, generating a 661 bp fragment carrying the 3' sequences of *npt II*, and a 3713 bp fragment containing 200 bp of the 5' sequences of *npt II* and the rest of the plasmid. This following conclusions were drawn from this analysis.

(1) When uncut DNA was loaded in the tracks, the hybridization signal comigrated with high molecular weight DNA (lanes 7 and 11).

(2) Each clone presented a hybridization signal of the expected sizes, i.e. at 1733 bp in Eco R1 digest, and at 3713 and 660 bp in Pst 1 digests, and the additional bands in lane 12 were shown to be generated by incomplete digestion of the DNA.

(3) Plasmid copy number varied between different clones, ranging from ca 5 to over 20 copies per haploid genome.

(4) Plasmid copy number was constant over 2 generations (compare lanes 3 and 4 with lanes 5 and 6).

(5) Three hybridization signals of low intensity and of unpredicted size, corresponding to putative flanking sequences, were detected in this analysis; one located at ca 1500 bp in lane 8 and the 2 others at ca 5800 and 7000 bp in lane 13. Overexposure of this autoradiograph confirmed these bands and revealed the presence of two additional ones at ca 6600 and 17000 bp in lane 9 (data not shown). Stability of copy number at different developmental stages was also demonstrated and similar results were obtained with Hg^R clones (data not shown). These data demonstrated that several copies of the transforming plasmid were stably integrated in the genome of *P. patens*.

Conclusion.

Mitotic and meiotic stability of the resistant phenotype, mendelian segregation of the resistance as a single character and molecular evidence for the presence of flanking sequences demonstrate that class I stable clones correspond to transgenic plants with multiple copies of the plasmid integrated at single independent genomic loci. This was the first demonstration of the successful integrative transformation of the moss *P. patens* (Schaefer, et al. 1991).

figure 4 link

Figure 4. Southern blot analysis of DNA extracted from different Kan^R stable strains. Lanes 1 and 2: untransformed pabA3. Lanes 3 and 4: DNA from HP23 L2 initial. Lanes 5 and 6: DNA from HP23 L2 first generation offspring. Lanes 7, 8 and 9: DNA from 11.01 initial. Lane 10: DNA from 11.01 first generation offspring. Lanes 11, 12 and 13: DNA from 7.01. Lane 14: genomic reconstruction with 5 equivalent copies of the Eco R1 fragment of pHP 23b. 2 µg of DNA were loaded in each lane, except in lanes 7, 10 and 11 which were loaded with 200 ng DNA. (U) uncut DNA, (E) Eco R1, (P) Pst 1 digested DNA. Arrows indicate the 1.5, 5.8 and 7 kb hybrid sequences. Specific activity of the probe was $7 \cdot 10^8$ cpm / µg DNA

2.2.2.5. Characterisation of class II unstable replicative transformants

Transformation frequencies.

As mentionned previously, these clones constituted the main class of resistant clones and were characterised by their high frequency of occurrence and the high mitotic instability of the resistant phenotype. This instability was associated with a reduced growth rate and alteration of the regeneration pattern of these clones under selective pressure, and with the apparition of sensitive and resistant sectors within the colony when selection was above 5 µg/ml Hygromycin B or 10 µg/ml G-418.

Initial transformation frequencies have already been discussed (see section initial transformation frequencies). However, since a constant decrease in the number of resistant clones, which could occasionally represent up to 80% of the initial counts, was monitored during the next stages of selective growth, it was not clear whether such high initial RTF reflected efficient transient gene expression or efficient genetic transformation associated with strong mitotic instability of the introduced sequences. Therefore, several hundred single protoplast derived resistant colonies, initially selected on different concentration of antibiotics, were isolated and further cultivated on PP NH_4 media supplemented with different concentration of antibiotics (Table 5). This experiment was realised to test the stability of the resistant phenotype, and the following observations were made.

(1) Resistant clones initially selected on low selective pressure and transferred to high concentration of antibiotics were lost at a high rate, with only 20 to 30 % of them surviving one month after the initiation of selection.

(2) A constant loss of resistant clones, initially selected on high concentration of antibiotics and transferred to the same selective media, was also monitored, but this loss only represented about 15 % of the initial number of clones.

(3) Optimum antibiotic concentration for the efficient selection of transformed clones and for a reliable stability of the unstable clones were established at 25 to 30 µg/ml for G-418 and at 15 to 20 µg/ml for Hygromycin B.

Additionally, when single clones were isolated, fragmented with a Polytron and further cultivated on selective medium, all of them could be propagated for years, indicating that the mitotic instability of these clones could be circumvented with appropriate subculture conditions. Another line of evidence, suggesting that the high RTF monitored here reflected efficient genetic transformation and not transient expression of the transforming sequences, came from the comparison of the transient GUS expression with the RTF monitored at different times following DNA uptake (Figure 5). This comparison indicated that the kinetics of transient gene expression and of the recovery of resistant clones were completely different, with highest RTF achieved when transient GUS expression had already dropped to about 25% of its maximum. It was therefore concluded that the high initial RTF monitored in these experiments effectively reflected the number of clones genetically transformed, but that these transformants displayed strong mitotic instability of the introduced sequences,

accounting for the constant loss of clones during further selective growth. Such high transformation frequencies provided evidence for efficient replicative transformation of *P. patens* protoplasts.

Table 5. Survival rates of single protoplast derived resistant colonies selected on increasing concentrations of antibiotics, isolated and further cultivated on PP NH_4 media supplemented with increasing concentration of antibiotics. Survival rates were monitored 2 weeks after the second transfer, i.e. 1 month after the initiation of selection.

Second selection	G-5		G-10		G-25		G-50	
Initial selection	Total tested	Survival. (%)	Total tested	Survival. (%)	Total tested	Survival. (%)	Total tested	Survival. (%)
G-5	263	100	98	68	81	32	78	21
G-10	91	100	209	86	98	42	95	32
G-25	103	98	112	87	239	67	118	49
G-50	116	99	115	97	123	85	306	64
Second selection	Hg-2.5		Hg-5		Hg-12.5		Hg-25	
Initial selection	Total tested	Survival. (%)	Total tested	Survival. (%)	Total tested	Survival. (%)	Total tested	Survival. (%)
Hg-2.5	243	99	106	89	109	43	103	20
Hg-5	107	98	257	90	106	57	110	36
Hg-12.5	125	98	122	97	249	84	125	59
Hg 25	52	100	54	100	78	97	201	84

Phenotypical analysis.

Mitotic instability of the resistance was monitored at all the developmental stages of regenerating colonies and are illustrated on Figure 6 (see also Figure 1). At the very early stages of protoplasts regeneration, resistant colonies displayed numerous cell divisions (Figure 6a), and the presence of resistant and sensitive sectors was occasionally monitored. However, filaments usually did not develop normally, giving rise to very compact colonies. Such regeneration pattern was often associated with early differentiation of gametophores on hygromycin supplemented media. When resistant colonies were further transferred to selective medium, regenerating-resistant and bleaching-sensitive sectors were routinely observed in the protonema and differentiated gametophores systematically died, indicating strong mosaicism in the resistant colonies (Figure 6b). Protoplasts isolated from these clones growing on selective medium were able to regenerate on selective medium. However, when these protoplasts were initially regenerated for ten days on non-selective medium and further transferred to selective medium, more than 90% of the regenerated colonies died (data not shown). When these

clones were submitted to the non-selective to selective assay, more than 95% of the protonema generated in 10 days on non-selective medium became moribund, with only a few discrete regenerating foci displaying restricted growth on selective media (Figure 6c). Rescue experiments were performed on differentiated gametophores isolated from resistant colonies grown on selective medium. All tested gametophores died on selective media, and regeneration was always restricted to the filaments associated to about 10% of the tested gametophores (Figure 6d). These data demonstrated that the resistant phenotype was mitotically unstable upon selective medium and was lost in 90% of the cells within 10 days of non-selective growth, and strongly suggested that maintenance of the transforming sequences was tissue specific, being restricted to the protonema of the resistant colonies.

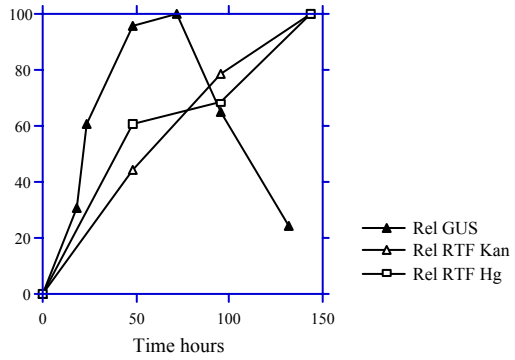

Figure 5. Comparison of transient gene expression with RTF monitored at different times after DNA uptake. Relative GUS specific activities (%) were calculated relatively to the maximum activity monitored at 72 hours after uptake. Relative RTF (%) were calculated relatively to the RTF monitored 6 days after uptake for each concentration of antibiotics, and the mean value was determined. Mean values for G-418 resistance RTF were 44.4 ± 8.1 % at day 2 and 78.6 ± 4.3 % at day 4, and for Hg resistance RTF 60.5 ± 16.7 % for day 2 and 68.3 ± 11.6 % at day 4..

Genetic analysis.

Despite numerous attempts (over 20 independent clones tested), meiotic transmission of the resistant phenotype has never been observed. Sporogenesis has never been successfully achieved under selective conditions since differentiated gametophores usually died when the culture was irrigated to favour fertilisation events, due to diffusion of the antibiotic in water. Mature sporophytes were only recovered from resistant clones regenerated on non-selective medium and no resistant spores were recovered from these (data not shown). These data were actually not surprising in view of the strong mitotic instability monitored in the protonema, and indirectly confirmed the tissue-specific maintenance of the resistance observed phenotypically.

figure 6 link

Figure 6. Phenotypical characteristics of class II unstable clones. (a) Early stage of regeneration characterised by numerous mitosis with restricted growth of the filaments and early differentiation of gametophores on Hg 25. (b) Strong mosaicism of a KanR colony Associated with bleaching of differentiated gametophores. (c) Population of KanR clones initially selected on increasing concentrations of G-418 for 10 days, transferred to non-selective medium for another 10 days, and transferred back to G-418 25. (d) Rescue experiment on a differentiated gametophore from a KanR clone. Regeneration of protonema is restricted to the filaments.

Preliminary molecular analysis.

Southern Blot analysis was performed to investigate whether the phenotypical loss of resistance monitored upon non-selective medium and in differentiated gametophores was associated with loss of the introduced sequences or with gene silencing. Total DNA was extracted from Hg^R clones SG1 and SG2 regenerated on selective and non-selective medium (these unstable HgR clones were isolated from exp.2). DNA was digested with Eco R1 and Bam H1, separated by agarose gel electrophoresis, transferred to nitrocellulose and hybridized with the ^{32}P-labelled 1 kb Bam H1 fragment of pGL 2, containing the coding sequence of *aph IV* (Figure 7). Bam H1 cuts twice in the plasmid, giving rise to a 1033 bp fragment containing the *aph IV* coding sequence and a 3425 bp fragment carrying the 35S promoter and polyA sequences of CaMV and the bacterial sequences. Eco R1 cuts three times in the plasmid, generating a 811 bp fragment containing the CaMV promoter and the 5' sequences of *aphIV*, a 1006 bp fragment containing the 3' end of *aphIV* and the polyA sequences and a 2641 bp fragment corresponding to bacterial sequences. The following conclusions were drawn from this analysis.

(1) The probe hybridized only with DNA extracted from material grown under selective conditions, demonstrating that the phenotypical loss of resistance monitored upon non-selective medium and in differentiated gametophores was correlated with a physical loss of the transforming sequences.

(2) The hybridization signals corresponded to the expected sizes, i.e. ca 1000 bp in Bam H1 digest and 800 and 1000 bp in Eco R1 digest, but the bands appeared to be poorly resolved, indicating a possible degradation of the transforming sequences.

(3) SG1 appeared to carry 20 to 30 copies, and SG2 about 10 copies of the plasmid per haploid genome; however, such copy number was probably underestimated due to the fact that these clones were shown to be mosaics, suggesting that copy number per resistant cell could be higher.

(4) Hybridization signal of unpredicted size, which would correspond to flanking or recombined sequences, were not monitored in this analysis, suggesting that the transforming sequences were neither integrated in the genome, nor recombined with other sequences.

In another Southern blot analysis performed on DNA extracted from a population of unstable clones, uncut DNA was loaded in the tracks and the hybridization signals were shown to comigrate with high molecular weight DNA, indicating that the transforming sequences were maintained as high molecular weight structures in these clones (data not shown). However, molecular analysis of this class of clones is so far insufficient.

figure 7 link

Figure 7. Southern blot analysis of DNA extracted from Hg^R clones SG1 and SG2. grown on selective and non-selective medium. Lanes 1 and 2: DNA from untransformed wild-type. Lanes 3 and 4: DNA from SG1 grown on selective medium. Lanes 5 and 6: DNA from SG2 grown on selective medium. Lanes 7, 8 and 9: DNA from SG1 grown on non selective medium. Lanes 10, 11 and 12: DNA from SG2 grown on non selective medium. Lanes 13, 14 and 15: genomic reconstruction with 1, 5 and 10 equivalent copies of the Bam H1 fragment of pGL 2. 2 µg of DNA were loaded in each lane. (B) Bam H1, (E) Eco R1, (H) Hind III digested DNA. Specific activity of the probe was $5 \cdot 10^8$ cpm / µg DNA.

Conclusion.

High frequencies of transformation and high mitotic instability, as evidenced by the reduced growth rate on selective medium, mosaicism of the resistant colonies and tissue-specific maintenance of the resistance provided good evidence for extrachromosomal replicative transformation in *P. patens*. Preliminary molecular characterisation established a correlation between phenotypical and physical loss of the resistance gene, and indicated that these sequences were maintained as high molecular weight structures in the resistant cells (data not shown). It was therefore concluded that bacterial plasmids introduced in moss protoplasts were probably converted to high molecular weight extrachromosomal structures which were most probably replicated in the protonema but not in the gametophore of these resistant clones. The origin of DNA replication would be provided by the bacterial sequences of the transforming plasmids and, since these plasmids did not provide functional elements involved in the mitotic segregation of replicated DNA, such concatenates would display a mitotic instability similar to ARS plasmids in *S. cerevisiae*, or to the transient resistant clones of *C. elegans* described in (Mello, et al. 1991).

2.2.2.6. Characterisation of class III fast growing replicative transformants

Transformation frequencies.

As mentioned previously, these clones were characterised by a growth upon selective medium sometimes as good as stable clones growth, but were distinguished from these since they were still displaying loss of the resistant phenotype in the absence of selective pressure. These clones were recovered at RTF ranging from 0.3 to $4.4 \cdot 10^{-5}$ (2 in 44000 in Exp.1, 10 in 565000 in Exp. 2, and 4 in 1320000 in Exp 3). These low RTF indicated that the transforming sequences had to undergo some modifications as compared to class II resistant clones to generate this class of resistant clones. Data from experiment 2 suggested that linear plasmids could be better templates than supercoiled ones for the generation of such clones, since 7 out of 10 class III clones were recovered with such plasmids. These clones were also more frequent in experiments in which protoplast survival was low (see Tables 2 and 3), suggesting that the presence of significant amounts of moss genomic DNA in the samples during DNA uptake could be required for the generation of this class of resistant clones.

Mitotic instability of the resistant phenotype was initially monitored in non-selective to selective assays. These experiments indicated that selective growth and mitotic stability of the resistant phenotype in these clones were actually improved as compared to unstable class II clones. Moreover, differences both in selective growth rate and in the loss rates of the resistance upon non-selective medium were readily observed between different clones, suggesting different levels of mitotic stability improvement (Figure 8a).

figure 8 link

Figure 8. Phenotypical and genetic characteristics of class III resistant clones. (a) Non-selective to selective growth assay on 8 class III and 2 class II (6.04 and 3.04) Kan^R strains. Class III clones displayed a range of growth improvement on selective medium and a range of loss of the resistance upon non-selective to selective conditions as compared to class II (left plates NS/S growth, right plates selective growth). (b) Rescue experiment on differentiated gametophores form Kan^R ABD2 S6 showing 10 sensitive and two partially resistant gametophores. (c) Non mendelian transmission of the character in the offspring of KanR clone 12.02. Germination of spores in non-selective to selective conditions is shown on the left plate.

When differentiated gametophores from 10 clones were submitted to rescue experiments, most tested gametophores died and regeneration was restricted to the protonema. Yet, some differentiated gametophores displaying partial or complete regeneration on selective medium were recovered from 6 of these clones (Figure 8b).

Stable meiotic transmission of the character was not monitored, but non-mendelian meiotic transmission was observed in 3 out of 10 tested clones (Figure 8c). These features will be illustrated by the characterisation of two of these clones, Kan^R ABD2 S6, which displayed a strong improvement, and Kan^R ABD2 L1, which displayed a weak improvement of mitotic stability as compared to class II resistant clones (Figure 8a).

Phenotypical and genetic characterisation of clone ABD2 S6.

This clone was characterised by a growth on selective medium comparable to transgenic clones. Non-selective to selective growth assay demonstrated loss of the resistance in about 50% of the regenerated protonema, but regenerating resistant foci still displayed a fast growth rate on selective medium. Differentiated gametophores were tested in rescue experiments and most of them displayed filament-restricted regeneration similar to class II unstable clones upon selective medium (Figure 8b). Yet, one complete resistant gametophore was recovered in these rescue experiments, suggesting stabilisation of the resistant phenotype.

This subclone (ABD2 S6 Resc.) was further propagated and re-tested in non-selective to selective assay. Loss of resistance in the absence of selection was readily monitored, but was dependent on the previous culture conditions, being hardly detectable when the inoculum was prepared from a selective culture and much more visible when the inoculum came from a non selective culture. This indicated that the loss of resistance following a short period of non-selective growth could be occasionally difficult to demonstrate in this clone, due to the improved mitotic stability of the transforming sequences in the protonema. This subclone was also selfed in order to test the meiotic stability of the character. No resistant spores were detected out of several thousands tested. Similar results were obtained with subclones derived from resistant gametophores recovered in clones Kan^R 2.01, 4.01, 12.02 and Hg^R 10.02 (data not shown). These data provided phenotypical evidence that the unstable phenotype can occasionally be transmitted and maintained in gametophores of this class of clones, and that this event was not associated with integration of the transforming plasmid in the genome.

In the same time, the original clone ABD2 S6 was successfully selfed on selective medium and 11 resistant spores out of 48000 were recovered. Such frequency of meiotic transmission, around 0.02 %, was clearly non-mendelian and was providing evidence for extrachromosomal transformation. One of these single spore derived colony (ABD2 S6 T1) was propagated and selfed, and both mitotic and meiotic stability of the resistance were tested. These experiments demonstrated that ABD2 S6 T1 was still displaying mitotic instability of the resistant phenotype, as monitored by non-selective to selective growth assay, and that the resistance was not meiotically transmitted, since the offspring of this clone was sensitive (data not shown).

Meiotic transmission of the unstable character at similar non mendelian frequencies was also monitored in clones Kan^R 12.02 and Hg^R 10.02. All spore-derived resistant colonies of clone Hg^R 10.02 were displaying mitotic instability, as monitored by non-selective to selective assays, and transmission of the resistant character to the second generation was not detected. In Kan^R 12.02, all spore-derived resistant colonies from the first generation were unstable. Three of these colonies (12.021, 12.022 and 12.023) were selfed again, and non mendelian transmission of the unstable character to the second generation was monitored in the offspring of 12.022 and 12.023. Yet, 100% resistant spores were recovered from selfed 12.021, and colonies were shown to maintain the resistant phenotype after their germination on non-selective medium, and in response to the non-selective to selective assay. These observations suggested a delayed integration event in 12.021 offspring. These results confirmed the observations made in ABD2 S6 that the unstable phenotype could occasionally be transmitted through meiosis, but also provided phenotypical and genetic evidence for the occurrence of integration events during germ line transmission of the unstable character, in a way similar to that observed in extrachromosomal transformants of *C. elegans* (Mello, et al. 1991) and *X. laevis* (Etkin and Pearman 1987).

Molecular characterisation of clone ABD2 S6.

Southern blot analysis was performed on DNA extracted from ABD2 S6 grown on selective medium and collected at different developmental stages (protonema only, and protonema with differentiated gametophores), and on DNA extracted from ABD2 S6 T1 grown on selective medium and collected with well differentiated gametophores (Figure 9). These DNA were digested with the restriction enzymes Bgl 2 or Pst 1 and hybridized with the ^{32}P labelled 800 bp Hind III fragment of pABD 2, carrying the coding sequence of *npt II*. Bgl 2 cuts once in the plasmid, generating a 5365 bp fragment corresponding to the linear plasmid. Pst 1 cuts three times in the plasmid, generating a 556 bp fragment within promoter sequences, a 1036 bp fragment containing the 3' sequences of *npt II*, and a 3673 bp fragment carrying the 5' sequences of *npt II* , the polyA sequences and the bacterial plasmid. The following observations were made from this analysis.

(1) The hybridization signal comigrated with high molecular weight DNA and no low molecular weight signal was detected when uncut DNA was loaded in the track, demonstrating that the transforming sequences were either maintained as high molecular weight elements or integrated in the moss genome.

(2) Hybridization bands of the expected sizes, i.e. 5365 bp in Bgl 2 digests and 1036 bp and 3673 bp in Pst 1 digests were recovered in protonema and gametophores of ABD2 S6, and the presence of one single major band in Bgl 2 digest suggested that the plasmid sequences were arranged in tandem head-to-tail structure in the concatenate.

(3) Additional bands were recovered in both digests and all of them were shown to be generated by partial digestion of the DNA: these bands were identical in protonema and gametophore of ABD2 S6, which could indicate that some restriction sites had been lost or modified during extrachromosomal array formation.

figure 9 link

Figure 9. Southern blot analysis of DNA extracted from clone Kan^R ABD2 S6 at different developmental stages, and from ABD2 S6 T1, a single-spore derived resistant colony obtained from selfed ABD2 S6. Lanes 1 and 2: DNA from untransformed pabA3. Lanes 3, 4 and 5: DNA from protonematal tissue of ABD2 S6. Lanes 7 and 8: DNA extracted from differentiated gametophores of ABD2 S6. Lane 9: DNA from differentiated gametophores of ABD2 S6 T1. Lane 13: genomic reconstruction with 5 equivalent copies of undigested pABD2. 2 µg of DNA were loaded in each lane except lane 3 which was loaded with 200 ng DNA. (U) undigested DNA, (B) Bgl 2, (p) Pst 1 digested DNA. Specific activity of the probe was $2 \cdot 10^8$ cpm / µg DNA. Top panel, 24 hours exposure, bottom panel 10 days exposure at -70°C

(4) Beside fragments of the expected sizes, a smeared hybridization was monitored in protonema tissue which could not be considered as non-specific, since DNA from untransformed moss did not display such hybridization. When this result was compared with Southern blot analysis of DNA extracted from extrachromosomal and integrative transformants of *C. elegans* (compare with figure 2 and 7 in (Mello, et al. 1991)), it seemed that this complex pattern was a general feature of extrachromosomal transformation. Such smear could be explained by the following mechanisms: (a) part of the replicated extrachromosomal arrays were not retained in the nucleus during mitosis and were subsequently degraded in the cytoplasm, (b) numerous and complex rearrangement of the transforming sequences have occured during array formation. Both mechanisms are not mutually exclusive and could account for the generation of this smeared hybridization signal.

(5) Plasmid copy number per haploid genome was estimated to be above 200 in protonema and around 10 to 20 in gametophores of ABD2 S6, confirming thus that the observed loss of resistance monitored in the gametophores was correlated with a physical loss of the introduced sequences.

(6) Two faint bands of 1036 and 3673 bp were detected in ABD2 S6 T1 DNA after overexposure of the autoradiograph, and their intensity indicated that copy number in this DNA was around 1 per haploid genome; such low copy number was considered as evidence for the tissue specific loss of the resistance gene in ABD2 S6 T1 gametophores, confirming that the transforming sequences were still unstable after meiotic transmission.

(7) Beside the large smeared hybridization mentioned previously, no low intensity hybridization signals of unpredicted size, indicating the presence of flanking sequences or of putative recombination of the concatenate with genomic sequences, were monitored in this analysis (the 11 kb signal in both Bgl 2 digests of ABD2 S6 was interpreted as representing a dimer of the plasmid generated by partial digestion or loss of restriction sites). This suggested that the transforming sequences were neither integrated in the genome, nor recombined with other DNA sequences. However, this should not be considered as a proof for the absence of such events, since Bgl 2 does not cut within the probe, allowing such events to be present without being detected in this analysis.

These results provided phenotypical, genetic and molecular evidence for (1) the mitotic instability of the resistance and the correlation between phenotypical and physical loss of the resistance gene in differentiated gametophores, (2) the occasional transmission of the unstable character to differentiated gametophores and through meiosis, (3) the presence of numerous copies of the transforming plasmid in protonematal tissue, and (4) the arrangement of the transforming sequences in tandem repeats in high molecular weight arrays. These data also provided phenotypical and genetic evidence that delayed integration events can occasionally occur during germline transmission of the unstable element.

Phenotypical characterisation of Kan^R clone ABD 2 L1.

This clone was characterised by a weak improvement of mitotic stability as compared to other clones of this class, loss of the resistance in more than 90% of the protonema in non selective to selective assay (see figure 8), a strict basal regeneration of gametophores in rescue experiments and absence of meiotic transmission of the unstable phenotype (data not shown). Yet, when differentiated gametophores from one year old selective and non-selective cultures were once again tested in a last rescue experiment, a resistant gametophore was recovered from the non-selective culture. This subclone, ABD2 L1 Resc., was further propagated and characterised. Non-selective to selective growth assay demonstrated stable maintenance of the resistance after a period of non-selective growth, all tested differentiated gametophores were able to regenerate a protonema upon selective medium and 100% resistant spores were recovered after selfing (data not shown). These data were already providing evidence for stable transformation of ABD2 L1 Resc. following delayed integration of the resistance gene in the moss genome during vegetative propagation.

Molecular characterisation of Kan^R clone ABD 2 L1.

Southern blot analysis was performed on DNA extracted from ABD2 L1 grown on selective medium and collected at different developmental stages (protonema only (P) and protonema with differentiated gametophores (G)), and on DNA extracted from ABD2 L1 Resc. grown on selective medium and collected with well differentiated gametophores. These DNA were digested with the restriction enzymes Bgl 2 or Pst 1 and hybridized with the ^{32}P labelled 800 bp Hind III fragment of pABD 2, carrying the coding sequence of *npt II* (Figure 10). The expected hybridization pattern has already been described for the Southern blot analysis of DNA from the Kan^R clone ABD2 S6 (see above). This analysis confirmed the conclusions drawn from the Southern blot analysis of DNA from S6, that is that the transforming sequences in class III resistant clones were maintained as high molecular weight arrays with plasmid sequences arranged in a head-to-tail tandem repeats, and that the observed loss of resistance monitored in gametophores of L1 was correlated with physical loss of the resistance gene. Yet significant differences were monitored between these two clones.

(1) Copy number per haploid genome in protonema of ABD2 L1 was estimated to be around 20, which is at least 10 fold lower than in ABD2 S6 protonema. This indicated either that copy number per resistant cells was lower in L1 than in S6, or that there was less resistant cell in L1 than in S6 protonema. The second alternative

appeared more likely, since it permitted to correlate this lower copy number with the reduced mitotic stability of the transforming sequences monitored in L1 as compared to S6.

(2) Copy number in DNA isolated from L1 gametophores was estimated to be lower than 5, confirming the physical loss of sequences at this developmental stage. Yet copy number in L1 Resc. gametophores was estimated to be around 40, indicating that the transforming sequences were developmentally stable in this subclone. Integration of the resistance gene in L1 Resc. was further indicated by the presence of 2 low intensity hybridization bands of ca 7000 bp and 23000 bp in the Pst1 digest, corresponding to putative flanking sequences.

figure 10 link

Figure 10. Southern blot analysis of DNA extracted from clone Kan^R ABD2 L1 and its stable subclone ABD2 L1 Resc. Lanes 1 and 2: DNA from untransformed pabA3. Lanes 3, 4 and 5: DNA from protonematal tissue of ABD2 L1. Lanes 7 and 8: DNA extracted from differentiated gametophores of ABD2 L1. Lanes 9, 10 and 11: DNA extracted from differentiated gametophores of ABD2 L1 Resc. Lane 13: genomic reconstruction with 5 equivalent copies of Pst 1 digested pABD2. 2 µg of DNA were loaded in each lane. (U) undigested DNA, (B) Bgl 2, (P) Pst 1 digested DNA. The arrow indicate the 7.5 kb recombined signal. Specific activity of the probe was $2 \cdot 10^8$ cpm / µg DNA

(3) A hybridization band of ca 7500 bp was detected in the Bgl 2 digest of DNA from L1 protonema, and this hybridization band provided evidence for the occurrence of recombination events between the transforming plasmid and possibly moss DNA sequences in the generation of class III clone. The intensity of the band corresponded to 1 to 3 copies per genome. However, since only a fraction of the protonematal cells were actually resistant, such copy number suggested that several copies of these recombined sequences were present per resistant cell. This band was also detected in the Bgl 2 digest of DNA from L1 Resc. gametophores, but with a copy number around 10 per genome, which would be too high to account for the presence of a flanking sequence. Two mechanisms could explain this apparent amplification: (a) the recombined sequence has been duplicated during formation of the extrachromosomal array, and then delayed integration occured randomly and/or (b) delayed integration occured by homologous recombination between the recombined extrachromosomal moss DNA sequence and its chromosomal homolog, resulting in a duplication of the recombined sequences associated with the generation of flanking sequences of higher copy number than one. The data presented here suggested the occurrence of both mechanisms, since no additional flanking sequences were detected in this digest.

The analysis of ABD2 L1 confirmed the conclusions drawn from the analysis of ABD2 S6, but also provided molecular evidence for the presence of recombined sequences in the extrachromosomal arrays, as well as for the occurrence of delayed integration of the transforming sequences in the genome during vegetative propagation. Moreover, these data indicated that delayed integration in the genome occured within these recombined sequences in ABD2 L1 Resc., suggesting that these sequences could be generated by recombination with moss genomic DNA, and that the presence of moss genomic DNA on the extrachromosomal arrays could facilitate delayed integration events by homologous recombination.

Conclusion.

Taken together, these data demonstrated that the transforming sequences in class III clones displayed a range of improved mitotic stability as compared to those in class II, up to a stage where the loss of resistance following a period of non-selective growth could be difficult to demonstrate in protonematal tissue. Such improvement was associated with a partial loss of tissue-specific maintenance of the resistant phenotype, allowing unstable resistance to be occasionally but reproducibly maintained in differentiated gametophores (monitored in 6 out of 10 tested clones). When complete resistant gametophores were isolated and further characterised, loss of the resistance following a period of non-selective growth was still monitored, and stable meiotic transmission of the character was not observed, demonstrating that the maintenance of the resistance in differentiated gametophores was usually not associated with integration of the transforming sequences in the genome. However, analysis of ABD2 L1 Resc., which was isolated as a complete resistant gametophore, demonstrated that delayed integration during vegetative propagation can also occur in these clones.

Meiotic transmission of the unstable character at non-mendelian frequencies was monitored in 3 out of 10 tested clones, and such frequencies provided evidence for extrachromosomal transformation. Phenotypical and molecular analysis of clone ABD2 S6 T1 demonstrated that the unstable character was effectively transmitted to the next generation, and phenotypical analysis of Hg^R 10.02 and Kan^R 12.02 offspring confirmed these observations. However meiotic transmission of the character associated with stabilisation of the resistant phenotype was also monitored in one of these clones (12.021), suggesting that the transforming sequences could become integrated in the genome during germ line transmission of the resistance gene.

Molecular analyses have established a correlation between phenotypical loss of resistance in differentiated gametophores and physical loss of the resistance gene, providing evidence for extrachromosomal replicative transformation. These analyses have also indicated the presence of a high copy number of the transforming plasmid per resistant cells (>100), that these plasmids were arranged in tandem repeats, and have provided evidence for the presence of recombined sequences in high molecular arrays, as well as for the occurrence of delayed integration of extrachromosomal sequences in the moss genome occurring within these recombined sequences. It was therefore concluded that class III resistant clones were extrachromosomal replicative transformants, and that the data presented here sustained the hypothesis that the improved mitotic stability of the extrachromosomal arrays monitored in these clones was associated with recombination of the transforming sequences with moss genomic DNA involved in DNA replication and/or mitotic segregation of replicated DNA. Additionally, these data also sustained the hypothesis that these genomic sequences could provide a target for homologous recombination mediated delayed integration events, accounting for the high frequency of occurrence of such events in this class (see also next section).

2.2.2.7. Delayed integration events

Spontaneous delayed integration during vegetative propagation and through germ line transmission of the unstable resistant character was demonstrated in 2 class III clones (ABD2 L1 and 12.021 T1). Spontaneous delayed integration events were also monitored during vegetative propagation of class II resistant clones, and these events were identified by the change from restricted to unrestricted growth upon selective medium. Hg 27 DI was one of the resistant clones isolated in experiment 3 to test the rate at which initial resistant clones were lost during further selective growth. About a month after transformation, this clone suddenly displayed unrestricted growth upon selective medium. This clone was further analysed and shown to display responses to the non-selective to selective assay and to rescue experiments similar to those of stable transgenic clones (data not shown). The final proof of delayed integration of the transforming sequences into the genome was provided by the successful targeting of the translocus of Hg 27 DI monitored in the second gene targeting experiment (see chapter 2.3, table 1 and 2). Yet, spontaneous delayed integration during vegetative propagation of class II clones was rare, since it has only been monitored once in these studies. This

indicated that the target sequences for delayed integration events were in most cases not present in the extrachromosomal array of class II clones.

Beside spontaneous delayed integration events, delayed integrations were also monitored following fragmentation of unstable class II and class III protonema. These events were identified by the apparition of fast growing foci in the inoculum on selective medium. The stable clone PCV - 35S rol B was obtained this way. Initially isolated as an unstable class II, this clone was fragmented with a polytron and replated on selective medium. Several fast growing foci were isolated and further characterised (Figure 11). Mitotic stability of the resistance was confirmed in non-selective to selective growth assay and by rescue experiments (Figure 11), and the final demonstration of integrative transformation in this clone was provided by the recovery of 100% resistant spores following selfing (data not shown). The frequency of occurrence of fragmentation-induced delayed integration was low in unstable class II clones, since such event has only been monitored once in these studies. Fragmentation-induced delayed integration events were monitored at a much higher rate in unstable class III resistant clones, since the apparition of fast growing foci was monitored in 3 out of 6 clones tested simultaneously in a non-selective to selective assay. It was postulated that the induction of wound responses and DNA repair following fragmentation could facilitate delayed integration in the genome.

figure 11 link

Figure 11. Fragmentation induced delayed integration in KanR clone pPCV-35S rolB. (a) Fast growing foci regenerating on selective medium. (b) Rescue experiment on differentiated gametophores isolated from fast (left) and slow growing (right) resistant colonies. All gametophores isolated from fast growing

foci regenerated a protonema on selective medium, whereas occasional basal regeneration occured in gametophores isolated from slow growing colonies.

Spontaneous and fragmentation-induced delayed integration events were monitored in both class II and III resistant clones, providing another evidence for extrachromosomal replicative transformation. The higher frequencies of such events monitored in class III also provided another indirect indication that the generation of class III unstable clones resulted from the recombination of the transforming plasmid with moss genomic sequences, and that these sequences provided a substrate for homologous recombination-mediated delayed integration events. Phenotypical changes from stable clones to one of the unstable classes has never been observed, indicating that integrative transformation can not be reversed. Changes from class II to class III have not yet been formally identified, but it is postulated that such event could occur following fragmentation of class II clones.

2.2.2.8. Transformation with YAC vectors

YAC vectors carrying the plant expression cassette from pHP 23b were tested in different conditions to transform P. patens. Initial transformation frequencies with YACs were 10 fold lower than those with pHP 23b or pGL 2, but 10 fold higher than those with plasmids pPVC002 and derivatives. Stable transformation frequencies were similar to those monitored with other plasmids (Table 6 and Figure 12).

Table 6. Transformation frequencies monitored following the introduction of YAC RC AB1 and AB2. Transformation conditions were as follows. AB sc: 15 µg supercoiled YAC DNA, AB lin: 15 µg Bam H1 linearized YAC DNA, AB 1+ λ: 4 µg linear YAC DNA with 12 µg Bam H1 digested lambda DNA, AB + SS. 4 µg linear YAC DNA with 10 µg sheared Salmon Sperm DNA.

III. YAC	Survival (%)	RTF (%)	No resistant per µg DNA	No transgenic plants	Stable RTF ($\cdot 10^{-5}$)
HP 23b	33	5.6	493	0	-
HP23 ΔSΔB	34	6.9	620	3	2.2
AB 1 sc	33	1.09	95	3	2.3
AB 1 lin	25	0.82	55	3	3.0
AB 1 + λ	26	0.60	153	3	2.9
AB 1 + SS	26	0.14	38	1	0.9
AB 2 sc	28	0.90	67	0	-
AB 2 lin	25	0.55	36	0	-
AB 2 + λ	24	0.38	91	0	-
AB 2 + SS	27	0.40	107	1	0.9

Yet, two different phenotypes were monitored in the initial plates during further selective growth, with two thirds of the resistant clones displaying reduced growth rate

typical of class II unstable clones, and one third displaying improved growth rate on selective medium, as monitored by the development of large and filamentous colonies (Figure 12b). Eighty-four fast growing clones were isolated and transferred to fresh selective plates. All of them displayed improved growth rate on PP NH_4 medium supplemented with G-418 25 or 50 µg/ml (Figure 12c). Rescue experiments were performed on several differentiated gametophores isolated from all these colonies, and complete resistant gametophores were recovered from 14 of these clones, corresponding to 17% of the tested clones (Figure 12d and Table 7).

Table 7. Summary of the data obtained in the phenotypical characterisation of resistant clones obtained following transformation with YAC vectors.

	Filament colonies	Frequency (%)	Number tested	Fast growing	RESCUE Dead	RESCUE Partial	RESCUE Complete
AB 1 sc	72	28	8	8	5	3	0
AB 1 lin	44	38	8	8	4	0	4
AB 1 λ	33	22	10	10	4	3	3
AB 1 SS	52	36	10	10	6	4	0
AB 2 sc	56	36	12	12	10	2	0
AB 2 lin	86	34	12	12	1	9	2
AB 2 λ	89	26	12	12	5	6	1
AB 2 SS	158	44	12	12	0	8	4

Improved growth rate on selective medium and occasional transmission of the resistant character to differentiated gametophores were established as phenotypical characteristics of class III unstable resistant clones. These data indicated RTF of class III resistant clones following transformation with YAC vectors were about 0.2 %, corresponding to a 100 fold increase as compared to non YAC plasmids, suggesting that YAC vectors were providing functional elements improving the mitotic stability of extrachromosomal arrays. It is proposed here that this functional element might be related to the SAR element associated with the ARS 1 element of *S. cerevisiae* present on YAC RC, since this element have been shown to specifically bind to tobacco nuclear scaffold (Hall, et al. 1991; Allen, et al. 1993). It could also be related to the CEN and TEL sequences of YAC vectors, since these elements also display SAR functions (Amati and Gasser 1988). Additionally, resistant clones carrying complete resistant gametophores were only recovered following transformation with linear YACs, suggesting that linear extrachromosomal molecules could be developmentaly more stable than circular ones, or that the CEN or the TEL sequences of the YAC vector provided additional functional elements necessary for stable maintenance of extrachromosomal elements in *P. patens* gametophores. To test these hypotheses, the different functional elements carried in YAC RC (ARS1, CEN4 and TEL), and non-functional derived sequences should be inserted in pHP 23b and pGL 2, and transformations should be performed with these modified constructs. The distribution of class I, II and III resistant clones recovered in these experiments would provide informations on the respective functions of these different elements in the

transformation of *P. patens*. Nevertheless, these preliminary data suggested that some elements carried by YAC vectors were providing functional elements improving mitotic stability of extrachromosomal arrays in *P. patens*.

figure 12 link

Figure 12. Transformation with supercoiled (left) and linear (right) YAC RC AB1. (a) Initial selection 3 weeks after transformation: one or two class I resistant clone can be identified following transformation with linear plasmid. (b) One and a half month after transformation, filamentous fast growing (class III) and globular slow growing (class II) resistant colonies can be seen on both plates. (c) Reduced growth rate of globular and fast growth rate of filamentous colonies plated on selective medium supplemented with 25 (left) or 50 (right) µg/ml G-418. (d) Rescue experiment showing basal regeneration of gametophores following transformation with supercoiled YAC and complete regeneration following transformation with linear YAC.

2.2.2.9. Transformation of P. patens with heterologous morphogenetic genes

The establishment of an efficient protocol of transformation in *P. patens* was an important step in the development of this organism as a model genetic system. However data presented so far did not provide evidence for functional comparability between mosses and higher plants in the study of plant development. The preliminary experiments described here were aimed to answer the following questions: (1) is it possible to use unstable transformants to investigate developmental processes, and (2) are heterologous morphogenetic genes biologically active in *P. patens* ? To answer these questions, transformations were performed with the 4 constructs listed in Table 8**.**

Table 8. List of plasmids carrying heterologous morphogenetic genes tested in P. patens

Plasmid name	Selectable cassette	Morphogenetic gene	References
pPCV002-rolA	NOS-npt II-pA Ocs	rol A	(Spena, et al. 1987)
pPCV002 CaMVBT	NOS-npt II-pA Ocs	35S-rolB	(Spena, et al. 1987)
pPCV002 CaMVC	NOS-npt II-pA Ocs	35S-rolC	(Spena, et al. 1987)
pPCV002 35S-ipt	NOS-npt II-pA Ocs	35S-ipt	Spena, unpubl.

The *ipt* gene of *Agrobacterium tumefaciens* T-DNA encodes isopentenyl transferase, an enzyme involved in the biosynthesis of the cytokinins zeatinriboside and zeatin. It was shown that transformation with this gene increased the cytokinin content in transgenic tobacco plants (Schmülling, et al. 1989). An exogenous supply of cytokinins in culture medium induces an over-production of gametophores in *P. patens* (Ashton, et al. 1979; Reski and Abel 1985). It was therefore predicted that biological activity of this gene in the moss should be associated with the generation of an OVE phenotype.

The *rolA*, *rolB* and *rolC* genes of *Agrobacterium rhizogenes* T-DNA were shown to induce pleiotropic morphological alterations in transgenic tobacco plants (Schmülling, et al. 1988), which were associated with alteration in the hormonal content and sensitivity of these plants (Schmülling, et al. 1993). The proteins encoded by the *rolB* and *rolC* genes were shown to hydrolyse indole and cytokinins glucoside conjugates respectively, suggesting that the biological activity of these genes might be related to an increase in the cellular concentration of free auxins or cytokinins (Estruch, et al. 1991a; Estruch, et al. 1991b). Since the normal development of *P. patens* is

mediated by a fine tuning of the auxin/cytokinin ratio, it was interesting to test the biological activity of these genes in transformed mosses.

Two independent transformations were performed with these constructs and plasmid pPCV002 was included in these experiments as a negative control. Relative transformation frequencies were around 10^{-3} for all constructs, which was in the range of RTF monitored previously with pPCV002 (data not shown). One stable clone was obtained with pPCV002 and one with the *rolB* construct. No phenotypical difference was observed between these two transgenic clones, indicating that the *rolB* gene had no direct morphogenetic effects on the development of *P. patens* (data not shown). Additional experiments should be performed to investigate the development of these plants in response to an exogenous supply of auxins and/or cytokinins to further investigate the biological activity of the *rolB* gene in *P. patens*. Class II unstable clones were recovered for all constructs. Resistant clones carrying the *rolB* or *rolC* construct were phenotypically similar to those carrying pPCV002, and unstable *rolC* transformants grown on selective medium supplemented with 6-benzylaminopurine 9-ß-D glucoside (a substrate for the *rolC* protein) did not display an OVE phenotype (data not shown). Yet, replicative transformants carrying the *rolA* or the 35S-*ipt* constructs were phenotypically different from those carrying pPCV002 (Figure 13). All resistant clones carrying the 35S-*ipt* gene displayed a clear OVE phenotype, associated with strong inhibition of protonematal development and massive overproduction of gametophores. The phenotype of resistant clones carrying the *rolA* construct was not so dramatic, but all of them showed increased production of secondary chloronemata as compared to those transformed with pPCV002. No direct explanation for this morphogenetic response can be proposed now, and additional experiments are clearly required to investigate the biological activity of the *rolA* gene in *P. patens*, including the generation of transgenic plants.

These data demonstrate that the biological activity of heterologous morphogenetic genes can be investigated in class II replicative transformants, and provide preliminary phenotypical evidence that the *ipt* gene from *A. tumefaciens* and the *rolA* gene from *A. rhizogenes* are biologically active in *P. patens*.

figure 13 link

Figure 13. Class II transformants obtained following transformation with pPCV002 (a), pPCV002-rolA (b), and pPCV-35S-ipt (c). Increased differentiation of secondary chloronemata can be seen in the resistant clone carrying the rolA gene, whereas the resistant clone carrying the ipt gene displays a clear OVE phenotype.

2.2.2.10. Transformation of another Bryophyte, Ceratodon purpureus

Finally, since the transformation of *P. patens* was shown to be essentially replicative, it was interesting to ask whether such transformation type was specific to *P. patens*, or if other Bryophytes would display a similar behaviour. To answer this question, transformation was performed on *Ceratodon purpureus*, a moss used to study phytochrome-mediated phototropic responses (Hartmann and Weber 1988; Hartmann and Weber 1990). However, this experiment should be considered as preliminary since protoplast isolation, transformation and regeneration were conducted under conditions similar to those for *P. patens* and, if protonema growth appeared normal, protoplast survival was very low and differentiated gametophores were not monitored on PP NH_4 medium. Genetic transformation was performed with plasmids pHP 23b and pABD 2. Protoplast survival ranged from 1.7 to 2.8 % and RTF ranged from 0.2 % to 6 % (data not shown), which matched well with the RTF monitored with *P. patens*. Loss of resistant clones during further selective growth was also monitored, and the growth rate of resistant colonies on selective medium was reduced as compared to non-selective medium. When these colonies were regenerated for three weeks on non-selective medium and then transferred back to selective medium, apparition of dead sectors was monitored in all tested colonies (Figure 14). These results indicated that the transforming sequences were mitotically unstable. However, both reduction in growth rate upon selective medium and loss of resistance in the absence of selection were reduced as compared to *P. patens* class II unstable clones, indicating that the mitotic stability of the transforming sequences was higher in *C. purpureus* protonema. These data provided preliminary evidence for extrachromosomal replicative transformation in *C. purpureus*, suggesting that this type of transformation could be a general feature of the transformation of Bryophytes.

Figure 14 link

Figure 14. Mitotic instability of kanamycin resistance associated with mosaicism in the protonema of the moss C. purpureus, following transformation with plasmid pABD2.

2.2.3. DISCUSSION

PEG-mediated transformation of P. patens protoplasts

The data presented in this chapter provide phenotypical, genetic and molecular evidence demonstrating both integrative and autonomous replicative transformation of the moss *P. patens* following PEG-mediated DNA transfer in protoplasts. Integrative transformants are recovered at frequencies in the range of 10^{-5}, and two main classes of probably replicative transformants have been identified according to the frequencies of transformation, the mitotic stability of the new character and its meiotic transmission. Phenotypical and genetic characteristics of these three classes of clones are summarized in Table 9.

Table 9. Main characteristics of the 3 classes of resistant clones recovered following PEG-mediated direct gene transfer into P. patens protoplasts.

Characteristics	Class I. Integrative transformants	Class II. Unstable replicative transformants	Class III. Fast growing replicative transformants
RTF	10^{-5}	10^{-1} to 10^{-3}	10^{-5}
Mitotic stability	100 %	< 5%	5 to 95 %
Growth rate upon selective medium	normal	reduced	± normal
Differentiation upon selective medium	normal	altered	± normal
Resistant gametophores	100 %	Not detected	10 %
Meiotic transmission	100 %	0 %	Occasional and non mendelian
Phenotypical changes	none	to I, rare	to I, frequent
Mosaic structures	none	always	frequent

The successful genetic transformation of *P. patens* offers now the possibility to use the molecular genetic approach to investigate plant cellular and developmental processes. Transformation of *P. patens* shares many similarities with transformation in the yeasts *S. cerevisiae* and *S. pombe*, in *X. laevis* and in *C. elegans*. Preliminary experiments performed with heterologous morphogenetic genes show that these genes are biologically active, indicating a level of functional comparability between mosses and higher plants. The potential applications of genetic transformation of *P. patens* will be discussed in the general discussion.

Integrative transformation in Physcomitrella patens

The data presented in this chapter brings additional information on integrative transformation as compared to our initial report (Schaefer, et al. 1991). RTF monitored

in these experiments are similar to the published frequencies despite the improvement of the uptake protocol, thus confirming that integrative transformation is rare in *P. patens*. Transgenic strains were recovered at similar frequencies with every plasmid tested, indicating that there are no special features associated with one or the other plasmid which would allow integrative transformation to occur. The data presented in the next chapter will demonstrate that these frequencies are improved by one order of magnitude with the presence in the genome of integrated sequences homologous to the transforming plasmids, suggesting that integration by homologous recombination occurs at higher rate than random integration in *P. patens*. Thus, the problem of low frequencies of integration events can be circumvented by the retransformation of transgenic strains with vectors carrying sequences homologous to the integrated plasmid, or by the insertion of genomic sequences in the transforming plasmid. Putative position effect, which is a classical feature of integrative transformation, was observed once in 18 transgenic strains analysed. In this case, it was associated with the inability of spores and protoplasts to germinate on selective medium. Insertional mutagenesis has not been monitored among transgenic clones isolated in these experiments, but this does not demonstrate that such event is not possible. The only evidence for insertional mutagenesis was observed during non-selective regeneration of the unstable class II clone SG2, which displayed two types of differentiated gametophores (data not shown). This putative mutant was not further characterised, since the transformation was performed in the presence of salmon sperm DNA as carrier, and it was not clear whether mutagenesis was induced by the integration of the transforming plasmid or of carrier DNA sequences. Finally, multiple integration events have not been monitored in the 9 independent transgenic strains which were successfully crossed with the self-sterile strain *nicB5ylo6*. However, these data are actually not surprising in view of the low fequencies of random integrative transformation events.

These data provided the first demonstration of successful integrative genetic transformation of the moss *P. patens* (Schaefer, et al. 1991).

Extrachromosomal replicative transformation in Physcomitrella patens

Characterisation of class II and III unstable resistant clones leads to the conclusion that these clones are extrachromosomal replicative transformants. This conclusion is sustained by the following arguments. (1) Transformation frequencies 10 000 fold higher than integrative transformation frequencies (class II). (2) Mitotic instability of the transforming sequences associated with reduced growth rate on selective medium and mosaicism of the transformants (class II and III). (3) High copy number per resistant cells and maintenance of the transforming sequences as high molecular concatenates (class II and III). (4) Loss of the resistant phenotype on non selective medium correlated with loss of the transforming sequences (class II and III). (5) Absence of hybrid fragments between the transforming plasmid and genomic sequences (class III). (6) Non-mendelian meiotic transmission of the unstable character (class III). (7) Occurrence of delayed integration events during both vegetative growth and germ line transmission of the resistance gene (class II and III). (8) Similarities of these features with the characteristics of extrachromosomal replicative transformants in *S. pombe* (Heyer, et al. 1986), *C. elegans* (Stinchcomb, et al. 1985; Mello, et al. 1991),

X. laevis (Etkin and Pearman 1987; Marini, et al. 1988), and Zebra fish (Stuart, et al. 1988).

However, one can consider that extrachromosomal replicative transformation has not been strictly demonstrated in these experiments, since such demonstration requires evidence for extrachromosomal maintenance of the transforming sequences, and evidence for the presence of an origin of DNA replication within these sequences. The identification of an extrachromosomal element can be achieved by in situ hybridization or Southern blot analysis of moss chromosomes separated by pulse-field gel electrophoresis (PFGE). Yet, the moss genome is composed of 26 chromosomes (Newton 1984), and it might therefore be difficult to demonstrate that the hybridization signal is independent of the 26 chromosomes. Alternatively, the recovery of ampicillin resistant colonies following transformation of *E. coli* with transformants uncut DNA provides evidence for the presence of extrachromosomal elements in this DNA. However, it has been shown that the transforming sequences are maintained as high molecular weight concatenates, and it is therefore predicted that these structures will not efficiently retransform *E. coli*. This assumption is sustained by the fact that the retransformation of *E. coli* has not been successfully achieved with uncut DNA isolated from replicative transformants of *S. pombe*, *X. laevis* and *C. elegans*.

The identification of an origin of DNA replication can be achieved by two dimensional gel electrophoresis or by the identification of the site of transition from continuous to discontinuous DNA synthesis (see (DePamphilis 1993) for references). However there are evidence that DNA replication of extrachromosomal arrays is initiated at one random site in each array, and not at one unique specific site in different arrays in *X. laevis* (see (DePamphilis 1993)). Therefore, the identification of a unique origin of DNA replication in extrachromosomal arrays might be difficult to achieve in this case.

It is therefore concluded that the phenotypical, genetic and molecular data presented in the results section, and the arguments presented above provide evidence strongly suggesting extrachromosomal replicative transformation in *P. patens*, and this would be the first time that such type of transformation is reported in plants. Bacterial plasmids are concatenated following their introduction in moss protoplasts to form high molecular extrachromosomal arrays which are most probably replicated in moss cells. The origin of DNA replication in the extrachromosomal arrays is either random, or provided by the Pvu II - Eco R1 2.3 kb fragment of pBR 322, since this is the only common fragment among all tested plasmids. The availability of such transformation provides the initial conditions to perform experiments designed to isolate and characterise moss genomic sequences involved in DNA replication, and these experiments may allow the development of a moss artificial chromosome (further discussed in the general discussion).

Generation of class III replicative transformants

Low frequency occurrence of class III clones suggests that the transforming sequences have to undergo an additional modification beside concatenation to generate this class of transformants. The improved mitotic stability and the clear cut between

class II and class III phenotypical characteristics suggests that the transforming sequences have acquired a functional element involved in DNA replication and/or mitotic segregation and/or nuclear retention of the transforming sequences. It is therefore postulated that the extrachromosomal sequences have recombined upon DNA uptake with functional moss genomic DNA fragments involved in these processes. Direct evidence for the recombination of the extrachromosomal arrays of class III with an as yet non-identified moss sequence have been provided by the Southern blot analysis of DNA from ABD2 L1 and ABD 2 L1 Resc. Indirect evidence for the recombination of transforming sequences with moss genomic DNA are also provided by the high frequency of delayed integration events monitored during vegetative propagation and meiotic transmission of the resistant genes in this class of transformants. It is proposed that these sequences could be functionally related to nuclear scaffold attachment region (SAR), and the following arguments sustain this hypothesis. During transformation, 70% of protoplasts died, which corresponds to ca. 280 ng of genomic DNA which could be simultaneously precipitated with plasmid DNA upon PEG addition. The actual amount of precipitated genomic DNA is probably smaller since not all dead protoplasts lyse during transformation. Nevertheless, this is a significant amount of moss DNA to provide substrate for the extensive extrachromosomal recombination and ligation events occurring during formation of the high molecular weight arrays. Therefore, the possibility for plasmid sequences to recombine with moss genomic DNA is quite high. However depending on the genomic DNA sequences involved, the consequences of such recombinations are different and the resulting phenotype can be predicted by analogy with transformation in *S. cerevisiae* (Struhl 1983).

(1) Recombination occurs with moss DNA sequences not involved in DNA replication. The transformant will share characteristics of class II transformants, and these sequences would only increase the probability of delayed integration by homologous recombination with their genomic homolog.

(2) Recombination occurs with moss DNA sequences involved in DNA replication. In this case, three different situations can be described.

(2 a) The recombined moss sequences carry centromere or telomere sequences. The transformed clone should display fast growth on selective medium associated with a high mitotic stability and low copy number of the transforming sequences. Yet the probability to recombine with such sequences is low, since they are not abundant in the genome, and it is considered that such event would not be detected in these analyses in view of the low frequency of occurrence of class III transformants.

(2 b) The recombined sequences carry one or several functional elements associated with moss chromosomal origins of DNA replication, such as an origin of bidirectional replication, a DNA unwinding element or transcription factor binding sites (for a description of eukaryotic origins of DNA replication, see (DePamphilis 1993)). The probability to recombine with these sequences is quite high, since they are abundant in the genome, but these functional elements are probably not required in this system, since these functions are provided by the transforming plasmid. Actually, one can even consider that a DNA unwinding element could be provided by the polyA sequences of the expression cassette, transcription factors binding sites by the promoter

of the expression cassette and an origin of DNA replication by the ColE1 oriC site, if such an element is required here. Moreover such transformants will probably display characteristics similar to class II resistant clones, preventing these clones to be phenotypically identified.

(2 c) The moss recombined sequences carry nuclear scaffold attachment sequences or nuclear retention elements. This assumption is considered as the most likely hypothesis to account for the generation of class III resistant clones. These sequences are abundant in the genome and the probability to recombine with those is quite high. These elements are evolutionary conserved between unrelated species including higher plants, as illustrated by the ability of the SAR associated with the yeast ARS 1 element to specifically bind tobacco nuclear scaffold (Hall, et al. 1991). Therefore, it appears that these elements are able to retain DNA molecules in the nucleus of different species during mitosis, a property which could be associated with improved mitotic stability of the transforming sequences. Indirect evidence is provided by the increased frequency of occurrence of class III resistant clones in transformations performed with YAC vectors. Another argument sustaining this hypothesis is the report that such elements are required on plasmids designed for the identification of mammalian origins of DNA replication in an extrachromosomal replication assay (Krysan, et al. 1989). However, demonstration of such a hypothesis clearly requires additional experiments. These experiments should include isolation and characterisation of the sequences which have recombined with extrachromosomal arrays in class III resistant clones, and transformation with bacterial plasmid carrying these sequences, transformation with bacterial plasmids carrying separate functional elements of YAC vectors, and isolation of nuclear scaffold-bound moss genomic DNA fragments and transformation with plasmids carrying these sequences.

It is therefore concluded that the generation of class III resistant clones is mediated by the recombination of the transforming sequences with moss genomic elements, and that these elements could be related to SAR elements. The improved mitotic segregation monitored in class III clones would consequently results from efficient retention of the extrachromosomal arrays in the nucleus.

Efficient replication of bacterial sequences in eukaryotic cells

Sequence independent replicative transformation reports demonstrate that, in some organisms, bacterial plasmids can be efficiently replicated in eukaryotic cells, and these reports raised interesting questions on the requirement for specific eukaryotic origins of replication sequences to replicate DNA. These results actually confirm the idea that some cells can initiate DNA replication at many DNA sites (DePamphilis 1993), but the reasons for such a behaviour is currently unknown. It has been postulated that this released specificity could be restricted to embryonic cells undergoing rapid cleavage events or not yet completely differentiated (DePamphilis 1993). Yet, it is relevant to notice that this behaviour might also be correlated with the ploidy state of the organism, and/or with its degree of evolution, since organisms displaying such behaviour are often haploid or haplodiplobiontic (filamentous fungi, *S. pombe*, *P. patens*, *Dictyostelium*, *Paramecium*) and/or relatively archaic (*X. laevis*, sea urchin, *P. patens*, *C. elegans*). An interesting finding in this report is the fact that autonomous

replication of foreign sequences appears to be restricted to the protonema. This observation may indicate that the choice for an origin of DNA replication is developmentally regulated, or that an increase in structural complexity could be associated with an increased specificity for the recognition of functional origins of DNA replication. In the next chapter, an argument will be presented to account for the high ratio of targeted to random integration events monitored in haploid organisms. In this view, it is interesting to notice that a relaxed specificity for the recognition of functional origins of DNA replication in haploid and archaic organisms could allow these species to maintain as extrachromosomal elements foreign DNA sequences conferring evolutionary advantages.

2.2.4. MATERIAL AND METHODS

Plant material

The techniques for tissue propagation, protoplast isolation and regeneration are described in Annex A. All transformations were performed with the wild type strain of *P. patens*, except experiment 2 which was performed with the auxotroph pabA3 (Ashton and Cove 1977). No differences were observed between these strains.

Plasmids

The different constructs used in these experiments are described in the result section (Table 1 and 8), and restriction maps of pHP 23b, pGL 2 and pABD 2 are presented in Annex A. Plasmids pLGVneo 2103, pABD 1, pABD 2, pHP 23b and pGL 2 were kindly provided by Dr. B. Hohn and Dr. J. Paszkowski (FMI Basel). Plasmids YAC RC AB1 and AB2 were a gift from G. Bonnema and N. Grimsley (INRA-CNRS Castanet-Tolosan, unpublished data). These vectors were constructed as follows: the Bam H1 and Sal 1 sites of pHP 23b were removed from the original vector, giving rise to plasmid pHP 23b ΔSΔB. An Eco R1 site was introduced in one of the Xho 1 site of YAC RC (Marchuk and Collins 1988), and the Eco R1 fragment of pHP 23b ΔSΔB, containing the complete plant expression cassette was inserted in this site. YAC RC AB 1 carry the plant expression cassette with the 35S promoter next to the telomere sequences and YAC RC AB 2 with the polyadenylation sequences next to the telomere. Plasmids pPCV002, pPCV002-rolA, pPCV002-CaMVBT, pPVC002-CaMVC and pPCV002-35S-ipt were a gift of Dr. A Spena (MPI Köln). All constructs were amplified and plasmid DNA was extracted and PEG purified according to standard techniques (Sambrook, et al. 1989).

Transformation procedure

In experiments 1 and 2, transformation and protoplast regeneration were performed according to the procedure described in (Schaefer, et al. 1991). All other transformations were performed with the optimum protocol established in the development of the transient gene expression assay. Except in experiment 3, protoplast regeneration was performed as follows: at the end of the transformation, samples were diluted stepwise with 8 ml protoplast liquid medium, transferred to a 5 cm Petri dish and left overnight in darkness. The next day, cells were sedimented by low speed centrifugation (600 g for 5 min) and half of the supernatant was replaced with protoplast liquid medium supplemented with 1.3% (w/v) agar. Four plates, containing protoplast solid culture medium and overlaid with an 8 cm cellophane disc, were then inoculated with 2 ml of protoplast suspension and regeneration was achieved in light. In

experiment 3, protoplasts were regenerated for 8 days in liquid medium to facilitate the experimental design, and then embedded in a top layer as described above.

Selection conditions, non-selective to selective growth test and rescue experiments

In experiment 3, selection was initiated by the addition of the appropriate amount of antibiotic to the liquid cultures. In all other experiments, selection was initiated 6 days after transformation by transferring the top layer to 9 cm Petri dish containing PP NH_4 solid medium supplemented with 50 µg/ml G-418 or 25 µg/ml Hygromycin B, corresponding to ca. 10 times LD_{50}. The cultures were further transferred to fresh selective plates every two weeks to maintain constant selective pressure. Initial RTF were monitored 10 days after the initiation of selection and additional counts were made during the next month. The non-selective to selective growth test was performed as follows: resistant colonies were fragmented for 30 seconds with a Polytron (position 4), and 2 ml of the suspension were inoculated and grown for ten days on both selective and non-selective PP NH_4 solid medium. At this stage each culture was transferred to selective medium and grown for an additional fortnight. Mitotic instability of the resistance was monitored by the apparition of dead sectors in the culture initially grown on non-selective medium. Rescue experiments were performed as follows: differentiated gametophores were plated on selective and non selective PP NH_4 solid medium and grown for 2 weeks. In some experiments, differentiated gametophores were cut in 1 to 2 mm segments and plated on selective medium with respect to their initial position.

Somatic fusion and genetic analysis

PEG-mediated somatic fusion of protoplasts was performed essentially according to (Grimsley, et al. 1977a), with the following modifications: protoplasts from a Kan^R and a Hg^R transgenic strains were resuspended at a concentration of $1 \cdot 10^6$ / ml in mannitol 0.48 M and mixed in a 1 to 1 ratio. 200 µl of the mixture was laid on the bottom of an empty Petri dish and left for 5 minutes to sediment the cells. 200 µl of PEG solution (PEG 1550, 60% w/v; $CaCl_2$, 125 mM; MES, 5 mM, pH 6.4) was then slowly added to the cells and the mixture was left for 20 minutes at room temperature. Samples were first progressively diluted with a washing solution (mannitol, 0.16 M; $CaCl_2$, 125 mM; MES, 5 mM, pH 6.4, 3 x 200 µl during the next 30 min.), then with 8 x 1 ml of protoplast liquid culture medium and the dishes were left overnight in darkness. The next day, cells were sedimented by low speed centrifugation, embedded in a top layer and regenerated under conditions similar to protoplasts after transformation. Selection for double resistant colonies was initiated 6 days after somatic fusion by transferring the top layers on PP NH_4 solid medium supplemented with 50 µg/ml G-418 and 25 µg/ml hygromycin B. Double resistant colonies were scored 2 weeks after the initiation of selection, and wild-type like diploid colonies were further regenerated and selfed. Mature sporophytes were isolated individually and

segregation analysis of the resistance genes was performed on spores isolated from single sporophyte.

Transgenic strains were selfed on both selective and non-selective medium, or crossed with the self-sterile strain *nicB5ylo6* according to the protocol described in chap 2.3. Segregation analysis was performed on spores isolated from single sporophytes.

Unstable transformants were selfed on selective and non-selective media and single sporophytes were analysed when present. Yet, to monitor putative low frequency meiotic transmission of the character, spores were also collected after crushing mature sporophytes with forceps and washing the culture with sterile water. Low frequency meiotic transmission of the character in class III clones was monitored this way.

Plant DNA analysis

Genomic DNA was isolated from fresh material with the CTAB procedure developed by Rogers and Bendich (Rogers and Bendich 1988). The yield of this procedure was rather low (ca. 1-3 µg DNA/g fresh weight), but this DNA was reproducibly digestible with restriction endonucleases. DNA was digested with the appropriate restriction endonuclease, fragments were separated by electrophoresis in a 0.7% (w/v) agarose gel, denatured and transferred by capillarity to nitrocellulose membrane (Schleicher and Schuel, BA 85) according to standard procedure (Sambrook, et al. 1989). Probes were isolated and purified from the transforming plasmid by conventional techniques (Sambrook, et al. 1989) and radio-labelled with ^{32}P-dNTP by the random hexamer priming method (Feinberg and Vogelstein 1983). Specific activities of the probes ranged from 10^8 to 10^9 cpm / µg DNA. High stringency hybridizations were conducted according to standard techniques and the final wash of the membranes was at 68^{o}C in 0.1 x SSC (Sambrook, et al. 1989).

2.3. GENETIC EVIDENCE FOR HIGH FREQUENCY GENE TARGETING IN *PHYSCOMITRELLA PATENS*.

Abstract.
Integrative transformation following PEG-mediated direct gene transfer to protoplasts occurs at a mean relative transformation frequency of 10-5 in the moss Physcomitrella patens. The plasmids pHP 23b and pGL 2 are identical except for the coding sequence and share a stretch of homologous sequence which extends over 3.3 kb. These plasmids were used to investigate the influence of homologous sequences on the rate of stable transformation and to test the possibility to perform gene targeting in this organism. Two experiments were performed in which seven independent transgenic strains, carrying multiple copies of either pHP 23b or pGL 2 integrated in single independent loci, were retransformed with the other plasmid and selected for double resistant transgenic plants. A 10 fold increase in mean stable relative transformation frequency was monitored in transgenic strains as compared to the wild type, with a maximum RTF of 10^{-3}. Segregation analysis was conducted by crossing 7 independent double resistant clones, issued from 6 independent initial transgenic strains, with the sensitive self sterile strain nicB5ylo6. The kanamycin and the hygromycin resistance markers were shown to be linked (< 0.5 cMorgan) in 6 of these clones whereas independent segregation was monitored in the seventh. These data provide strong genetic evidences that gene targeting occurs at higher frequency than random integration in the moss Physcomitrella patens. The ratio of targeted to random integration has been estimated around 10 to 1. Such ratio is several orders of magnitude higher than previous reports of gene targeting in plants, and open the way to the development of efficient reverse genetics in this model organism.

Abbreviations. ES cells: mouse embryonic stem cells, PEG: polyethylene glycol, RTF: relative transformation frequency, npt II: neomycin phosphotransferase, aph IV: hygromycin phosphotransferase, wt: wild-type, Hg: hygromycin B, G-418: geneticin, PCR: polymerase chain reaction.

2.3.1. INTRODUCTION

In most eukaryotes, the integration of foreign DNA sequences in the genome occurs at more or less random locations by illegitimate recombination. However, the presence of genomic sequences on the introduced plasmid can facilitate DNA integration at its own chromosomal locus by homologous recombination, a process known as gene targeting. Initially monitored in *Saccharomyces cerevisiae* (Hinnen, et al. 1978), this event is a prerequisite to the application of one of the most powerful tools of modern molecular genetics, namely reverse genetics. In classical genetics, a population of individuals is randomly mutagenised, and individual mutants are then phenotypically and genetically characterised with the hope that the mutated gene could be identified. In reverse genetics, a gene, even of unknown function, can be specifically mutagenised *in vitro* and reintroduced to its own chromosomal location in order to study its function (reviewed in (Struhl 1983; Capecchi 1989b)). This approach permits determined modification of any cloned gene and replacement of the wild-type sequence

with the mutated one with almost 100% efficiency since the classical "position effect" problems encountered following random integration are avoided.

However the successful application of reverse genetics is dependent on the ratio of homologous to illegitimate recombination events during integrative transformation, and this ratio is extremely variable among different eukaryotes. Integration of foreign DNA sequences occurs almost exclusively by homologous recombination in *Saccharomyces cerevisiae* (reviewed in (Rothstein 1991)), *Trypanosoma brucei* (ten Asbroek, et al. 1990; Eid and Sollner-Webb 1991), *Leishmania major* (Cruz and Beverley 1990) and chicken B cell lines (Buerstedde and Takeda 1991). In *Schizosaccharomyces pombe* (Grimm and Kohli 1988), *Aspergillus nidulans* (Miller, et al. 1985), *Neurospora crassa* (Asch and Kinsey 1990) and *Dictyostelium discoideum* (De Lozanne and Spudich 1987), integration by homologous recombination is usually more frequent than random integration, though illegitimate recombination events can predominate for certain loci. In most other eukaryotes tested, the ratio of homologous to random integration falls to very low levels, ranging from 10^{-2} to 10^{-5} in mammalian cells (Bollag, et al. 1989) and from 10^{-4} to 10^{-5} in plant protoplasts (reviewed in (Lichtenstein and Barrena 1993)).

Therefore, if the use of reverse genetics is now established as routine in yeast, and accounts for an important part of its success as an experimental model system for the last 15 years, it is still in its infancy in animals and plants, since the gene-targeted mutant has to be identified among the totality of transgenics generated by illegitimate recombination (methods and strategies are discussed in (Bollag, et al. 1989; Offringa 1992; Lichtenstein and Barrena 1993)). In this respect, major progress has been realised in animal systems with the use of mouse embryonic stem cells (ES cells) which can be pre-screened in culture for gene targeting prior to reintroduction in mouse embryo (reviewed in (Capecchi 1989a; Joyner 1991)). Recent studies with these cells have shown that the frequency of gene targeting is influenced by parameters such as length of homology (from 2 to 14 kb) (Deng and Capecchi 1992), plasmid form (linear rather than supercoiled) and type (insertion versus replacement vectors) (Deng and Capecchi 1992; Thomas, et al. 1992), origin of homologous sequence (isogenic) (Deng and Capecchi 1992) and the characteristics of the targeted locus itself. These parameters were similar to the factors modulating efficient integrative transformation in *S. cerevisiae* (Rothstein 1991). However, optimization of gene targeting conditions did not significantly improve homologous recombination frequencies in ES cells, and the reasons for the low ratio of homologous to random integration observed in most higher eukaryotic cells is currently not known. An argument has been presented by Capecchi which postulates that this lower ratio could actually reflect an increase in the efficiency of non-homologous recombination in these cells, which would have been selected during evolution to increase DNA repair efficiency (Capecchi 1990). An alternative hypothesis, based on the different possible consequences of integrative transformation in haploid and diploid cells will be presented in the discussion.

In plants, gene targeting has been successfully achieved in tobacco following PEG-mediated direct gene transfer (Paszkowski, et al. 1988) or Agrobacterium-mediated transformation (Lee, et al. 1990; Offringa, et al. 1990), and in *Arabidopsis*

thaliana following PEG-mediated direct gene transfer (Halfter, et al. 1992). In three of these studies (Paszkowski, et al. 1988; Offringa, et al. 1990; Halfter, et al. 1992) the target sequence was a single artificial locus carrying multiple copies (2 - 10) of a defective antibiotic resistance marker, which was restored following the introduction of homologous sequences complementing the deficient marker. The extent of homology between the target sequence and the incoming DNA was rather short, ranging from 0.4 to 1 kb and the ratio of homologous to random integration ranged from 10^{-4} to 10^{-5}. On the other hand, Lee and coworkers have successfully targeted a natural locus in the tobacco genome, the acetolactate synthase gene (ALS), and modified the wild-type sequences with a deleted, non-functional fragment from the ALS gene carrying a single amino-acid mutation conferring resistance to chlorsulfuron (Lee, et al. 1990). In these experiments, the extent of homology between the target sequence and the incoming DNA was about 2 kb and the ratio of homologous to random integration was also around 10^{-5}. These data clearly demonstrate the feasibility of gene targeting in plants following both *Agrobacterium* and PEG-mediated transformation, but the ratio of homologous to illegitimate recombination are too low to routinely perform reverse genetics experiments in plants. Prospects and possible improvements in this field have been recently discussed (Lichtenstein and Barrena 1993) and will not be further addressed here.

The low frequencies of illegitimate integration events, the high frequencies of replicative transformation and similarities with transformation in yeasts led us to investigate the effects of the presence of homologous sequences between the genome and the introduced DNA on integrative transformation frequencies, in order to test the possibility to perform gene targeting experiments in *P. patens*. Integrative transformation of the wild-type strain of *P. patens* occurs at a mean relative transformation frequency (RTF) of 10^{-5}. To test the influence of homologous sequences on transformation rates, seven independent transgenic stains, carrying multiple copies of either pHP 23b or pGL 2 integrated in their genome, were retransformed with the other plasmid in 2 independent experiments. Since plasmids pHP 23b (35S-*npt II*) and pGL 2 (35S-*aph*) are identical except for the coding sequence (both are pDH 51 derivatives (Pietrzak, et al. 1986)), they share a stretch of homology which extends over 3.3 kb. On the other hand, genetic analysis of transgenic mosses have shown that these plants carry multiple copies of the plasmid integrated at independent single genomic loci. Such artificial loci are thus similar to the plant loci targeted in the experiments mentioned above and were used as targets in the gene targeting experiments described here. The data will show that stable relative transformation frequencies (sRTF) were increased by at least one order of magnitude in transgenic strains as compared to the wild type and that the kanamycin and hygromycin markers used in these experiments were genetically linked in the offspring of 6 out of 7 double-resistant transgenic plants, isolated from 6 independent initial transgenic strains, as demonstrated by segregation analysis. These data provide strong genetic evidence that gene targeting occurs at higher frequencies than random integration in the moss *P. patens* and open the way to the development of efficient reverse genetics in this model organism.

2.3.2. RESULTS

2.3.2.1. Sequence homology between an introduced plasmid and an artificial genomic locus increases stable transformation frequencies.

P. patens wt and seven independent transgenic stains were transformed with supercoiled plasmid pHP 23b or pGL 2 in two independent experiments. A reproducible increase of stable RTF was monitored in all transgenic strains, with sRTF ranging from $2.7 \cdot 10^{-5}$ to $1 \cdot 10^{-3}$, as compared to the 10^{-5} monitored in wt (table 1). Increase in sRTF was apparently not associated with a higher survival of the protoplasts, nor with a higher initial RTF (compare HP23 L2 in exp.1 and 15.01 in exp. 2), and was readily observed 2 or 3 weeks after the initiation of selection (figure 1), indicating that integration most probably occured upon transformation and not later, by delayed integration during protoplast regeneration. Stable integration in these clones was confirmed by the following data: (1) 39 out of 39 tested clones displayed unrestricted growth on both G-418 and Hygromycin B supplemented media, whereas the 6 stable clones obtained in wild-type were either hygromycin or kanamycin resistant (figure 2), (2) 23 out of 23 tested clones maintained their resistance following a 2 week-growth period on non-selective medium and did not display any signs of mitotic instability when transferred back to selective medium (data not shown), and (3) 11 out of 11 tested clones gave rise to 100% double-resistant offspring following self-fertilisation (table 2, figure 3).

A total of 151 double-resistant transgenic clones were isolated in these 2 experiments, which is 5 times more than the total number of transgenic plants regenerated in all other transformation experiments performed so far. The mean sRTF obtained in transgenic strains was $2 \cdot 10^{-4}$ which corresponds to a 20-fold increase as compared to the mean sRTF of 10^{-5} monitored in previous experiments with wild-type, or to a-10 fold increase as compared to the internal wild-type controls of these experiments. This reproducible increase was considered to be due to the presence in the genome of the recipient strains of sequences homologous to the transforming plasmids and it was postulated that these higher transformation rates were reflecting efficient targeted integration through homologous recombination. Since (1) plasmids pHP 23b and pGL 2 were not suitable for restriction and/or PCR analysis of homologous recombination events, (2) supercoiled plasmids, as used in these experiments, are usually considered as insertion vectors leaving the target sequence unchanged, thereby diminishing the possibility of detecting rearrangements in the sequences, and (3) no selectable markers were associated with the region of homology, thus preventing the possibility of monitoring a gene disruption event at the phenotypical or molecular level, a segregation analysis of these double resistant transgenic plants was performed to test the gene targeting hypothesis.

Table 1. Initial and stable RTF in wt and transgenic strains as monitored in 2 independent gene targeting experiments. The initial number of protoplasts was 350 000 per sample in exp.1 and 300 000 in exp.2. The increase factor (a) was calculated on a mean stable RTF in wt of 10^{-5}.

Table1 link

figure 1 & 2 link

Figure 1. Initial selection of wt (left) and HP23 L2 (right) transformed with pGL 2. Pictures were taken 20 days after the initiation of selection. More than 10 stable clones are clearly visible on the HP23 L2 but no stable clone are seen on the wt plate.

Figure 2. Unrestricted growth of transgenic strains form exp.1 growing on non-selective (top), G-418 (bottom left) or Hygromycin B (bottom right) supplemented media. Top row: HP23 b in wt (Kan^R Hg^S). Second row: GL 2 in wt (Kan^S Hg^R). Third and fourth rows: HP 23b in 15.01 (Kan^R Hg^R). Bottom row: GL 2 in HP23 L2 (Kan^R Hg^R).

2.3.2.2. Segregation analysis of double-resistant transgenic plants

Seven independent double-resistant transgenic plants, isolated from 6 different initial transgenic strains, were successfully crossed with the self-sterile strain *nicB5ylo6*. Two sporophytes collected on the *ylo* colony (crossed) and one sporophyte collected on the transgenic plant (selfed) were analysed by testing over 100 spores per sporophyte. Data from this segregation analysis are presented in table 2 and figure 3 illustrates the segregation of clone GH 1 and GH 3/2 following cross- and self-fertilisation. In six of these clones, half of the offspring were sensitive and the other half resistant to both G-418 and Hygromycin B, whereas the offspring of clone GH 3/2 was 21.4 % sensitive, 22.8 % Kan^R, 27.7 % Hg^R and 28.2 % double resistant (table 2). Such segregation demonstrated that the kanamycin and the hygromycin resistance markers were tightly linked in 6 clones (genetic distance < 0.5 centiMorgan), whereas the distribution observed in clone GH 3/2 corresponds to the 1;1;1;1 segregation ratio expected in the case of a second independent integration event. Segregation of the *ylo* marker was also scored. In all the strains, half of the spores regenerated yellow colonies and the other half generated wild-type colonies independently from the antibiotic resistance markers (table 2). Such distribution corresponded to the expected segregation of an unlinked additional marker. A statistical analysis was performed and CHI square values were calculated for the segregation of the resistance markers (table 2). CHI square values determined for 5 out of 6 cosegregating strains allowed the hypothesis of linkage to be accepted at the 85% confidence level. The absence of any single-resistant spores in the offspring of targeted clones indicated that processes such as trans-inactivation (Matzke and Matzke 1993b), or intrachromosomal recombination between closely linked repetitive sequences (Peterhans, et al. 1990), which could induce the loss of one of the marker, were not detectable in the 1200 siblings analysed here. Finally, a preliminary ratio of homologous to random integration could be calculated form these data, 6 out of 7 corresponding to 86 %. Such high ratio would be in the same range as the ratio monitored in the transformation of yeasts and Trypanosomatideae, and has never been reported for any plant so far.

Table 2. Segregation analysis of double resistant transgenic clones crossed with the strain nicB5ylo6. Chi square values were determined on the total number of tested spores for each strain. Clone GH 5 has not been successfully selfed, probably because it is a pab A3 derivative and only one sporophyte was recovered on the ylo colony crossed with GH 7.

Table 2 link

figure 3 link

Figure 3. Single spore derived replicates were plated on G-418 (left) or Hygromycin B (right) supplemented media and grown for 15 days. From top to bottom: GH 1 selfed, GH 1 cross nicB5ylo6, and GH 3/2 cross nicB5ylo6. The kanamycin and hygromycin markers are linked in GH 1 and segregate independently in GH 3/2.

2.3.3. DISCUSSION

The data presented here clearly demonstrate that integrative transformation frequencies of the moss *P. patens* were increased by the presence in the genome of sequences homologous to the transforming plasmid, and that this increase was correlated with genetic linkage of the resistance markers used in these experiments. These data provide strong genetic evidence that the second plasmid was more frequently integrated at the same locus as the first one than anywhere else in the genome, and that targeted integration by homologous recombination occured at a higher rate than random integration in the moss *P. patens*. However, the absence of molecular analysis does not allow an unambiguous conclusion, and there are two other possibilities of interpreting these data which will be discussed here.

(1) It is possible to imagine that the stock of each plasmid was contaminated by the other one and that the genetic linkage of the markers monitored in the segregation analysis was due to extrachromosomal recombination between the plasmids prior to genomic integration. This possibility is dismissed by the following arguments: (a) wild-type transgenic strains were resistant to only one antibiotic, (b) such a mechanism could not account for the increase in transformation frequencies, since large plasmids were found to transform *P. patens* at similar frequencies than small ones, and (c) the segregation of such event should be 50% double resistant, 25% single resistant (recipient strain phenotype) and 25% sensitive, which did not correspond to the observed segregation.

(2) An alternative explanation would be that artificial genomic loci, such as the ones targeted in the recipient transgenic strains, displayed some as yet unidentified determinant (such as an altered chromatine structure for example) which increased integration rates of foreign DNA at this locus upon subsequent transformation. Such a mechanism could not be ruled out by these data, but the following arguments made this hypothesis rather unlikely: (a) such a mechanism has never been reported so far for plant transformation, (b) similar frequencies of homologous recombination have been monitored in an artificial and a natural locus in tobacco (Lee, et al. 1990; Offringa, et al. 1990), and (c) such putative determinant would probably be associated with mitotic and meiotic instability of the locus, which did not fit with the strong mitotic and meiotic stability observed in all transgenic strains of *P. patens* analysed so far, nor with the faithful meiotic transmission of the double-resistant markers monitored in this segregation analysis. However, such a possibility can not be completely discarded without a molecular analysis.

Efficient gene targeting by homologous recombination is the most likely mechanism to account for the increase in stable transformation frequencies and for the genetic linkage of the resistance markers monitored in these experiments, and the next part of the discussion will be presented in regard to this mechanism.

Maximum stable RTF achieved in both experiments were in the range of 10^{-3}, or about 3 clones per µg DNA. Such frequencies correspond to the rate of integrative transformation by homologous recombination in *S. cerevisiae* (Struhl 1983) and to

fairly good frequencies of random integration following PEG-mediated DNA uptake in plant protoplasts (see (Negrutiu, et al. 1990)). However it is difficult to discuss these sRTF since supercoiled plasmids were used in these experiments, and it is known that linear templates are better substrates for gene targeting events. It is therefore believed that the sRTF monitored here were possibly underestimated, and that additional experiments, performed with linear DNA molecules are required to assess this parameter. For the same reason, it is too early to discuss the influence of the targeted locus on transformation frequency, though significant differences were monitored between different strains. It is more relevant to note that cosegregation was monitored in each recipient strain tested, thereby demonstrating that targeting was efficiently achieved in every tested locus, independently of the transformation frequency. Such efficiency could be associated with the fact that these targeted artificial loci were carrying multiple repeats of an actively expressed plasmid, and that homologous recombination might be favoured in such structures, since mitotic intrachromosomal homologous recombination occurs at high rates in such genomic structure in *S. cerevisiae* (Petes and Hill 1988) and in tobacco (Peterhans, et al. 1990). The length of homologous sequences has also been shown to be an important factor affecting gene targeting frequencies. In the experiments described here, homology between the introduced plasmid and the target sequence was 3.3 kb, and the data indicated that such a length was sufficient to promote efficient targeting to an artificial locus. However, such observations might not be true for a natural locus and additional experiments are clearly required to assess this parameter.

To answer these questions, the following experiment is proposed: a series of plasmids containing a functional selectable marker (gene 1), a deleted second selectable marker (gene 2) and fragments of increasing size of different moss genomic loci (isolated from different lambda genomic clones) should be constructed. Control plasmid should be designed the same way, but with genomic DNA from another plant. Wild-type *P. patens* should be transformed with these plasmids linearized and selected for gene 1 resistance. It is predicted that plasmids with moss genomic sequences should transform wild-type with a higher efficiency than controls, thereby confirming that the increase in sRTF monitored here was not due to the presence of an artificial locus in the genome. Simultaneously, the influence of the targeted locus and of the length of homologous sequences on gene targeting efficiency could be addressed. These transgenic clones should then be regenerated and retransformed with DNA sequences homologous to and complementing the second deleted selectable marker and selection should be done for gene 2 resistance. Molecular analysis of the reconstruction of the deleted marker could then be performed in these second transformants. Such an experiment should allow a rapid definition of optimum conditions to perform efficient gene targeting in the genome of *P. patens*.

An estimation of the ratio of targeted to random integration can be made from the data obtained in these experiments. Mean sRTF monitored in transgenic strains were 10 fold higher than sRTF in wt strain, and this increase most probably reflected efficient integration by homologous recombination. This indicates that integration by homologous recombination occured an average of 10 times more often than random

integration in the genome of *P. patens*. Segregation analysis has shown that only one out of the seven tested double-resistant plants displayed the 1;1;1;1 segregation ratio expected from an independent integration event. These genetic data indicated that gene targeting was achieved in 86 % of the cases. An independent estimation could be done on strain Hg 27DI, where both independent and cosegregation were monitored. This ratio would be 50 % of the tested clones (1 in 2), or 66.6 % of the total number of double resistant clones recovered in this strain (2 in 3, assuming that the stable clone which was not crossed was actually targeted). These independent estimations are fairly convergent, and one can reliably conclude that integration by homologous recombination occurs about 10 times more often than random integration in the genome of the moss *P. patens*. Such a high ratio of homologous to random integration is about 10 000 times higher than previous reports of gene targeting in plants, and provides the essential requirement to develop efficient reverse genetics in *P. patens*. When one realizes the importance of the reverse genetic approach 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 give to the moss *P. patens* a valuable advantage as model system for the genetic dissection of plant cellular and developmental processes.

A high ratio of homologous to random integration in the moss *P. patens* was a rather unexpected result. The question of why do some eukaryotic cells achieve integrative transformation by homologous recombination when most others do not led to the formulation of the following hypothesis. This hypothesis is based on the following observation: a ratio of homologous to random integration above 20 % has only been monitored so far in haploid cells (*S. cerevisiae*, *S. pombe*, *A. nidulans*, *N. crassa*, *D. discoideum* and *P. patens*), and in some diploid cells which in parallel use gene conversion to perform their own cellular functions (*Trypanosoma* and *Leishmania* for the production of variant surface glycoproteins, reviewed in (Pays and Steinert 1988) and chicken B cell lines for the production of IgG, reviewed in (Thompson 1992)). Such observation suggests that efficient gene targeting is associated either with haploidy, or with the presence of an efficient cellular gene conversion system. On the other hand, the following assumptions were made on the different putative consequences of integrative transformation on the integrity of haploid and diploid genomes.

1. "Organisms with a dominant haplophase do not accept to integrate foreign DNA in their genome".

The integration of foreign DNA sequences in a haploid genome is potentially immediately mutagenic, and organisms with a dominant haplophase have possibly developed strategies to avoid such events. This could account for the low frequency of illegitimate integrative transformation monitored in *P. patens* and in *S. cerevisiae* (Schiestl and Petes 1991). Such strategies might involve the possibility of maintaining autonomously replicating extrachromosomal elements such as plasmids or high

molecular weight episomal arrays, accounting for the fact that replicative transformation is sometimes easy to achieve in such organisms. Arguments sustaining such an assumption are found in the ability of *P. patens*, *C. purpureus*, *S. pombe* (Heyer, et al. 1992) and some filamentous fungi (Timberlake and Marshall 1989) to replicate bacterial plasmids. However the presence of homologous sequences on the introduced DNA would increase the probability of integration through homologous recombination, possibly by unrestricted formation of stable double strand structures between the incoming DNA and the homologous genomic sequences, and their subsequent integration during DNA replication or transcription. Homologous sequences would therefore be required to allow integrative transformation to occur, resulting in a higher ratio of homologous to random integration in these cells.

2. "Organisms with a dominant diplophase accept the integration of foreign DNA in their genome, but protect themselves against efficient homologous recombination"

The integration of foreign DNA sequences in somatic diploid cells does not lead to mutagenesis, and it has been clearly demonstrated that these cells actually integrate foreign DNA quite easily at random location in their genome. This indicates that a mechanism preventing integrative transformation with foreign DNA is not present in diploid cells. On the other hand, if these cells were able to perform efficient homologous recombination, it would rapidly lead to mitotic recombinations between homologous allelic sequences, repetitive DNA and multigene families, resulting in multiple chromosome rearrangements and ultimately to cell death, unless these cells were able to protect themselves against such recombination processes. Such protection could be achieved by the absence of the enzymes and machinery involved in homologous recombination. But this was considered to be unlikely since these enzymes are also involved in DNA repair (Capecchi 1990), and are therefore probably essential to the cell. It is therefore postulated that diploid cells have developed a protection mechanism by which homologous genomic sequences are specifically recognised and modified, possibly at the chromatine level, in order to prevent extensive mitotic recombination to occur. Consequently, integrative transformation in diploid cells occurs essentially by illegitimate recombination, and gene targeting is a rare event.

In diploid cells using gene conversion for their own cellular functions, such a process should be either independent of the homology sensing mechanism, or have an additional level of information, such as sequence specific recognition sites and endonucleases, to be successfully achieved. The second alternative has been selected for example in V(D)J recombination for the production of IgG (Gellert 1992), whereas gene conversion in Trypanosoma appears to be sequence specific independent (Pays and Steinert 1988). However, the generation of IgGs in chicken is a two step process which involves initially V(D)J recombination followed by sequence independent intrachromosomal gene conversion (reviewed in (Thompson 1992)). Therefore it seems that both Trypanosomatidae and chicken B cells have developed efficient gene conversion systems which work independently of the cellular homology sensing mechanism. Consequently, this independence would allow these cells to integrate foreign DNA sequences in their genome preferentially by homologous recombination,

accounting for the high ratio of homologous to random integration events monitored in Trypanosomatidae and in chicken B cells.

Obviously, the next question is which cellular process could account for this diploid cell specific ability to recognise and modify homologous sequences within a genome ? It was considered that genomic imprinting was a good candidate to fulfil these functions. Genomic imprinting is being more and more accepted as a fundamental and widespread process that determines, in ways not predicted by the laws of Mendel, the expression of a particular allele, set of genes or even complete chromosome in a diploid cell. It was initially identified as a process responsible for the correct dosage of paternal and maternal genome expression in the development of plant and mammalian embryos (reviewed in (Solter 1988; Moore and Haig 1991)), and for the inactivation of X chromosome in mammalian cells (reviewed in (Lyon 1992)). In plants, genomic imprinting has also been associated with paramutation and more recently with the trans-inactivation phenomena monitored in some transgenic plants (reviewed in (Matzke and Matzke 1993b)). A central feature of imprinting is that it involves the recognition and the modification, possibly at the chromatin level, of homologous allelic or ectopic sequences within a genome. Such modification results in the inactivation of one or both alleles or homologs in a way which is transmissible in somatic cells, but which can nevertheless be reversed. Such inactivation has been sometimes associated with modification of the DNA (such as methylation (Solter 1988; Matzke and Matzke 1993b)) and/or the chromatin structure (such as a lack of histone H4 acetylation, (Jeppesen and Turner 1993)), but the mechanisms responsible for such inactivation are not yet clearly identified (discussed in (Matzke and Matzke 1993b)). The idea that genomic imprinting could be associated with a general homology sensing cellular mechanism in diploid eukaryotic cells has been recently discussed by Matzke (Matzke and Matzke 1993a). In this view, one of the function of genomic imprinting would be the modification of homologous sequences in diploid genomes in order to prevent deleterious mitotic recombinations to occur within these sequences. In this context, it is worth noting that some reports of trans-inactivation of trans-gene expression in plants (Matzke and Matzke 1993b), which have been associated with genomic imprinting but which can not be explained by gene dosage requirement, could actually be a consequence of such homology sensing mechanism.

It is therefore proposed that efficient gene targeting in haploid cells would be associated with the physical capacity of introduced DNA to pair with homologous genomic sequences during DNA replication, allowing integrative transformation to occur despite the fact that haploid cells try to protect themselves against it. On the other hand, inefficient gene targeting in diploid cells would be associated with a protection of diploid cells against homologous recombination, a protection which would be mediated by a homology sensing mechanism sharing many characteristics with genomic imprinting. The consequences of such hypothesis is that efficient gene targeting could only be achieved in diploid cells which have a gene conversion machinery working independently from the homology sensing mechanism, or in diploid cells where this

homology sensing mechanism is depressed. Though speculative, this working hypothesis could be tested in experiments designed to answer the following questions.

(1) Since genomic imprinting has been shown to be sometimes associated with changes in DNA methylation (Solter 1988; Matzke and Matzke 1993b), is cytosine methylation inhibiting efficient homologous recombination in diploid cells ? To answer this question, gene targeting experiments should be performed in protoplasts isolated from plants treated with 5-azacytine, an inhibitor of methylation, and in protoplasts isolated from DNA hypomethylated mutants. In this context, it might be very interesting to perform gene targeting experiments in the *ddm* mutants of *A. thaliana* recently described (Vongs, et al. 1993). The only phenotype monitored in these mutants was a distortion of meiotic segregation, and this phenotype could indicate that meiosis or gametogenesis were impaired as a consequence of a disturbed cellular homology sensing mechanism. Such experiments might allow to establish a link between efficient gene targeting and cytosine methylation. Parallel experiments should be performed with animal cells in order to assess the general validity of the results, and experiments with methylation inhibitors should focus on cell lines which perform efficient gene conversion, in order to investigate the relations between DNA methylation, genomic imprinting, gene conversion and gene targeting.

(2) Is integrative transformation systematically preferentially achieved by homologous recombination in haploid cells ? Gene targeting experiments should be performed in other mosses such as *C. purpureus* and *F. hygrometrica* as well as in other fungi and haploid protozoans. The ratio of homologous to random integration should be determined in these organisms in order to assess the general validity of this assumption.

Though speculative, the model proposed here to account for the low frequencies of gene targeting events monitored in diploid cells can be experimentally tested. If this model is confirmed, it may allow the development of strategies designed to improve the efficiency of the reverse genetic approach in the dissection of biological processes in diploid cells.

2.3.4. MATERIAL AND METHODS

Plant material, plasmids and antibiotics

The following *P. patens* strains were used in this study:

- Untransformed wild type strain (Kan^S Hg^S).

- Untransformed self-sterile strain *nicB5ylo6* (Kan^S Hg^S)(Ashton and Cove 1977)

- Carrying pHP 23b: Kan^R Hg^S strains HP23 L2 and G 418-1 in wt

- Carrying pGL 2: Kan^S Hg^R strains Hg-1, Hg-2, Hg-3, Hg 27 DI in wt and 15.01 in pabA3. All these strains are described in the previous chapter.

The techniques for tissue propagation, protoplasts isolation and culture are described in annex A. Plasmids pHP 23b (35S-NPT II) (Paszkowski, et al. 1988) and pGL 2 (35S-APH IV, described in (Schaefer, et al. 1991)) are both pDH 51 derivatives (Pietrzak, et al. 1986) conferring resistance to kanamycin and Hygromycin B respectively, and are described in the previous chapter. These plasmids were amplified and PEG-purified according to standard techniques (Sambrook, et al. 1989). G-418 and Hygromycin B were purchased from Gibco and Calbiochem respectively and were added to culture medium after sterilisation.

Transformation and selection of stable double resistant clones

Protoplasts were transformed according to the optimized protocol with supercoiled plasmid pHP 23b or pGL 2. At the end of the transformation, samples were diluted stepwise with liquid culture medium, transferred in 5 cm Petri dish and left overnight in darkness. The next day, cells were sedimented by low speed centrifugation (600 g for 5 min), half of the supernatant was replaced with top layer agar (PP-NH_4 + mannitol + 1.3% agar) and each sample was plated on 4 plates of PP-NH_4 + mannitol solid culture medium and regenerated for 6 days. Surviving colonies were counted at this stage and selection was applied by transferring the top layers on PP-NH_4 solid culture medium supplemented with 40 mg/l G-418 or 25 mg/l Hygromycin B. Transfer on fresh selective medium was then repeated every 2 weeks to maintain selective pressure constant. Initial relative transformation frequencies were determined 10 days after the initiation of selection and stable transgenic clones were identified by their unrestricted growth on selective medium during the next month and counted. These clones were isolated and their stable double resistant phenotype was confirmed by testing their ability to display unrestricted growth both on G-418 and Hygromycin B supplemented media, by non-selective to selective growth tests and by meiotic transmission of the characters upon selfing. Relative transformation frequencies (RTF) are expressed as the ratio between the number of resistant clones and the number of regenerating colonies after transformation.

Selfing, crossing and segregation analysis

Strain *nicB5ylo6* present the advantage of self-sterility associated with auxotrophy for nicotinic acid, associated with a convenient phenotypical yellow marker (*ylo)* allowing easy identification of this strain in crossing experiments (Ashton and Cove 1977). A protonematal inoculum of each double resistant transgenic strain and of strain *nicB5ylo6* were transferred side by side on PP-NO_3 medium in glass culture tubes and regenerated at 26°C until some 50 gametophores were completely differentiated (colony diameter, ca 1 cm). 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 resuspend the spores and kept 3 weeks at 4°C to ensure high spore germination rates. Spore capsules collected on the transgenic colony were generated by self-fertilisation and the ones collected on the *nicB5ylo6* colony by cross-fertilisation. One tenth of each spores suspension was inoculated on PP-NH_4 medium and grown for 10 days in 9 cm Petri dishes. At this stage, well defined single spore derived colonies were isolated and plated on PP-NO3 medium for an additional week (52 colonies / 9cm Petri dish). Segregation of the *ylo* phenotype was recorded at that stage. Replicates of the master plates were made by inoculating a fragment of each colony to similar positions on PP-NO_3 medium supplemented with either G-418 (40 mg/l) or Hygromycin B (25 mg/l). Segregation of antibiotic resistance was monitored within the next 2 weeks and segregation of the *ylo* character in double resistant transgenic siblings was recorded during the next month.

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3. GENERAL DISCUSSION

The results presented all along this dissertation may appear disperse and often incomplete, but the main purpose in this kind of pioneer work was to test the feasibility and the potential applications of several different molecular approaches in *P. patens*, rather than to fully characterised one or the other of these processes. Therefore investigations have been performed in many different directions. Additionally, some of the results obtained in this work were totally unexpected and it was not always evident to find a plausible explanation to account for these data. Discussions are therefore sometimes speculative, but the exceptional behaviour of *P patens* in response to the introduction of DNA in protoplasts, and the lack of a well defined model describing these rare autonomous replicative transformation events in eukaryotes justifies it. Moreover, the singularity in these responses led to the development of a general concept accounting for the different fates of foreign DNA molecules observed following their introduction in different eukaryotic cells. This general discussion will recapitulate the main results obtained during this work and discuss their potential applications.

3.1. Development of molecular genetic techniques in P. patens

Optimization of the PEG-mediated DNA uptake procedure published in 1991 (Schaefer, et al. 1991) has permitted the establishment of a sensitive and versatile transient gene expression assay in protoplasts of *P. patens*. GUS specific activities monitored following transfections with a 35S GUS constructs are at least hundred-fold above background and in the range of those measured in a transgenic tobacco plant transformed with a similar construct. Transient expression of two constructs driven by heterologous light-inducible promoters has been reproducibly monitored, and the expression of one of them was shown to be regulated by moss phytochrome. These physiological evidence indicate that some of the mechanisms involved in the regulation of gene expression in plants are conserved between higher plants and mosses, and open interesting perspectives for further development of transient gene expression assays in *P. patens* protoplasts.

The optimum PEG-mediated uptake procedure was adapted to introduce antibodies in moss protoplasts in order to test the feasibility of a transient *in vivo* immunodepletion assay. The idea was to specifically inactivate cellular proteins with antibodies, and to be able to couple this approach with transient gene expression experiments. Our data demonstrate that this assay can be used to immunodeplete *in vivo* the product of transient gene expression, and that anti-tubulin antibodies introduced in moss protoplasts seriously affects cell survival, regeneration and ploidy. The mechanisms underlying these responses are currently not known and requires further investigations. Nevertheless, the possibility to immunoinactivate *in vivo* cellular proteins with specific antibodies should allow the development of new strategies to investigate cellular processes. It is predicted that this approach can be directly applied

to other plant protoplasts, and possibly to other cells such as yeasts and bacteria, allowing *in vivo* immunodepletion assays to become widely used in biological experimental research.

Finally, an efficient protocol for the genetic transformation of *P. patens* has been established, and both low frequency integrative and high frequency replicative transformants have been recovered. The establishment of genetic transformation in *P. patens* fills a major methodological gap in the development of this plant as a model genetic system. Preliminary experiments performed with heterologous morphogenetic genes have indicated that these genes are biologically active in *P. patens*, and that the phenotype can be monitored in both types of transformants. These data indicate that some of the mechanisms involved in plant development are conserved between higher plants and the moss *P. patens*. Investigations of moss integrative transformation provided strong genetic evidence that integration by homologous recombination was more frequent than random integration in the genome of *P. patens*, opening the way to the development of efficient reverse genetics in this model system. Investigations of replicative transformation indicated that this type of transformation can be used for the identification of genomic elements involved in moss DNA replication, opening the way to the development of an experimental model system to investigate plant DNA replication. These unusual findings for plant transformation and their potential applications are discussed in the next section.

3.2. Transformation in P. patens

Integrative transformation occurs at reproducible low frequencies with all plasmids tested so far. The possibility to integrate foreign DNA in the moss genome offers the opportunity to investigate the biological activity of heterologous genes as well as the possibility to generate tagged mutants by insertional mutagenesis. Yet, the low frequencies of these integrative events prevents the direct generation of large number of transgenic strains, but this disadvantage can be efficiently circumvented by the retransformation of transgenic strains with homologous plasmids, taking advantage of the higher frequencies of targeted to random insertional events in *P. patens* monitored in artificial loci.

Replicative transformants are recovered at high frequencies and two classes of clones have been identified. This is the first report of replicative transformation in plants. The characteristics of these clones are compared with those of replicative transformants in *S. cerevisiae*, in *S. pombe* and *C. elegans* (Struhl 1983; Heyer, et al. 1986; Mello, et al. 1991).

Class II replicative transformants are the most abundant resistant clones and display a phenotypical behaviour similar to yeasts transformed with ARS plasmids. However, preliminary molecular characterisation indicated that the extrachromosomal transforming sequences are maintained as high molecular weight structures, thereby preventing this type of transformants to be used for the development of shuttle vectors between moss cells and *E. coli*. These clones are therefore more similar to the

replicative transformants of *S. pombe* following transformation with bacterial plasmids described by Heyer, or to the transient transformants of *C. elegans* described by Mello.

Class III replicative transformants are recovered at low frequencies and display a phenotypical behaviour similar to yeast transformed with ARS-CEN or ARS-TEL vectors. Yet, molecular analysis has demonstrated that the extrachromosomal sequences are maintained as high molecular weight structures, and these clones resemble more to the extrachromosomal transformants of *C. elegans*, *X. laevis* (Etkin and Pearman 1987), and Zebra fish (Stuart, et al. 1988). Improved mitotic stability of the resistance gene and high rates of delayed integration events suggest, by analogy with yeast transformation, that the transforming sequences have recombined with moss genomic sequences functionally involved in DNA replication and/or mitotic segregation. Further molecular analysis are required to confirm this hypothesis. Yet, an indirect evidence has been obtained in preliminary transformation performed with YAC derivatives. A strong increase in the frequency of occurrence of this class of clones as compared to transformation with bacterial plasmids was monitored, indicating that the functional elements carried by YAC vectors induce a similar phenotype.

Experiments performed to investigate the possibility of performing gene targeting in *P. patens* demonstrate that integration by homologous recombination is more frequent than random integration in the moss genome. This result came as a complete surprise, since such a high ratio of targeted to random integration has never been reported in plants and is rare in other eukaryotes. These results offers a unique opportunity to develop efficient reverse genetic approaches in this plant.

Finally, preliminary experiments performed with another moss, *C. purpureus*, provided phenotypical evidence indicating that replicative transformation could be a general feature of genetic transformation in Bryophytes.

The overall characteristics of moss transformation and comparison with transformation in other eukaryotes led to the formulation of a general concept about gene engineering and gene targeting. This concept is based on the different putative consequences of integrative transformation in organisms with a dominant haplophase versus diplophase, and on the control of recombination events between homologous DNA sequences in haploid and diploid genomes. The model predicts that the genetic transformation of a eukaryotic cell will not be achieved by similar mechanisms in organisms with a dominant haplophase or diplophase.

Organisms with a dominant haplophase will display low frequencies of integrative events by illegitimate recombination, since such event is immediately mutagenic and these organisms have developed strategies to avoid it. Yet, these species are easily amenable to replicative transformation, in order to maintain the possibility to acquire new characters with evolutionary advantages. However, the presence on the transforming plasmid of sequences homologous to genomic sequences would circumvent this defense mechanism and allow these plasmids to become integrated by homologous recombination, resulting in a higher rate of targeted to random integration events in these organisms.

On the other hand, diploid cells will integrate easily foreign DNA sequences in their genome, since this event has no mutagenic effects for the species. Yet, replicative transformation is not efficient in these organisms since it is possibly an archaic mechanism of genetic transformation, and diploid cells display high specificity for the recognition of DNA replication origin. The most important point is that diploid cells are not competent for efficient gene targeting, since an efficient homologous recombination system would induce numerous intrachromosomal homologous recombination events between genomic homologous sequences, leading more or less rapidly to extensive chromosomal rearrangement and ultimately to cell death. Therefore, diploid cells must have developed an efficient homology control mechanism, and it is proposed that this function is fulfilled by genomic imprinting. All the data on genetic transformation in eukaryotes fit well with this concept, and one can nearly say that the unusual features of moss transformation could have been predicted according to this explanation.

This concept has also evolutionary implications. In organisms with a dominant haplophase and/or relatively archaic, an efficient replicative transformation system could easily permit horizontal gene transfer, a mechanism which may have been involved in the evolution of living species. Alternatively, replicative transformation may also have allowed organisms to survive hard environmental conditions by transient genetic transformation without consequences for the next generation.

3.3. Towards the development of new moss genetics

The main features of *P. patens* transformation monitored in these experiments should allow the following approaches to be developed.

(1) The possibility to monitor different phytochrome-mediated responses in *P. patens* protoplast is unique among plant protoplasts. It is therefore predicted that these cells can be advantageously used to investigate the mechanisms underlying phytochrome actions. This is an important question in plant physiology which lacks a suitable single-cell system to be studied. The roles of the different phytochrome molecules has been successfully studied in the last years, taking advantage of the large number of tagged photomorphogenetic mutants generated in *Arabidopsis thaliana* (reviewed in (Kendrick and Nagatani 1991; Chasan 1993; Pepper, et al. 1993)). Yet, the transduction pathways of phytochrome-mediated responses are still largely unknown, and have so far only been accessible at the level of single cells in the phototropic responses of *Ceratodon purpureus* protonematal cells (Hartmann and Weber 1988; Herth, et al. 1990), in the swelling of wheat protoplasts in response to red light irradiation (Bossen, et al. 1988; Bossen 1990; Shacklock, et al. 1992), and in microinjection experiments where possible intermediates of the transduction pathway were introduced in hypocotyl cells of wild-type and phytochrome deficient mutants of tomato (Neuhaus, et al. 1993). The availability of a protoplast system displaying several phytochrome-mediated responses could advantageously complement the tools available to study these questions. Moreover, the simultaneous development of an *in vivo* immunodepletion assay should permit the development of alternative immunological approaches to investigate these cellular and developmental events. New mutagenesis

and screening for photomorphogenetic mutants should be performed to complete the tools available to study these processes.

(2) High frequency autonomous replicative transformation, the clear phenotypical differences between class II and class III replicative transformants, and the physiological data obtained with YAC vectors strongly suggest that the ratio of class II versus class III replicative transformants can be used as a criteria for the identification of moss genomic fragments carrying functional sequences involved in DNA replication and/or mitotic segregation. Transformation with vectors in which random fragments of moss DNA have been inserted should be performed, and the vectors generating class III resistant clones at high rates further characterised. Since SAR elements have been postulated to be involved in the generation of class III resistant clones, and since these elements are abundant in the genome, it is predicted that within 25 to 50 random 5 kb genomic fragments, the probability to clone a functional SAR is quite high. With such an approach, it is predicted that moss genomic sequences involved in DNA replication and/or mitotic segregation could be rapidly identified and characterised, allowing experiments designed to investigate plant DNA replication to be performed. To our knowledge, there are no equivalent system in higher plants where such processes can be so advantageously studied. In the same time, experiments aimed at the isolation of nuclear scaffold bound moss DNA sequences should be performed, and their biological activity in transforming vectors investigated. The same experiments should be performed with the functional SAR, TEL and CEN element carried by YAC vectors. It might also be extremely interesting to isolate micro- and B-chromosomes from other moss species and to test the possibility to develop transformation vectors based on these elements (for further references, see (Newton 1984)). It is predicted that such experiments might rapidly lead to the development of Moss Artificial Chromosomes (MAC). These vectors should then be tested in higher plants. This approach might lead to the development of a plant artificial chromosome, a tool which would have broad applications in plant genetic engineering and in plant breeding.

(3) High frequency replicative transformation opens the possibility to develop shuttle vectors for moss cells and *E. coli*, and experiments should be performed to investigate this possibility. However, it might be not straightforward to develop such vectors when one consider the type of replicative transformation monitored in *P. patens*. Extrachromosomal plasmids are not maintained as single monomers, but are concatenated to form high molecular weight structures. Therefore these extrachromosomal arrays will probably not efficiently transform back bacteria. Actually, shuttle vectors have so far not been developed in other organisms displaying autonomous replicative transformation associated with high molecular weight extrachromosomal structures such as *X. laevis*, *C. elegans* or Zebra fish. Yet, this approach should be investigated and the successful development of shuttle vectors for *P. patens* would allow the isolation of nuclear genes by extrachromosomal complementation of mutants, following a strategy similar to the one developed for bacteria and *Neurospora crassa* (Akins and Lambovitz 1985).

(4) Direct applications of the establishment of transformation in *P. patens* can also take advantage of the high frequencies of gene targeting. The isolation of known developmental genes can be achieved with higher plants heterologous probes. Genomic

sequences have to be isolated and characterised. These sequences should permit gene disruption and gene replacement experiments to be performed, with the advantage that the targeted plant will probably not have to be identified among a large number of transgenic plants generated by random insertional events. Alternatively, one can construct a genomic library with an average size around 5 kb and randomly clone these fragments in transformation vectors. Tagged mutagenesis can be achieved this way. Yet, to improve the ratio of coding sequences in the transforming vectors, one can screen this library with cDNA probes and introduce only the genomic clones which hybridize with the cDNA probes in transformation vectors. To improve the specificity of the screening, it is proposed that substracted cDNA should be used as probes to select genomic clones specific for the investigated process. This approach should also rapidly leads to the generation of tagged mutants by homologous recombination mediated insertional mutagenesis. The power of the reverse genetic approach does not need to be emphasized, and the possibility to develop such an approach in *P. patens* provides a valuable new tool for the development of this plant as a model system to study cellular and developmental processes.

(5) The generation of tagged mutants is a major goal in the development of *P. patens* as a model genetic system, and a lot of work have been investigated to generate transposon-tagged mutants (Cove, et al. 1991). However, this approach has not yet been successful and might be not very efficient in view of the low frequencies of integrative events monitored following transformation. It is believed that the approach proposed above, using gene targeting, may be more straightforward for the generation of tagged-mutants.

(6) Finally, there is a strong requirement for the generation of a genetic map of the genome of *P. patens*. It is considered that the RAPD approach (Williams, et al. 1990) will probably be the most suitable one, since it is specially efficient to map haploid genomes, and efforts should be done in that direction.

3.4. Potential of P. patens as a model genetic system.

The development of *P. patens* as a model genetic system to study plant development has a high potential, but is still at its infancy. Established plant model genetic systems, such as *Arabidopsis thaliana* (Meyerowitz 1989), maize, *Antirrhinum* or tomato are miles ahead, and one can wonder why investigate so much efforts in the development of mosses. Structural and developmental advantages have been presented in the introduction. These are associated with (1) the simple morphology of the plant associated with the possibility to follow cellular and developmental processes *in vivo* at the level of individual cells, (2) the similar responses of mosses and higher plants to environmental and growth factors, and (3) the facilitated genetic analyses associated with the dominance of the haplophase in the life cycle. The development of molecular genetic approaches for *P. patens* fills a major methodological gap in this view. Moreover, the extraordinary responses of *P. patens* to gene transfer, i.e. low frequency random integrative transformation, high frequency replicative transformation, and a high ratio of targeted to random integration events has so far no equivalent in other

plant model system. These features open realistic perspectives to study fundamental processes and to elaborate strategies which are not yet accessible in other model systems. The potential applications of these discoveries discussed above indicate that *P. patens* can be advantageously used to study plant biological processes with the combination of the most powerful approaches of modern molecular genetics. It is therefore predicted that the gap between *P. patens* and higher plants model systems could rapidly be compensated by the new approaches which can be developed in this plant. It is therefore concluded that the moss *Physcomitrella patens* provides a valuable model system to study plant cellular and developmental processes which conveniently complete the tools available in modern plant biology.

ANNEX A

Standard conditions for the culture of P. patens in Lausanne

Growth conditions

The standard conditions for the culture of *P. patens* in the lab are as follows. Cultures are grown in the culture room at 26°C in discontinuous white light (16 hours / day). Light is provided by fluorescent tubes Sylvania GRO-LUX WS at quantum irradiance of 50 to 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Protonematal cultures are made in 9 cm Petri dishes poured with solid culture medium and overlaid with cellophane disks (W.E. Cannings, Bristol, UK). Sporogenesis is performed in Magenta boxes, or in culture glass tubes. Cultures are irrigated with sterile water and transferred at 17°C in illuminated temperature-controlled growth chambers (Polytron, Weiss Technik AG) for three weeks to induce gametogenesis.

Culture media (see also (Knight, et al. 1988) for further informations)

Culture media used all along this study are derived form the recipe described in (Ashton, et al. 1979).

PP NO_3: This medium is used for phenotypical analyses and for sporogenesis.

Macro elements:	
$CaNO3 \cdot 4H_2O$	0.8 g/l
$MgSO_4 \cdot 7H_2O$	0.25 g/l
$FeSO_4 \cdot 7H_2O$	0.0125 g/l
Micro elements	
$CuSO_4 \cdot 5H_2O$	0.055 mg/l
$ZnSO_4 \cdot 7H_2O$	0.055 mg/l
H_3BO_3	0.614 mg/l
$MnCl_2 \cdot 4H_2O$	0.389 mg/l
$CoCl_2 \cdot 6H_2O$	0.055 mg/l
KI	0.028 mg/l
$Na_2MoO_4 \cdot 2H_2O$	0.025 mg/l
Glucose	5 g/l

Add 1 ml per litre of a 1000 x stock solution of KH_2PO_4 / KOH buffer (dissolve 25 g/l KH_2PO_4 and titrate to pH 7 with 4M KOH). Add 7 g/l Agar agar (Merck 1614) and sterilise by autoclaving (20 min, 120°C)

Supplements for auxotrophs

Thiamine HCl	500 µg/l
Para-aminobenzoic acid	250 µg/l
Nicotinic acid	1 mg/l

Supplements are added to the culture medium prior to sterilisation

PP NH_4.

Add 500 mg/l NH_4 tartrate to PP NO_3. This medium is used for the production of chloronemata enriched protonema used to isolate protoplasts. The presence of ammonium tartrate in the medium increases the yield of secondary chloronemata, but does not allow the completion of the life cycle. Add 7 g/l Agar agar and sterilise by autoclaving (20 min, 120^{o}C)

Protoplast solid culture medium.

Supplement PP NH_4 solid culture medium with 66 g/l mannitol and check osmolarity (around 480 mOsmol). Sterilise by autoclaving.

Protoplast liquid culture medium.

Supplement PP NH_4 culture medium without agar with 66 g/l mannitol and check osmolarity (around 480 mOsmol). Sterilise by autoclaving. A precipitate forms during autoclaving (probably calcium phosphate). Sediment the precipitate and use the clear supernatant.

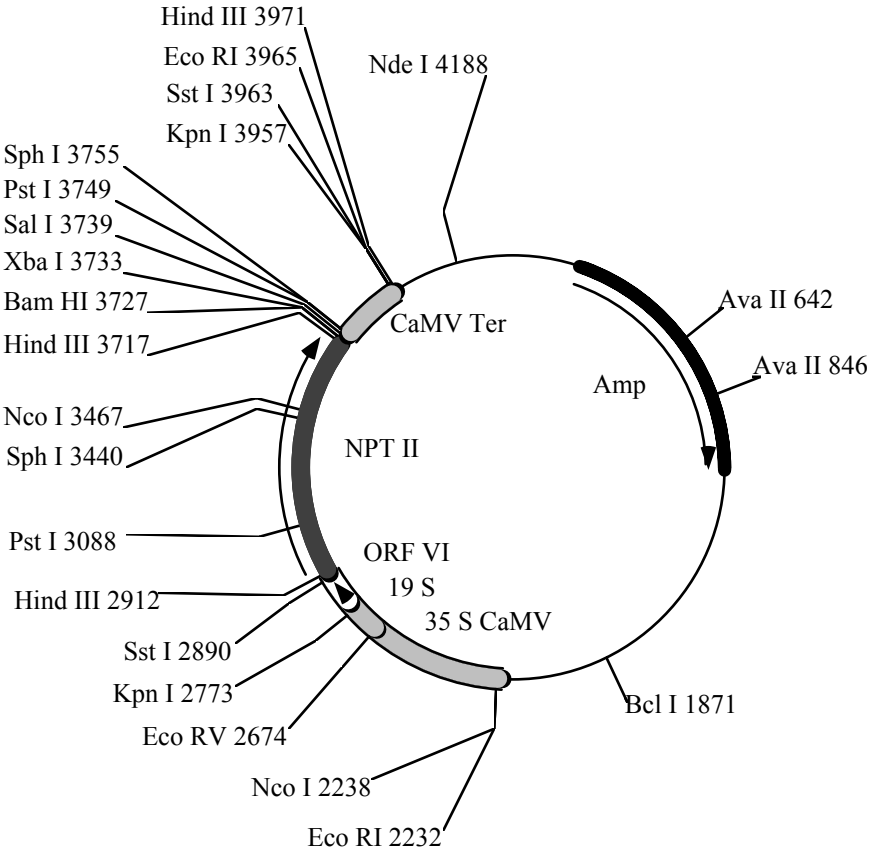
Protoplast isolation and regeneration (adapted from (Grimsley, et al. 1977b)).

Collect protonema from 5 to 10 cultures grown for maximum 6 days on PP NH_4 and transfer to a 9 cm Petri dish containing 10 ml 0.48M mannitol. Add 15 ml of a sterile solution of 2% (w/v) non purified Driselase (Fluka 44585) and incubate at room temperature for 30 min. with occasional gentle mixing. (Driselase is dissolved in 0.48M mannitol, centrifuged at 10000 rpm for 10 min. to remove debris, and sterilised by passage through a 0.45 µm filter). Filter the preparation through a 100 µm stainless steel sieve and leave for an additional 15 min. to complete digestion. Filter through a 50 µm stainless steel sieve and transfer to sterile 10 ml glass tubes. Harvest the cells by low speed centrifugation (600 rpm for 5 min.) and gently resuspend the pellet in mannitol 0.48M. Repeat centrifugation and resuspend the cells in mannitol 0.48M. Take one aliquot of the suspension, dilute ten times in mannitol 0.48M and counts protoplasts with an hematocytometer. The yield is usually around 10^6 viable protoplasts per initial culture plate. Repeat centrifugation and resuspend in mannitol 0.48M at a concentration of ca 10^5/ml. Add one volume of protoplast top layer (protoplast liquid culture medium or mannitol 0.48M with 1.3% agar, kept molten at 45^{o}C in a water

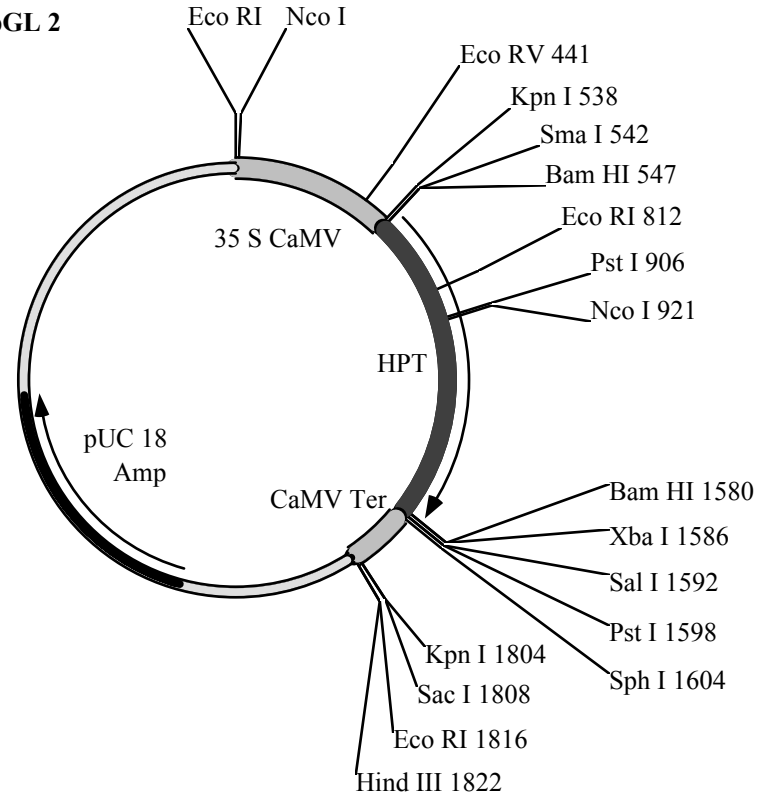
bath) and dispense 2 ml aliquot on 9 cm Petri dishes containing protoplasts solid culture medium overlaid with a cellophane disk and regenerate in light in culture room.

Restriction maps of plasmid pHP 23b, pGL2 and pABD 2

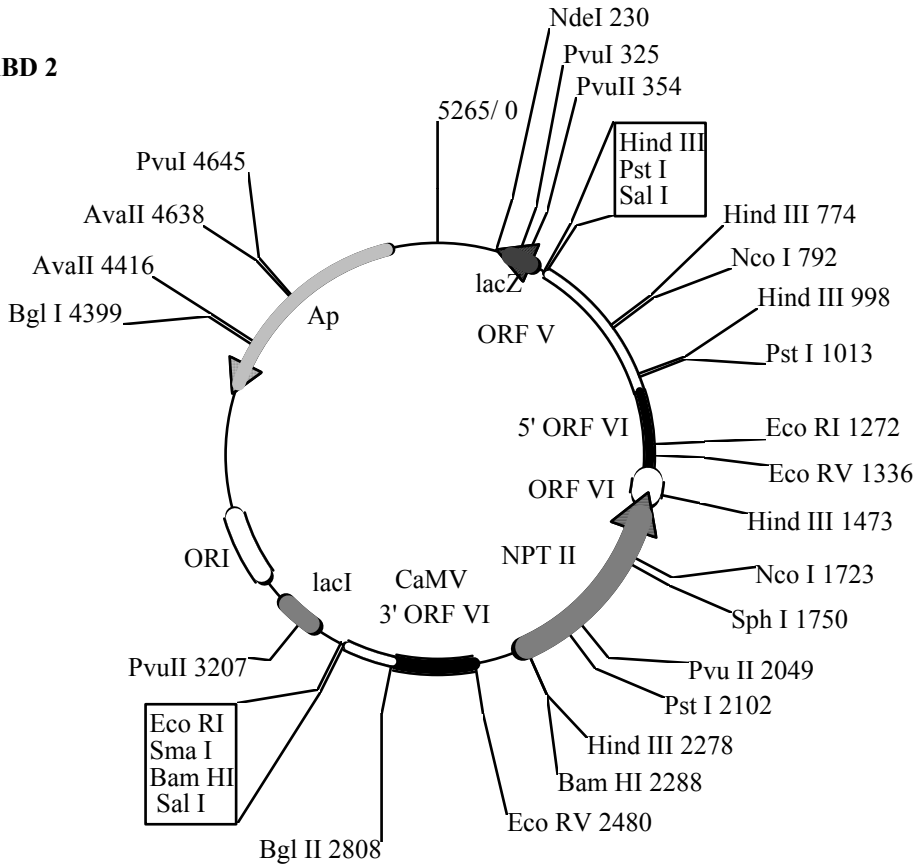
pHP 23b



pGL 2


Eco RI
Nco I
Eco RV 441
Kpn I 538
Sma I 542
Bam HI 547
Eco RI 812
Pst I 906
Nco I 921
35 S CaMV
HPT
pUC 18
Amp
CaMV Ter.
Bam HI 1580
Xba I 1586
Sal I 1592
Pst I 1598
Sph I 1604
Kpn I 1804
Sac I 1808
Eco RI 1816
Hind III 1822

p ABD 2


NdeI 230
PvuI 325
PvuII 354
5265/ 0
Hind III
Pst I
Sal I
PvuI 4645
AvaII 4638
AvaII 4416
Bgl I 4399
Ap
lacZ
ORF V
Hind III 774
Nco I 792
Hind III 998
Pst I 1013
5' ORF VI
Eco RI 1272
Eco RV 1336
ORF VI
Hind III 1473
ORI
lacI
CaMV
3' ORF VI
NPT II
Nco I 1723
Sph I 1750
PvuII 3207
Pvu II 2049
Pst I 2102
Eco RI
Sma I
Bam HI
Sal I
Hind III 2278
Bam HI 2288
Eco RV 2480
Bgl II 2808

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In the following pages you will find photographies and table which were originally present in the manuscript; for convenience they have been moved here in the Adobe PDF available on our web site

JPZ October 11, 2001

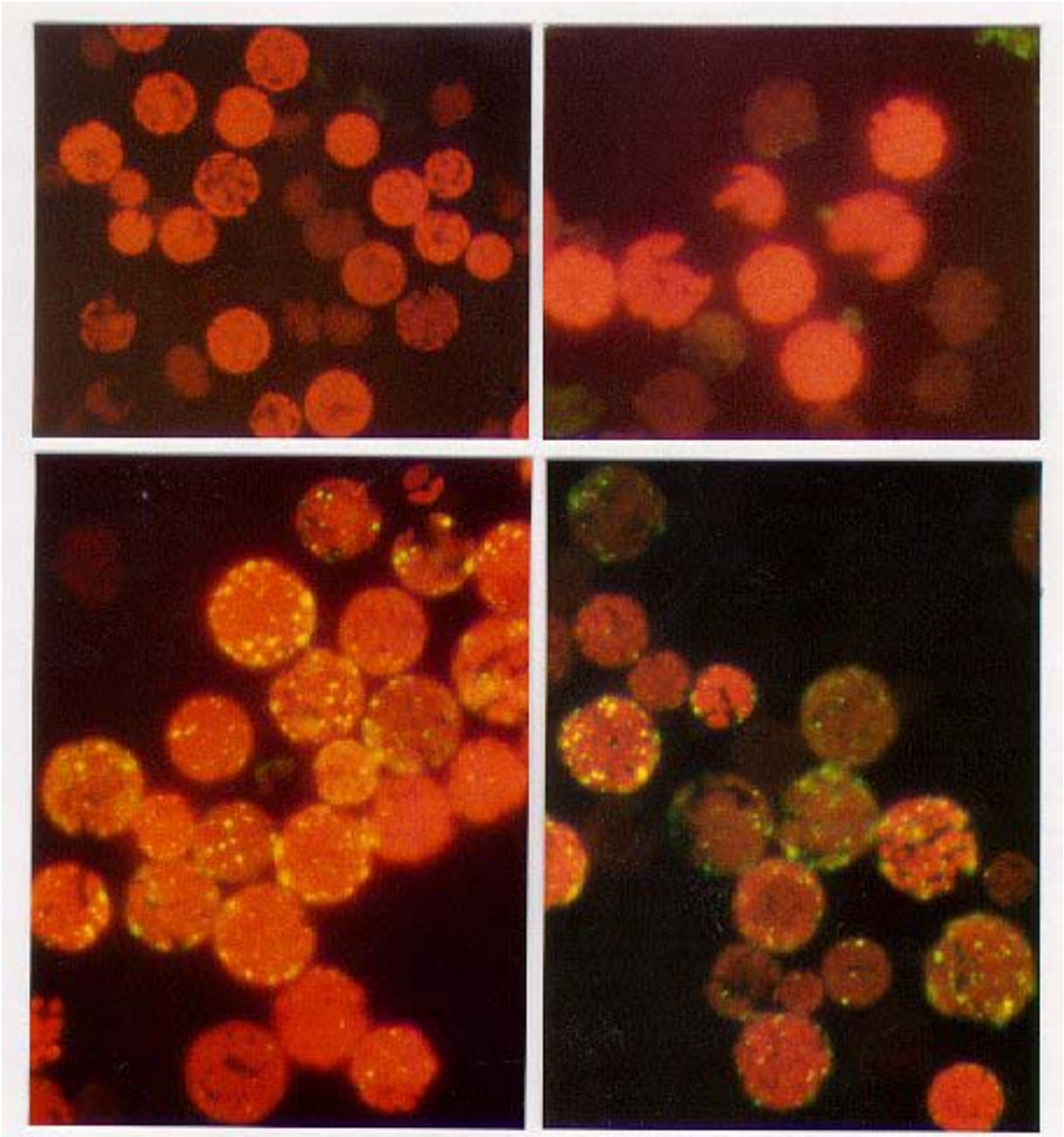

Figure 4. FITC-labelled protoplasts of P. patens as observed 6 hours after PEG-mediated IgG uptake. From top left to bottom right: untreated protoplasts, protoplasts incubated with FITC-IgGs but without PEG, treated sample in UV light (excitation 390 - 490 nm, emission cut off 515 nm), treated sample in UV light (excitation 450 - 490 nm, emission cut off 515 nm). Samples were heat shocked prior to the addition of PEG and illustrate heavily labelled protoplasts.

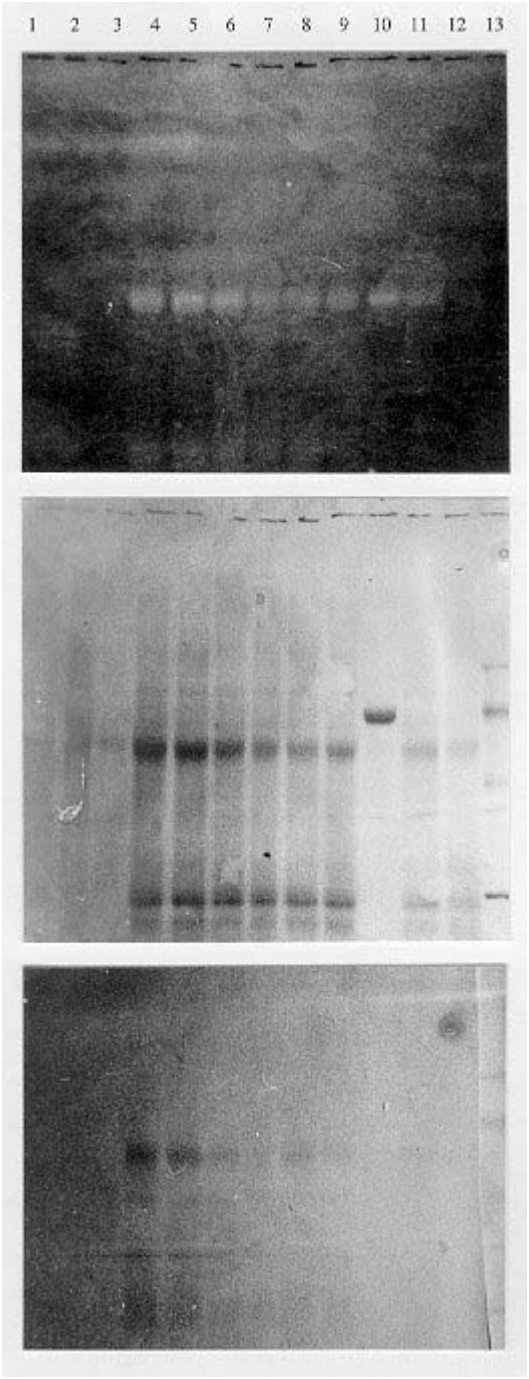
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Figure 8. Illustration of the dark-green and the sparse phenotype of colonies regenerated following PEG-mediated IgG uptake. Top: wild-type-, middle: sparse-, and bottom: dark green colonies.

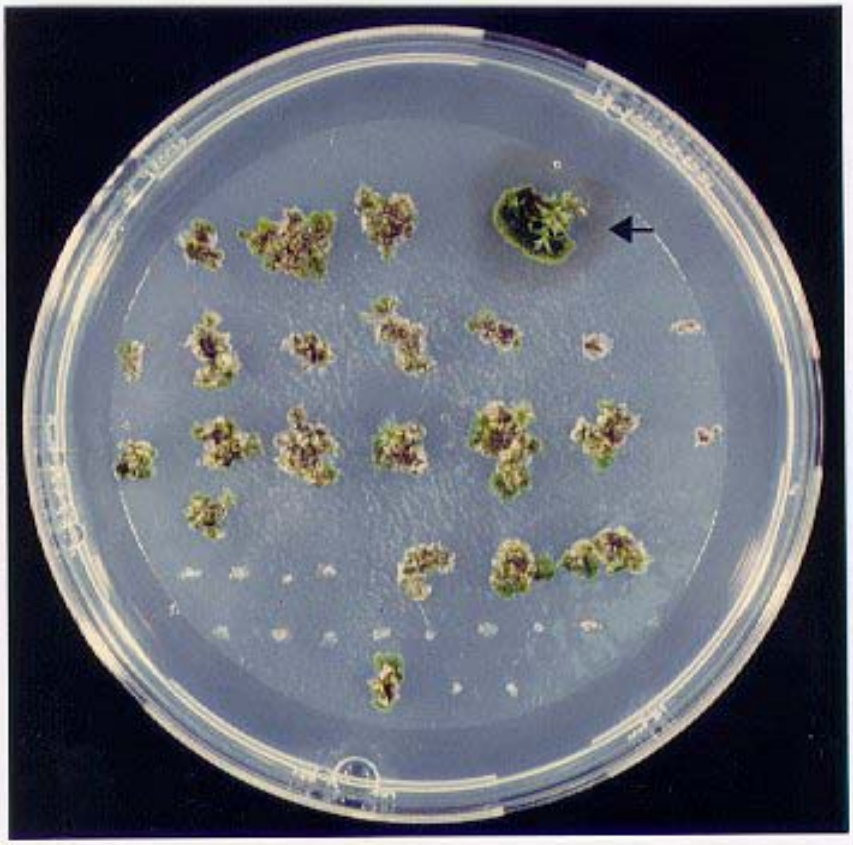


Figure 1. Five weeks old kanamycin resistant clones selected on G-418 50 µg/ml. A fragment of a stable clone (Kan R 7.01, arrow) displaying unrestricted growth can be identified among numerous dead or mosaic class II resistant clones.

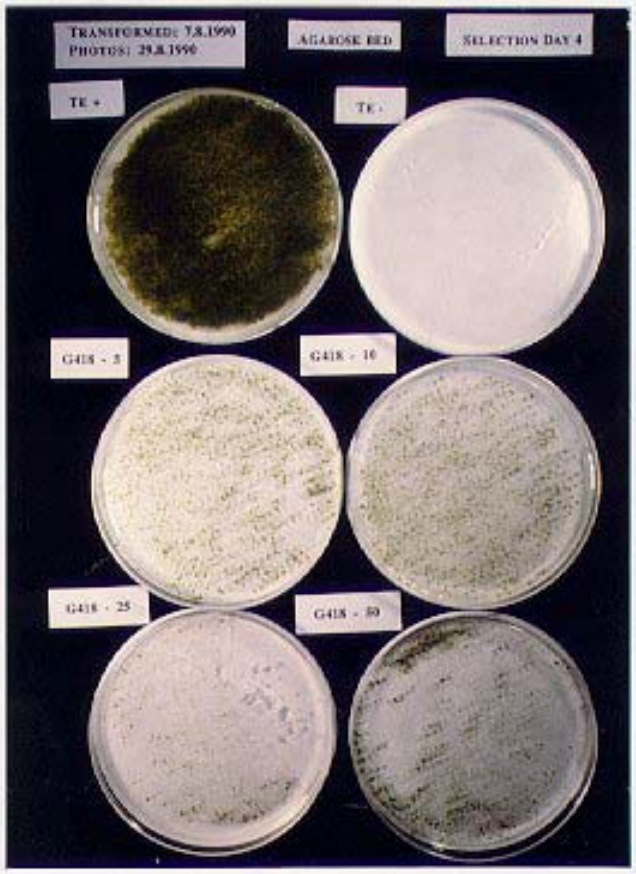
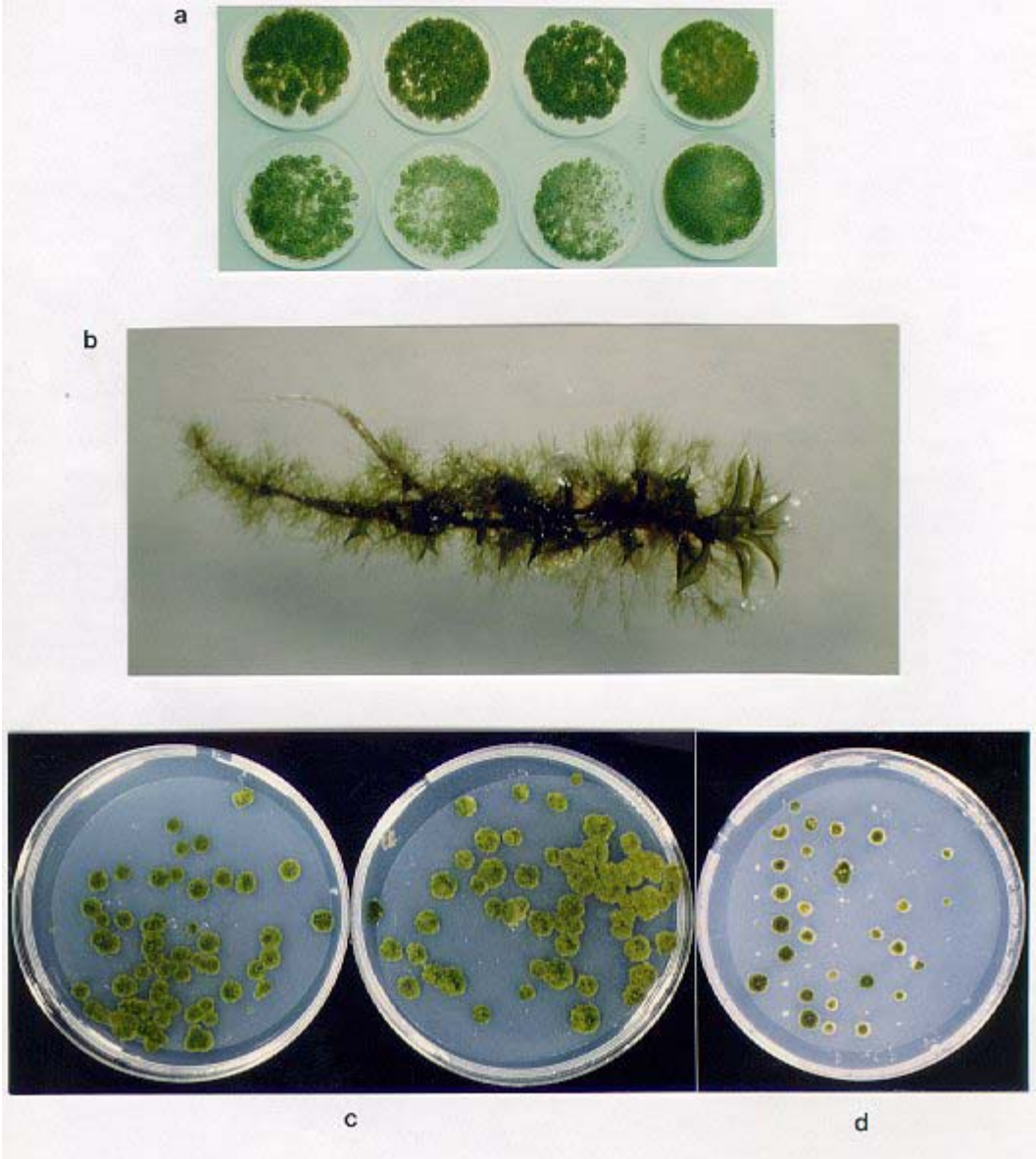


Figure 2. Initial transformation frequencies. Protoplasts were transformed with supercoiled pHP 23b and selected 4 days after DNA uptake on increasing concentrations of G-418. Each plate represent 1/4 of a sample. Pictures were taken 18 days after the initiation of selection. Top plates, untreated protoplasts regenerating on non-selective medium TE + or selective TE - medium.

Figure 3. Phenotypical and genetic characteristics of transgenic clones. (a) Non selective to selective growth assay on clones Kan^R 7.01, 8.01, 11.01 and 12.01 (from left to right). Top row: non-selective to selective growth, bottom row: selective growth. (b)Rescue experiment: the whole gametophore regenerate a protonema. (c) 100 % meiotic transmission of the character. Spores from the fourth generation of HP 23 L2 were germinated under selective (left) and non-selective to selective conditions (right). (d) Independent 1:1 segregation of the Kan^R and ylo marker in spores of HP 23 L2 x nicB5ylo6 germinated under non-selective to selective conditions.



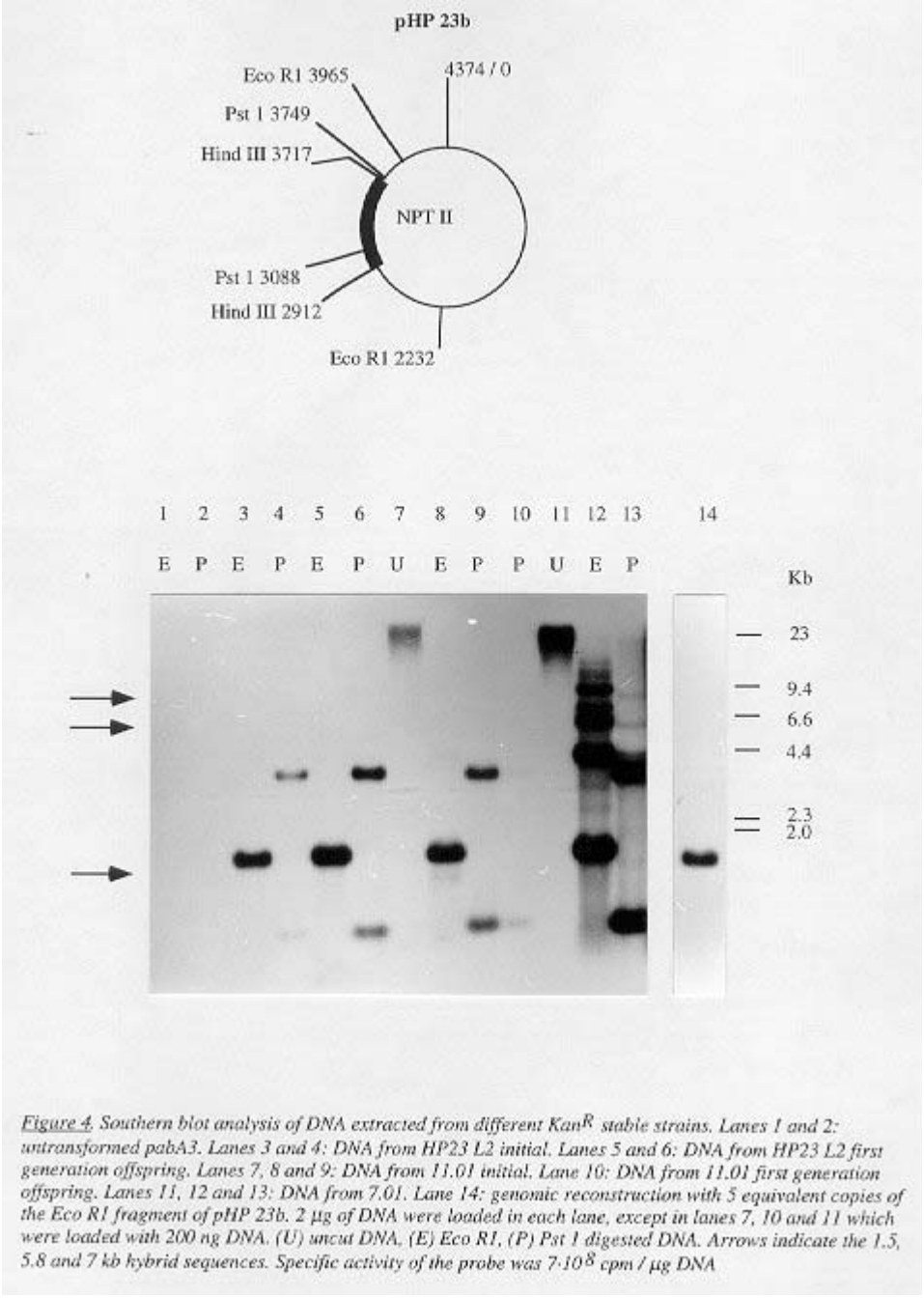


Figure 4. Southern blot analysis of DNA extracted from different Kan^R stable strains. Lanes 1 and 2: untransformed pabA3. Lanes 3 and 4: DNA from HP23 L2 initial. Lanes 5 and 6: DNA from HP23 L2 first generation offspring. Lanes 7, 8 and 9: DNA from 11.01 initial. Lane 10: DNA from 11.01 first generation offspring. Lanes 11, 12 and 13: DNA from 7.01. Lane 14: genomic reconstruction with 5 equivalent copies of the Eco R1 fragment of pHP 23b. 2 µg of DNA were loaded in each lane, except in lanes 7, 10 and 11 which were loaded with 200 ng DNA. (U) uncut DNA, (E) Eco R1, (P) Pst 1 digested DNA. Arrows indicate the 1.5, 5.8 and 7 kb hybrid sequences. Specific activity of the probe was $7 \cdot 10^8$ cpm / µg DNA

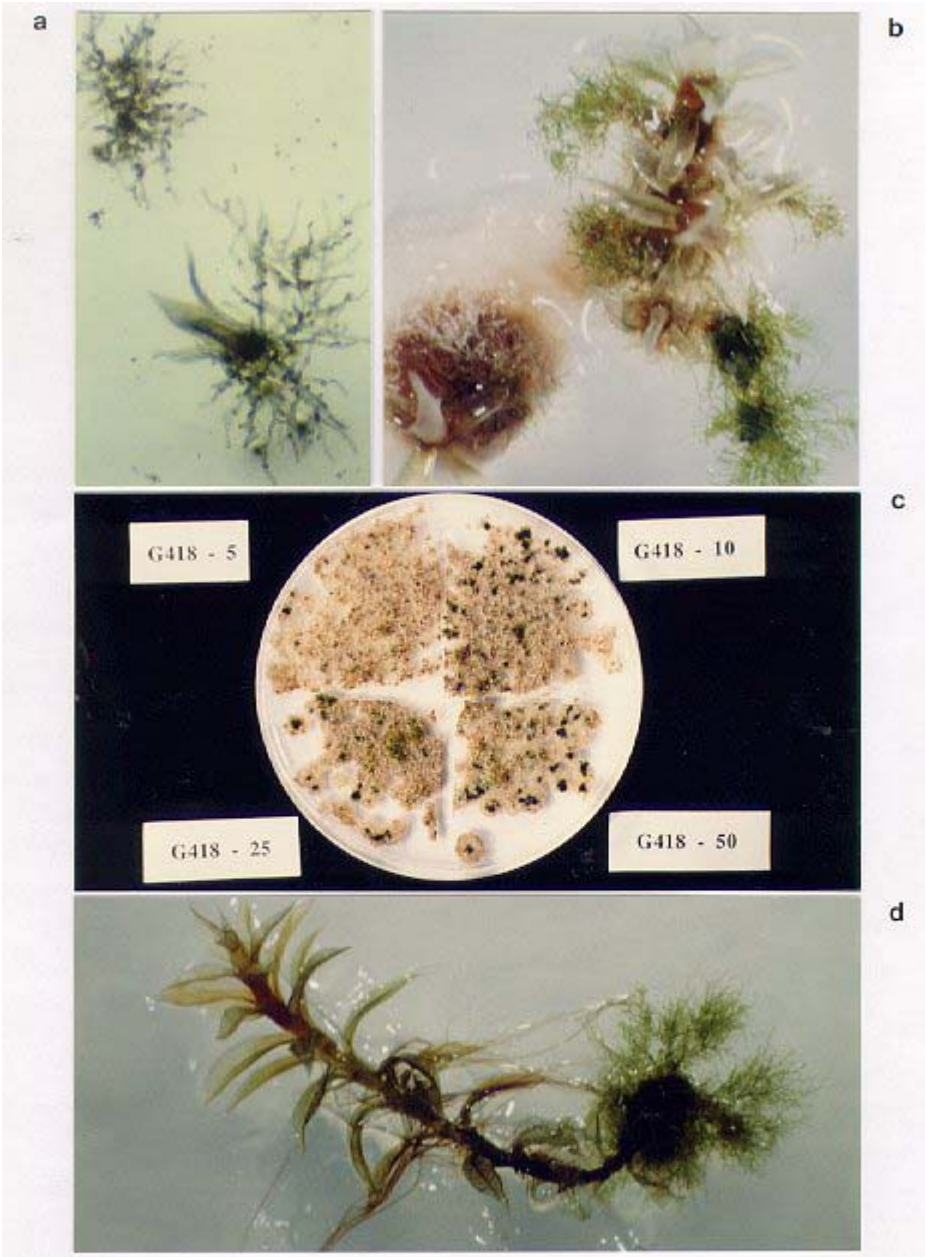
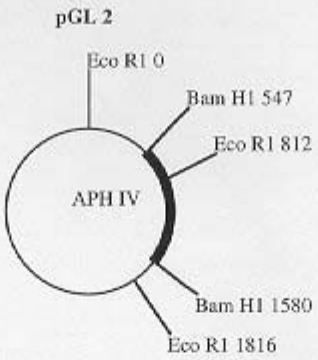


Figure 6. Phenotypical characteristics of class II unstable clones. (a) Early stage of regeneration characterised by numerous mitosis with restricted growth of the filaments and early differentiation of gametophores on Hg 25. (b) Strong mosaicism of a KanR colony Associated with bleaching of differentiated gametophores. (c) Population of KanR clones initially selected on increasing concentrations of G-418 for 10 days, transferred to non-selective medium for another 10 days, and transferred back to G-418 25. (d) Rescue experiment on a differentiated gametophore from a KanR clone. Regeneration of protonema is restricted to the filaments.



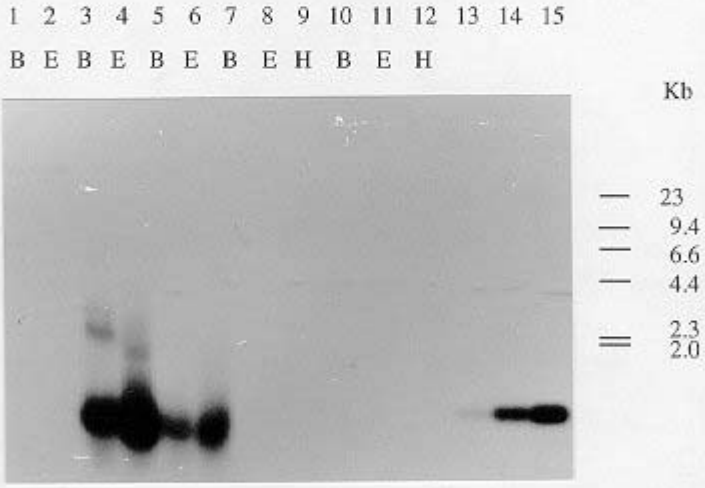
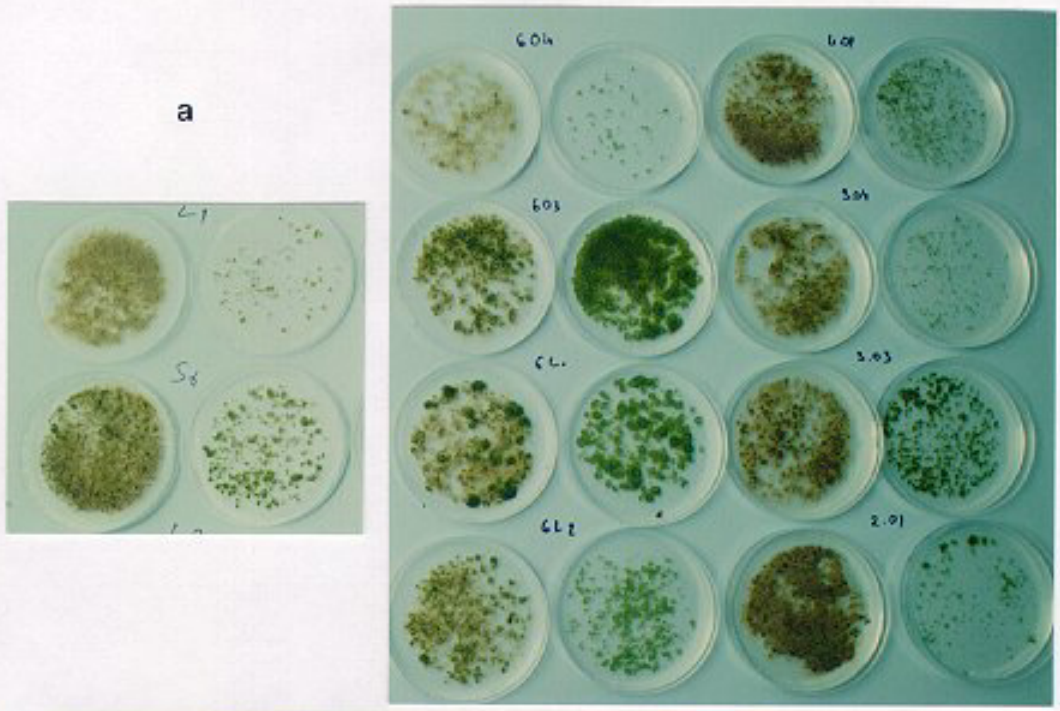


Figure 7 . Southern blot analysis of DNA extracted from Hg ^{R} clones SG1 and SG2 grown on selective and non-selective medium. Lanes 1 and 2: DNA from untransformed wild-type. Lanes 3 and 4: DNA from SG1 grown on selective medium. Lanes 5 and 6: DNA from SG2 grown on selective medium. Lanes 7, 8 and 9: DNA from SG1 grown on non selective medium. Lanes 10, 11 and 12: DNA from SG2 grown on non selective medium. Lanes 13, 14 and 15: genomic reconstruction with 1, 5 and 10 equivalent copies of the Bam H1 fragment of pGL 2. 2 µg of DNA were loaded in each lane. (B) Bam H1, (E) Eco R1, (H) Hind III digested DNA. Specific activity of the probe was 5·10 ^{8} cpm / µg DNA.

Figure 8. Phenotypical and genetic characteristics of class III resistant clones. (a) Non-selective to selective growth assay on 8 class III and 2 class II (6.04 and 3.04) Kan^R strains. Class III clones displayed a range of growth improvement on selective medium and a range of loss of the resistance upon non-selective to selective conditions as compared to class II (left plates NS/S growth, right plates selective growth). (b) Rescue experiment on differentiated gametophores form Kan^R ABD2 S6 showing 10 sensitive and two partially resistant gametophores. (c) Non mendelian transmission of the character in the offspring of KanR clone 12.02. Germination of spores in non-selective to selective conditions is shown on the left plate.



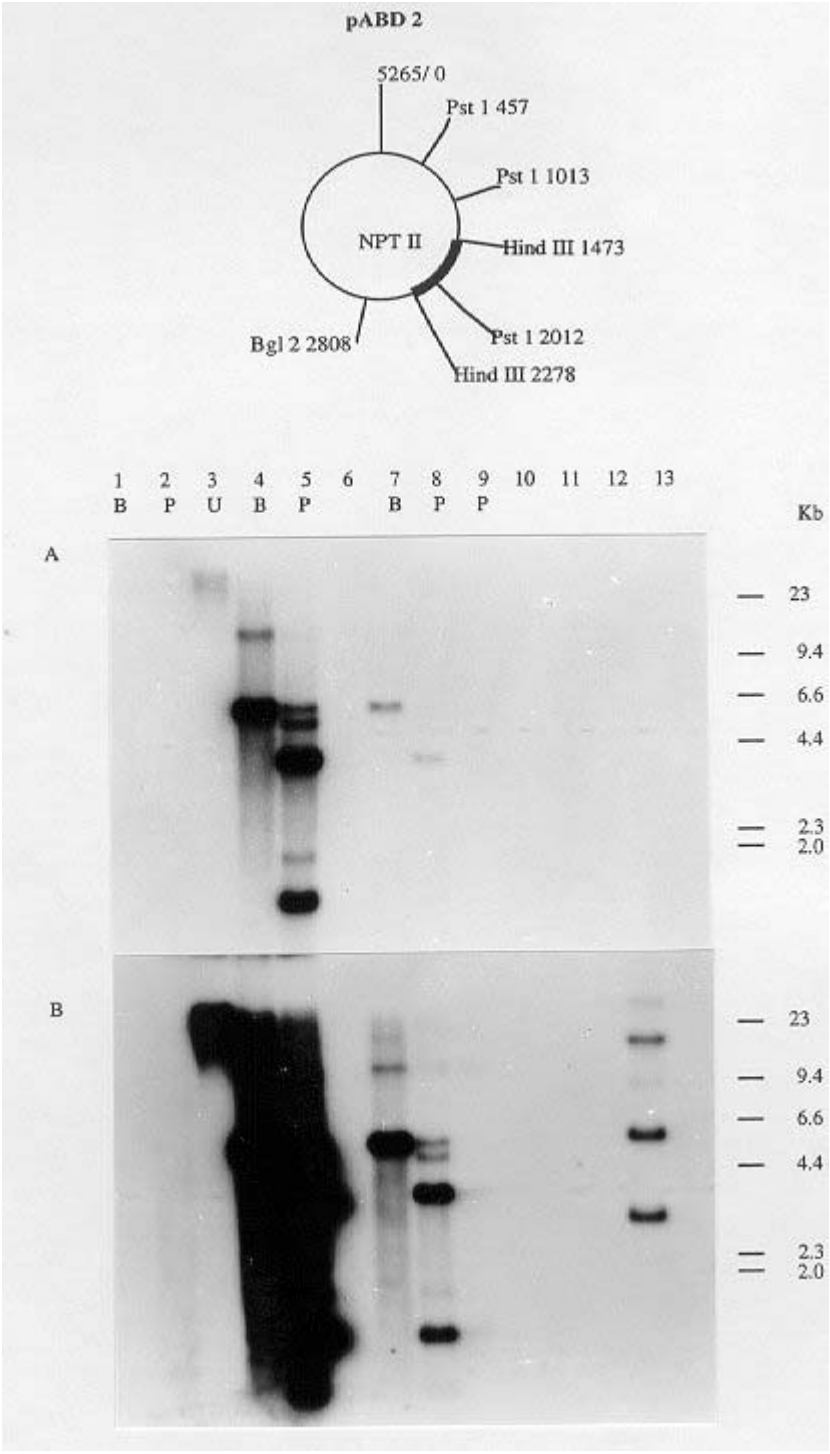
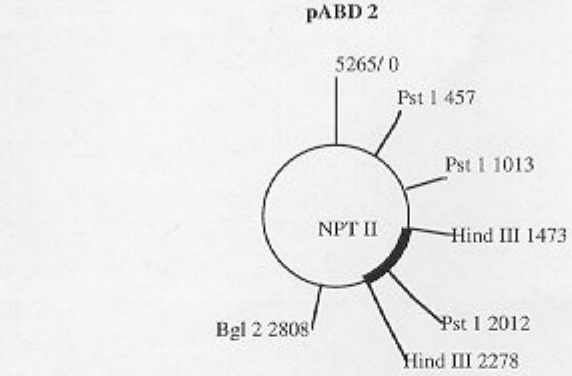
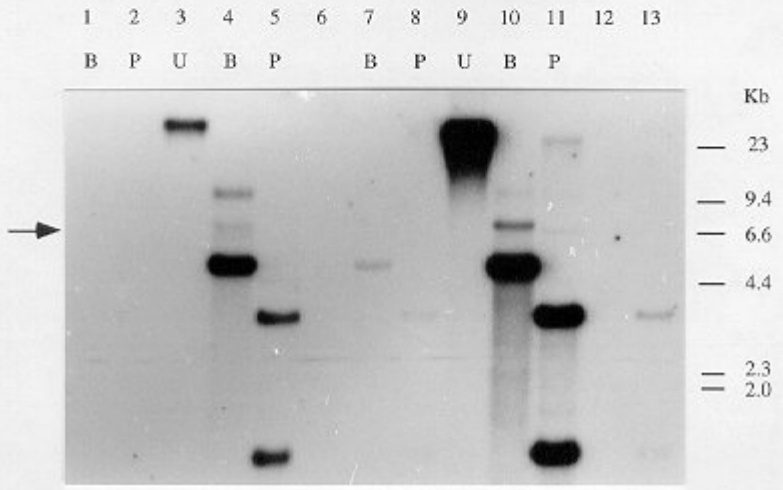

pABD 2
5265/ 0
Pst 1 457
Pst 1 1013
NPT II
Hind III 1473
Bgl 2 2808
Pst 1 2012
Hind III 2278
1 2 3 4 5 6 7 8 9 10 11 12 13
B P U B P B P P
Kb
A
23
9.4
6.6
4.4
2.3
2.0
B
23
9.4
6.6
4.4
2.3
2.0

Figure 10. Southern blot analysis of DNA extracted from clone Kan^R ABD2 L1 and its stable subclone ABD2 L1 Resc. Lanes 1 and 2: DNA from untransformed pabA3. Lanes 3, 4 and 5: DNA from protonemata1 tissue of ABD2 L1. Lanes 7 and 8: DNA extracted from differentiated gametophores of ABD2 L1. Lanes 9, 10 and 11: DNA extracted from differentiated gametophores of ABD2 L1 Resc. Lane 13: genomic reconstruction with 5 equivalent copies of Pst 1 digested pABD2. 2 µg of DNA were loaded in each lane. (U) undigested DNA, (B) Bgl 2, (P) Pst 1 digested DNA. The arrow indicate the 7.5 kb recombined signal. Specific activity of the probe was 2·10^8 cpm / µg DNA





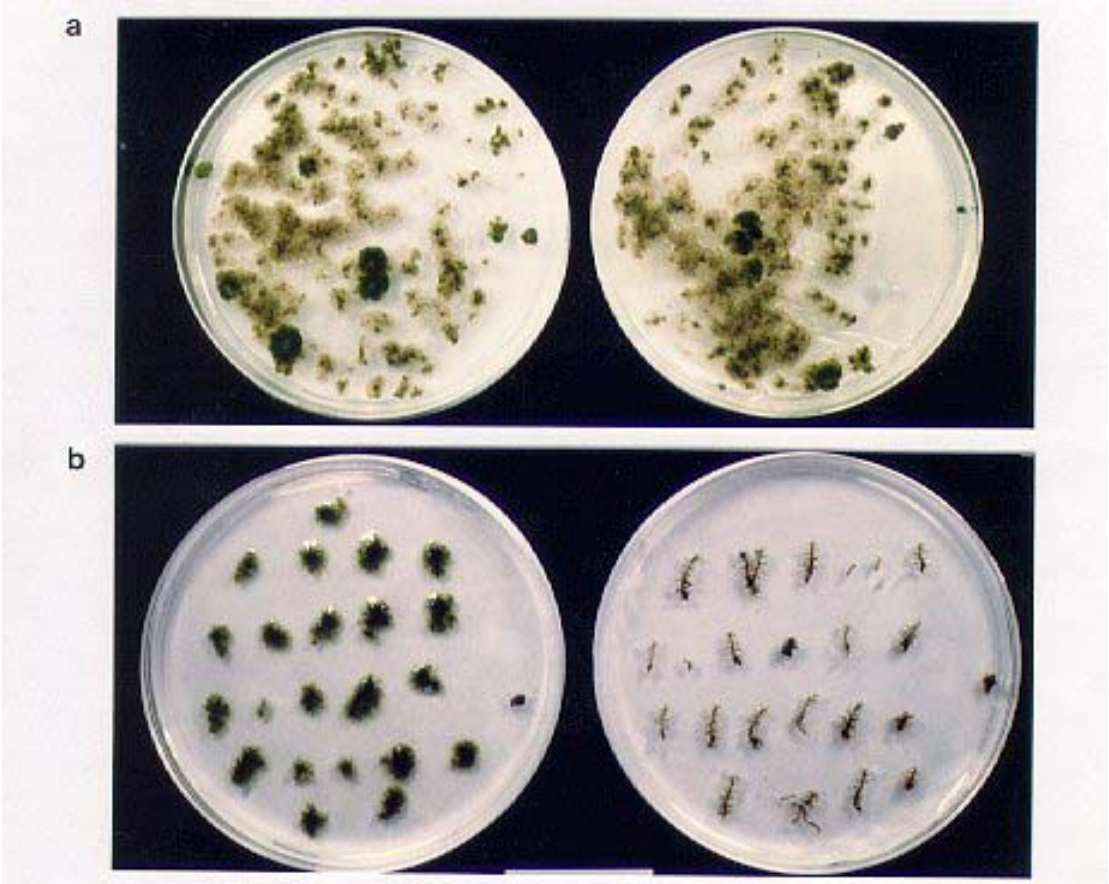


Figure 11. Fragmentation induced delayed integration in KanR clone pPCV-35S rolB. (a) Fast growing foci regenerating on selective medium. (b) Rescue experiment on differentiated gametophores isolated from fast (left) and slow growing (right) resistant colonies. All gametophores isolated from fast growing foci regenerated a protonema on selective medium, whereas occasional basal regeneration occured in gametophores isolated from slow growing colonies.

Figure 12. Transformation with supercoiled (left) and linear (right) YAC RC AB1. (a) Initial selection 3 weeks after transformation: one or two class I resistant clone can be identified following transformation with linear plasmid. (b) One and a half month after transformation, filamentous fast growing (class III) and globular slow growing (class II) resistant colonies can be seen on both plates. (c) Reduced growth rate of globular and fast growth rate of filamentous colonies plated on selective medium supplemented with 25 (left) or 50 (right) µg/ml G-418. (d) Rescue experiment showing basal regeneration of gametophores following transformation with supercoiled YAC and complete regeneration following transformation with linear YAC.

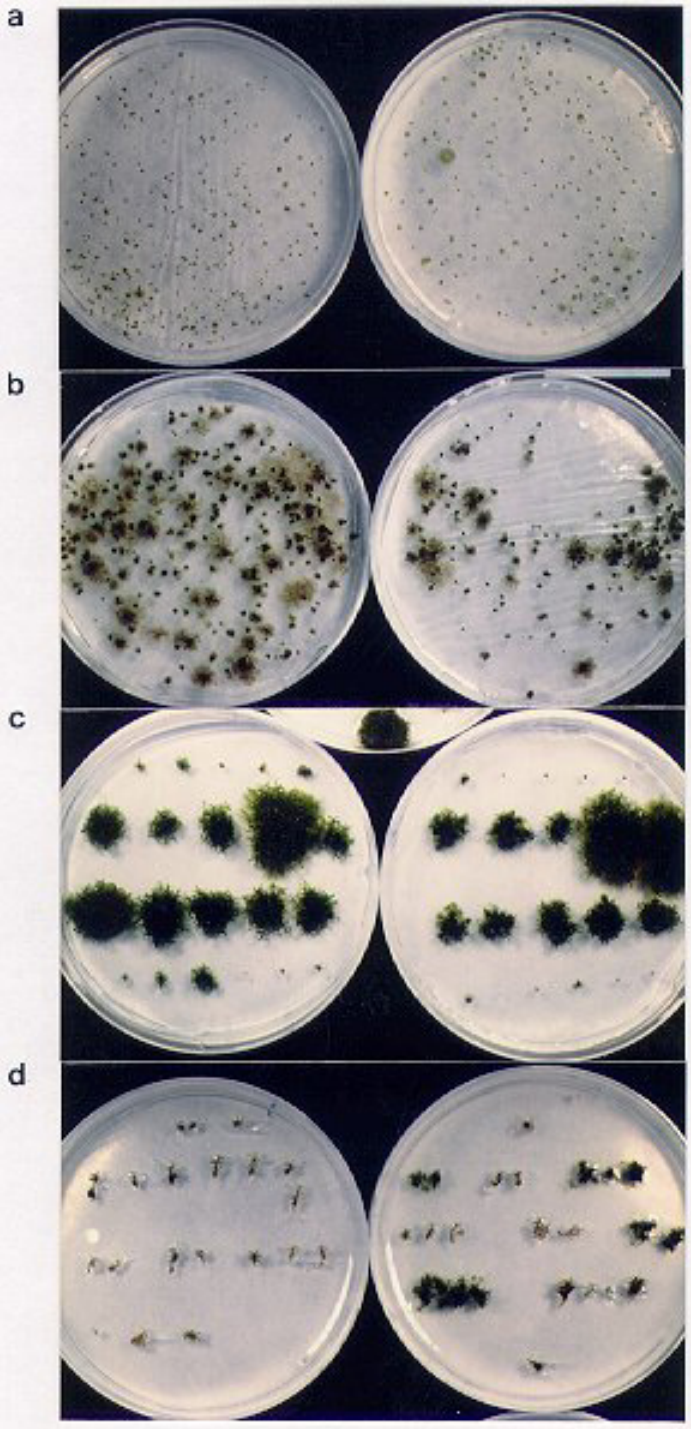
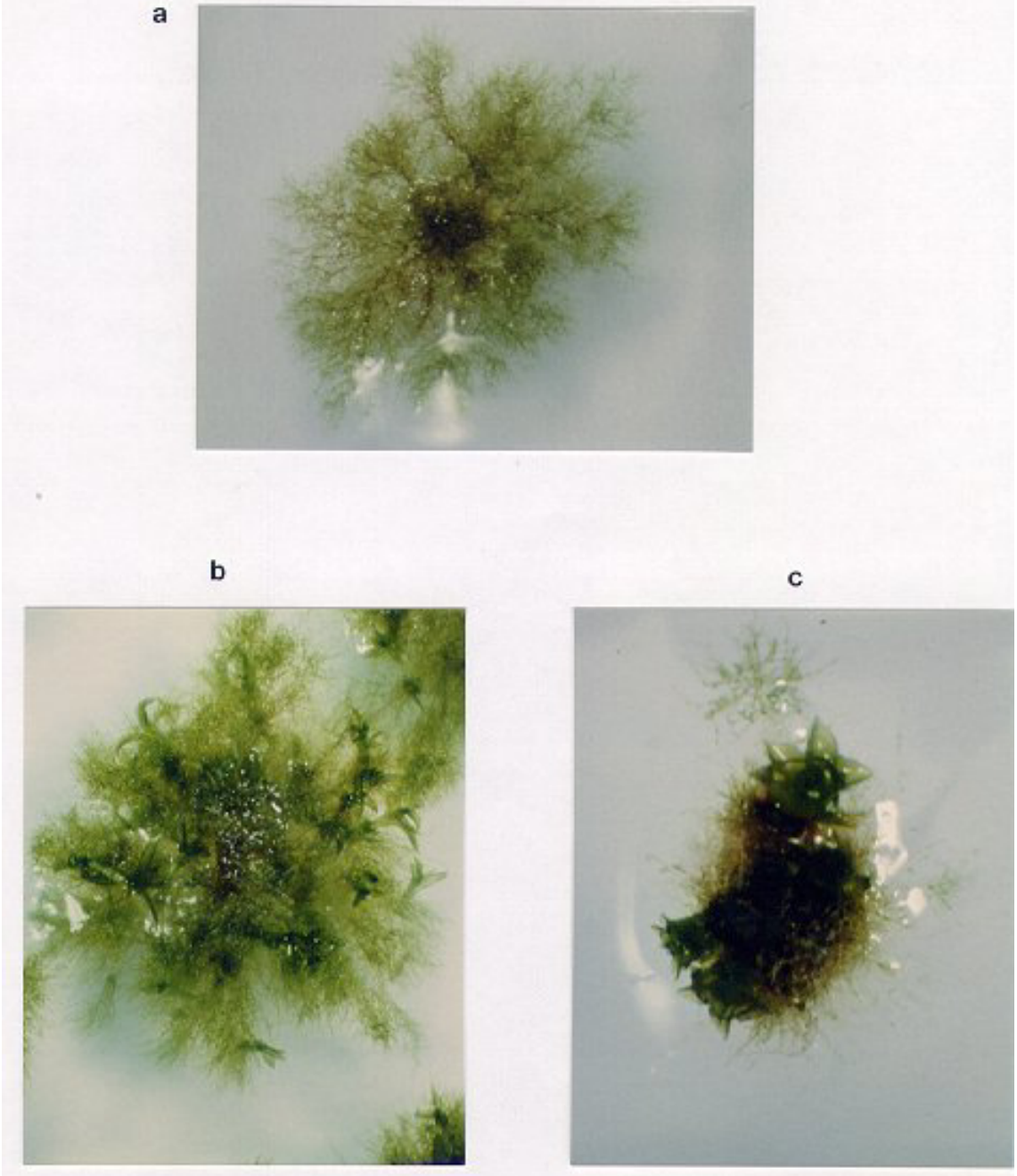


Figure 13. Class II transformants obtained following transformation with pPCV002 (a), pPCV002-rolA (b), and pPCV-35S-ipt (c). Increased differentiation of secondary chloronemata can be seen in the resistant clone carrying the rolA gene, whereas the resistant clone carrying the ipt gene displays a clear OVE phenotype.



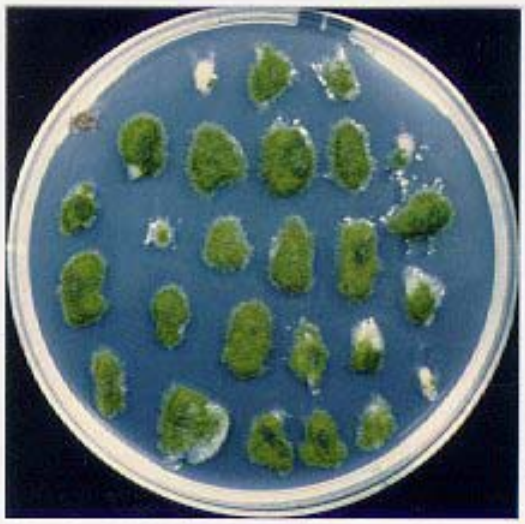


Figure 14. Mitotic instability of kanamycin resistance associated with mosaicism in the protonema of the moss C. purpureus, following transformation with plasmid pABD2.

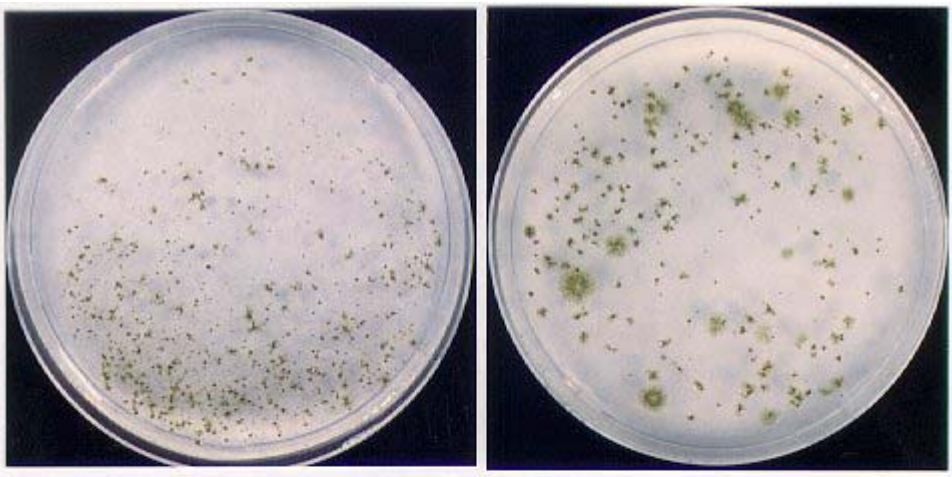


Figure 1. Initial selection of wt (left) and HP23 L2 (right) transformed with pGL 2. Pictures were taken 20 days after the initiation of selection. More than 10 stable clones are clearly visible on the HP23 L2 but no stable clone are seen on the wt plate.

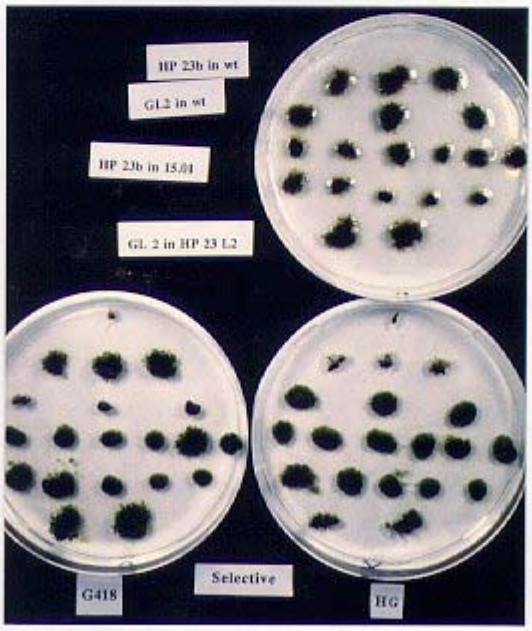


Figure 2. Unrestricted growth of transgenic strains form exp.1 growing on non-selective (top), G-418 (bottom left) or Hygromycin B (bottom right) supplemented media. Top row: HP23 b in wt (Kan^R Hg^S). Second row: GL 2 in wt (Kan^S Hg^R). Third and fourth rows: HP 23b in 15.01 (Kan^R Hg^R). Bottom row: GL 2 in HP23 L2 (Kan^R Hg^R).

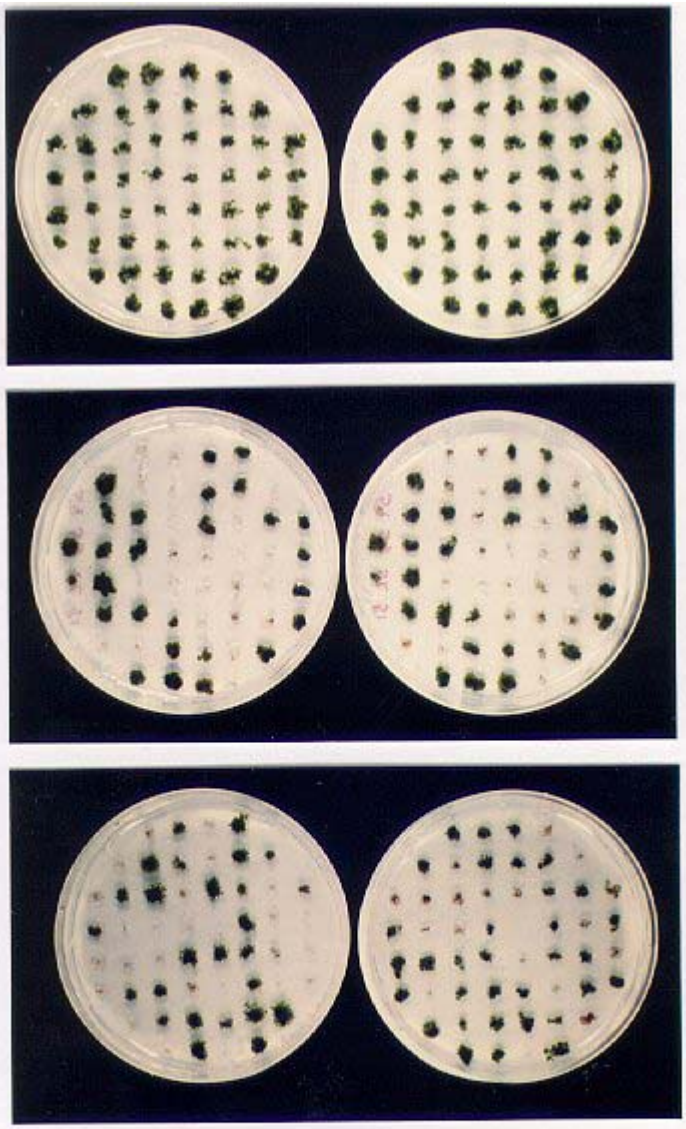


Figure 3. Single spore derived replicates were plated on G-418 (left) or Hygromycin B (right) supplemented media and grown for 15 days. From top to bottom: GH 1 selfed, GH 1 cross nicB5ylo6, and GH 3/2 cross nicB5ylo6. The kanamycin and hygromycin markers are linked in GH 1 and segregate independently in GH 3/2.

Table 1. Initial and stable RTF monitored in wt and transgenic P. patens in 2 independent gene targeting experiments. The initial number of protoplasts was 350 000 in exp. 1 and 300 000 in exp. 2. The increase factor (a) was calculated on a mean stable RTF in wild-type of 10^{-5}.

Strain	Plasmid	Surviving cells	%	Initial RTF %	Stable	Stable RTF ($\cdot 10^{-5}$)	Increase factor (a)	Phenotype	Selfed	Crossed (name)
Exp. 1										
wt	GL 2	86000	24.6	13.1	3	3.5	-	Kan^S Hg^R	-	-
wt	HP 23b	85000	24.3	12.9	3	3.5	-	Kan^R Hg^S	-	-
HP23 L2	GL 2	110000	31.4	3.9	58	52.7	52.7	Kan^R Hg^R	3	($M8_{(n)}$)
15.01	HP 23b	64000	18.3	8.1	8	12.5	12.5	Kan^R Hg^R	-	-
15.01	HP 23b	73000	20.1	2.1	5	6.9	6.9	Kan^R Hg^R	-	-
Exp. 2										
wt	GL 2	65000	21.7	3.1	0	-	-	-	-	-
wt	HP 23b	57000	19.0	14.4	0	-	-	-	-	-
HP23 L2	GL 2	100000	33.3	2.9	15	15.0	15.0	Kan^R Hg^R	3	1 (GH 7)
G 418-1	GL 2	60000	20.0	2.1	5	8.0	8.0	Kan^R Hg^R	1	1 (GH 6)
15.01	HP 23b	50000	16.7	24.1	49	98.0	98.0	Kan^R Hg^R	-	1 (GH 5)
Hg 1	HP 23b	71000	23.7	10.1	2	2.8	2.8	Kan^R Hg^R	-	-
Hg 2	HP 23b	53000	17.7	13.4	2	3.8	3.8	Kan^R Hg^R	1	1 (GH 1)
Hg 3	HP 23b	80000	26.7	12.5	4	5.0	5.0	Kan^R Hg^R	1	1 (GH 2)
Hg 27DI	HP 23b	113000	37.7	3.6	3	2.7	2.7	Kan^R Hg^R	2	2 (GH 3/1, GH 3/2)
TOTAL	wt	293000			6	2.0				
	Transgenics	769000			151	19.6	19.6			

Table 2. Segregation analysis of double-resistant transgenic clones crossed with the strain nicB5ylo6. Chi sqare values were determined on the total number of spores tested for each strain. Clone GH 5 was not successfully selfed, probably because it is a pabA3 derivative. Only one sporophyte was recovered on the ylo colony crossed with GH 7.

Strain		Total	Sensitive	Kan^R	Hg^R	Kan^R Hg^R	Double R %	CHI square	$P_{(3)}$	ylo in double R	% ylo in double R
GH 1	Cross 1	104	50	0	0	54	51.9			28	51.9
	Cross 2	103	47	0	0	56	54.4	0.82	0.84	27	48.2
	Self	104	0	0	0	104	100	-		0	-
GH 2	Cross 1	104	48	0	0	56	53.8			26	46.4
	Cross 2	103	49	0	0	54	52.4	0.82	0.84	24	44.4
	Self	104	0	0	0	104	100	-		0	-
GH 3/1	Cross 1	104	46	0	0	58	55.8			27	45.8
	Cross 2	104	45	0	0	59	56.7	3.25	0.37	27	44.3
	Self	104	0	0	0	104	100	-		0	-
GH 5	Cross 1	104	53	0	0	51	49.0			24	47.1
	Cross 2	104	57	0	0	47	45.2	0.69	0.87	20	44.4
	Self	NT	-	-	-	-	-	-		-	-
GH 6	Cross 1	68	38	0	0	30	44.1			NT	-
	Cross 2	104	50	0	0	54	51.9	0.76	0.86	25	46.3
	Self	104	0	0	0	104	100	-		0	-
GH 7	Cross 1	134	62	0	0	72	53.7	0.75	0.86	35	48.6
	Cross 2	NT	-	-	-	-	-	-		-	-
	Self	104	0	0	0	104	100	-		0	-
GH 3/2	Cross 1	104	26	22	26	30	28.8			NT	-
	Cross 2	102	18	25	31	28	27.5	2.89	0.42	NT	-
	Self	104	0	0	0	104	100	-		0	-