Molecular Tools to Study *Physcomitrella patens*

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Abstract: **The moss *Physcomitrella patens* has become a suitable model plant system for the analysis of diverse aspects of modern plant biology. The research strategies have been influenced by the implementation of state-of-the-art cell culture and molecular biology techniques. The forthcoming completion of the *Physcomitrella* genome sequencing project will generate many open questions, the examination of which will rely on a diverse set of molecular tools. Within this article, we intend to introduce the essential cell culture and molecular biology techniques which have been adopted in recent years to make *Physcomitrella* amenable to a wide range of genetic analyses. Many research groups have made valuable contributions to improve the methodology for the study of *Physcomitrella*. We would like to apologise to all colleagues whose important contributions could not be cited within this manuscript.**

Key words: **Cell culture, bioreactor, targeted knockout, sporophyte induction, transformation.**

Introduction

The non-vascular, multicellular land plant *Physcomitrella patens,* a member of the bryophyte family, was originally chosen as a model system to study developmental processes in plants (Cove and Knight, 1993; Lorenz et al., 2003; Sakakibara et al., 2003; Repp et al., 2004). The simple morphology of the moss has made such analyses easier and the studies have led to valuable contributions in the field. Recently, many research groups have initiated experimental studies on *Physcomitrella* which have been facilitated by the application of almost all modern molecular biology techniques in the moss system (Nishiyama et al., 2000; Hiwatashi et al., 2001; Egener et al., 2002; Bezanilla et al., 2003; Heintz et al., 2004; Sarnighausen et al., 2004). The establishment of these techniques has intensified the use of *Physcomitrella* in plant biology research. *Physcomitrella* combines several characteristic traits that provide advantages over other model plant systems. A number of these features also form the basis for considering *Physcomitrella* as a valuable production platform for molecular farming, i.e., the production of recombinant pharmaceutical proteins (Decker and Reski, 2004). *Physcomitrella* plants are amenable to *in vitro* plant tissue culture techniques and can be grown under axenic conditions on inorganic media devoid of any phytohormones or vitamins (Reski and Abel, 1985; Nishiyama et al., 2000). The plants are photoautotrophic and do not require any carbon source in the medium and can either be cultivated on solid medium or in liquid culture. Plant liquid cultures are usually composed of undifferentiated cells originating from plant callus tissue. These cells show high levels of somaclonal variation (Scowcroft et al., 1987), making them unfavorable for all applications requiring long-term cultivation against a stable genetic background. In contrast, liquid cultures of *Physcomitrella* consist of differentiated plants, preventing genetic variations, and thus providing stable conditions for any production processes performed in the moss system. One outstanding character of *Physcomitrella* is its high degree of homologous recombination observed in the nuclear DNA. First genetic evidence for homologous recombination in *Physcomitrella* was obtained by the transformation of a transgenic *Physcomitrella* line harbouring a plasmid containing a resistance marker gene with a second plasmid containing a different selection marker gene. Co-segregation and molecular analysis confirmed that the second plasmid was integrated at the site of insertion of the first plasmid by homologous recombination (Kammerer and Cove, 1996; Schaefer and Zrÿd, 1997). The rate of homologous recombination in *Physcomitrella* was found to be several orders of magnitude higher than in any other characterized plant species. Recently, Brucker et al. (2004) reported comparable rates of homologous recombination in another moss, *Ceratodon purpureus.* This unique feature allows precise manipulations of the genomic DNA by gene replacement using suitable gene disruption constructs. Experiments performed by several independent groups have shown that the targeted disruption of a genomic locus in *Physcomitrella* correlates with a mutant phenotype that reveals the biological function of the disrupted gene (Girke et al., 1998; Strepp et al., 1998; Girod et al., 1999; Imaizumi et al., 2002; Koprivova et al., 2002; Lorenz et al., 2003; Olsson et al., 2003; Koprivova et al., 2004; Mittmann et al., 2004). Another striking feature of *Physcomitrella* is the predominant haploid phase of its life cycle. Mosses undergo a heteromorphic *Generationswechsel,* the alternation of two generations which are distinct from each other in terms of nuclear DNA amounts and morphology (Fig. **1**). Starting from a germinating haploid spore, the gametophytic phase is initiated by

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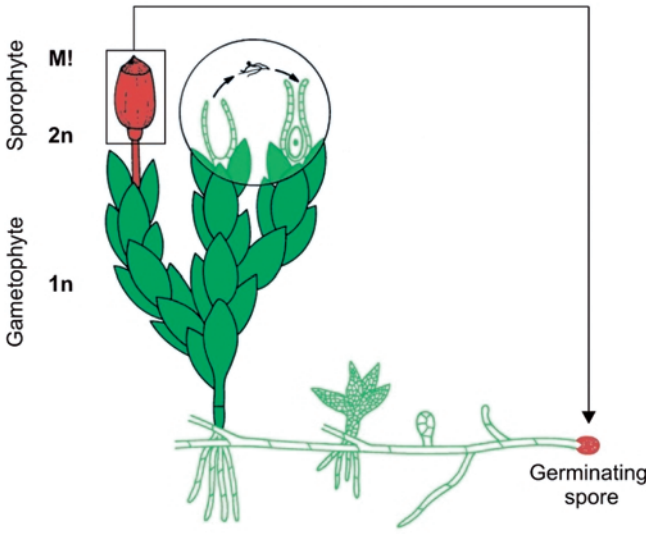

Fig. 1 The heteromorphic *Generationswechsel* of mosses. A haploid spore germinates and grows into the filamentous protonema cells. Starting with a three-faced apical cell, bud formation is initiated, which gives rise to the leafy adult gametophyte. In monoecious moss species, both sex organs (antheridia and archegonia) are present on one and the same plant. Fertilization of the egg cell takes place in the presence of water. From the fertilized egg the sporophyte grows out of the archegonium. The diploid sporophyte is highlighted by the surrounding rectangle. Within the spore capsule the cells undergo meiosis and new spores are formed.

the growth of a filamentous germ tube. The germ tube grows out to form protonema, which is sub-classified into chloronema and caulonema cells. Chloronema cells are characterized by a large number of chloroplasts and cell walls perpendicular to the growth axis, while caulonema cells contain less chloroplasts and have oblique cell walls. Further development proceeds by the formation of buds which are initially composed of a three-faced apical cell. This bud forms the initial meristem for development of the leafy adult gametophyte. The next developmental phase results in the formation of sex organs. As the sex organs emerge from the adult gametophyte, it is also termed a gametophore. The monoecious moss species *Physcomitrella* bears both male (antheridia) and female (archegonia) sex organs on one plant. Male gametes (spermatozoids) are produced within antheridia and female gametes (oogonia or egg cells) are produced in archegonia. Fertilization is achieved by swimming of spermatozoids through a surface water film and down to the neck of the archegonium, which normally contains one egg cell. The zygote develops into an embryo, which grows out to produce the diploid sporophyte. The two moss generations are physically connected because the sporophyte grows on top of the gametophyte. Within the spore capsule (sporangium), the diploid cells undergo meiosis and produce a large number of haploid spores, resulting in completion of the life cycle. For routine laboratory use of *Physcomitrella,* the plants do not have to pass through the complete life cycle because the moss can be propagated by vegetative growth under *in vitro* culture conditions. The predominant haploid phase, together with the high rate of homologous recombination, make *Physcomitrella* a most suitable system to initiate forward and reverse genetic approaches to study gene functions related to almost all aspects of plant biology. Furthermore, the gene disruption will not be counterbalanced by a second allele which results in immediately visible and genetically stable mutant phenotypes. The growing interest in *Physcomitrella* has led to the initiation of functional genomics projects, including EST sequencing. Over 100 000 public ESTs (Nishiyama et al., 2003) and additional 110 000 proprietary ESTs (Rensing et al., 2002) are available in different databases representing more than 95% of the *Physcomitrella* transcriptome. Enquiries for access to our proprietary EST data are particularly encouraged. Analysis of these data has already indicated their value in obtaining further information about the diversity of land plants. When comparing the codon usage of *Arabidopsis* and *Physcomitrella,* there is a clear tendency to equalize the fraction of used codons from the pool of possible codons. Analysis of gene families in *Physcomitrella* and *Arabidopsis* indicates that the average gene family in *Arabidopsis* seems to be nearly twice as big as in *Physcomitrella* (Rensing et al., 2002). The reduced gene redundancy found in *Physcomitrella* is another advantage for gene function analyses, as the disruption of a gene is more unlikely to be complemented by another member of the same gene family. Analysis of phenotypes of transgenic *Physcomitrella* lines which have been generated in large-scale forward genetics approaches has underlined the value of *Physcomitrella* for such functional studies. These experiments revealed a high rate of deviating mutant phenotypes, exceeding the number of altered phenotypes in comparable studies performed in *Arabidopsis* (Nishiyama et al., 2000; Bouche and Bouchez, 2001; Egener et al., 2002). In the following sections we will describe the procedures necessary for the preparation of plant material, *Physcomitrella* transformation, analysis of transformants, and generation of knockout plants. Additional data and detailed protocols are available from the following web-sites: www.plant-biotech.net; www.biology.wustl.edu/moss/; www2.unil.ch/lpc/docs/pdf/PPprotocols2001.pdf; www.nibb.ac.jp/~evodevo/.

Physcomitrella Cell Culture Conditions

Physcomitrella plants can be cultivated either on solid medium or in liquid culture (Fig. **2**). Upon cultivation on solid medium, the plants undergo normal developmental progression resulting in the formation of leafy gametophores. Starting from protonema tissue, gametophore development is initiated by the formation of buds consisting of a three-faced cell. Plant tissue cultures of gametophores can be maintained by sub-culturing the gametophores at monthly intervals. Liquid cultures can be started either from protonema tissue or gametophores, by inoculating liquid medium with the respective tissue. *Physcomitrella* shows a high capacity for regeneration and therefore mechanical disruption of the tissue leads to predominant growth of protonema tissue in liquid medium, providing a routine method for sub-culturing (Grimsley et al., 1977). Liquid cultures can be maintained in Erlenmeyer flasks in small volumes or plants can be grown in semi-continuous bioreactor cultures for large-scale production of moss material (Hohe and Reski, 2002). At any stage, moss material from liquid cultures can be used to set up cultures on solid medium. Protonema cultures may also be maintained by inoculating protonemal filaments onto cellophane sheets overlying solid medium. For routine use of *Physcomitrella* plants, it is recommended that plant cultures are kept on solid medium as a backup system or spores are kept which can be used to initiate new culture lines. Medium-term storage of *Physcomitrella* strains can be achieved

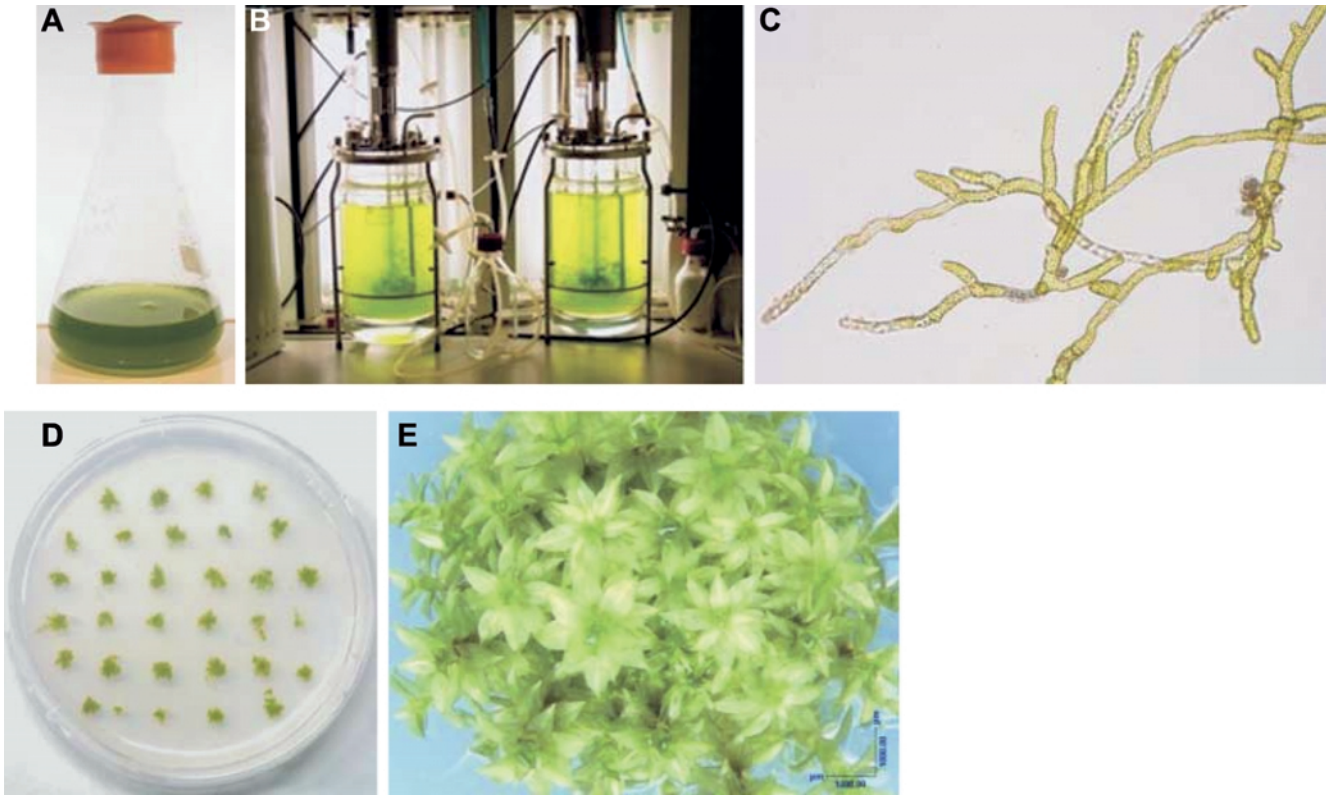

Fig. 2 Cell culture of *Physcomitrella* plants on solid and in liquid medium. (**A**) Small-scale liquid culture in a 500-ml Erlenmeyer flask. (**B**) Bioreactor cultures for highly standardized growth conditions and up-scaling purposes. (**C**) Protonema filaments representing the predominant tissue type in liquid cultures. (**D**) *Physcomitrella* plants grown on solid medium in a petri dish. (**E**) Close-up of a single plant grown on solid medium showing the leafy structures of the gametophores.

by cultivating plants on solid medium at low temperatures (4 – 15 °C) and low light conditions (20 µmol m^{-2} s^{-1}). Long-term storage of *Physcomitrella* strains was shown to be reliably working via cryopreservation (Schulte and Reski, 2004).

Small-Scale Cultivation of *Physcomitrella* Plants

Physcomitrella can be grown on agar-containing medium in Petri dishes on basal mineral media devoid of any organic compounds. The media used by different groups only vary in minor constituents. The two media which are most commonly used for standard growth of *Physcomitrella* plants are Knop medium (Reski and Abel, 1985) and BCD medium (Ashton and Cove, 1977). For the cultivation of metabolic or auxotrophic mutants, addition of supplements to the medium may be necessary. A medium enriched in this manner is described by Egener et al. (2002). *Physcomitrella* plants are grown axenically on these media. The plants are cultured in Petri dishes in a growth chamber at 25 ± 1 °C under a 16/8 h light/dark photoperiod with a light intensity of 50 – 70 µmol m^{-2} s^{-1}. Maintenance of the plants is achieved by monthly sub-culturing of the plant tissue onto fresh medium. For small-scale propagation of moss material, *Physcomitrella* plants can be cultivated in liquid medium in Erlenmeyer flasks (Fig. **2A**). When initiating a primary liquid culture, a small flask is inoculated with two to three *Physcomitrella* gametophores, followed by disruption of the plant material using a suitable blender. To promote fast growth of the moss material, 1 % (w/v) sucrose can be added. Cultivation of plants in the presence of sucrose for long time periods should be avoided as the cultures show deviating differentiation compared to growth in basal mineral medium. At regular disintegration of the protonema filaments these cultures can be kept as long-term suspension cultures.

Large-Scale Cultivation of *Physcomitrella* Plants in Bioreactors

Physcomitrella bioreactor cultures are useful for scale-up and to establish highly standardized growth conditions, as environmental parameters may affect growth kinetics, gene expression patterns, and differentiation. The cultivation of *Physcomitrella* in bioreactors has been reported previously (Reutter and Reski, 1996; Cove et al., 1997). For long-term cultivation of *Physcomitrella* on a laboratory scale, a semi-continuous bioreactor has been developed by Hohe et al. (2002 a).

Standard Knop medium is appropriate for the growth of *Physcomitrella* in bioreactors. The cultures are grown in stirred glass tank bioreactors (Fig. **2B**) with working volumes up to 12 l, equipped with a marine impeller. The cultures are aerated and grown at 25 °C under a photoperiod regime of 16/8 h (light/dark) with light supplied at the surface of the vessels. The cultures can be run semi-continuously, i.e., the suspension has to be harvested and replaced by an equal amount of fresh medium daily. The density of the cultures is controlled by determining the dry weight. For long-term cultivation of *Physco-*

mitrella suspension cultures, the cell density in the bioreactor should be maintained between approximately 150 mg/l and 200 mg/l dry weight. The growth rate of the suspension cultures can be increased by aeration with air that is enriched with CO_2 (Hohe et al., 2002 a). Under these conditions, the growth rate can be doubled but, under non-controlled pH conditions, the pH of the culture may decrease markedly. The growth rate of liquid cultures is not affected within a pH ranging from 4.5 to 7.0, while the development of protonemal filaments is influenced by other pH values. Generally, cultures grown at low pH mainly consist of chloronema filaments, while increased pH values favour caulonema development (Hohe et al., 2002 a). With respect to the standardized production of recombinant pharmaceutical proteins, a further upscaling of cultivation volumes was realized by Lucumi et al. (2003), who developed a tubular reactor with a pilot plant scale of 30L.

Induction of Sporophyte Development

Beside vegetative propagation of *Physcomitrella,* sexual reproduction can be induced under specific cell culture conditions. Engel (1968) had already reported the influence of temperature on sexual reproduction of *Physcomitrella.* Sporophyte formation was induced between 15 – 19 °C, whereas no sporophytes developed between 20 – 23 °C. Based on these results, the influence of light intensity, light/dark period, and temperature on development of gametangia and sporophytes was further investigated (Hohe et al., 2002 b). The highest numbers of spores were obtained upon culture of *Physcomitrella* plants at 15 °C, 8 h light, with an intensity of 20 µmol m^{-2} s^{-1}. The spores can be harvested and kept for long-term storage or they can immediately be plated on solid medium to initiate a new line. The initiation of new *Physcomitrella* lines may be necessary when decreasing qualities of used lines are observed (e.g., reduced protoplast yield).

To induce sporophyte development, gametophores are transferred onto supplemented medium (300 mg/l Fetrilon and 200 mg/l glucose) and grown at 15 °C under reduced light intensity and an 8/16 h (light/dark) regime for gametangia induction. After four to six weeks, the dishes are flooded with autoclaved tap water and cultivated for another six to eight weeks under the same growth conditions for development of mature spore capsules. The sporophytes are harvested and the spores can be released from the spore capsules by mechanical disruption of the capsules. To induce germination of the spores, they are transferred onto solid standard growth medium and grown under normal growth conditions. For long-term storage of up to several years, the spores are dried under sterile conditions and kept dry at 4 °C in the dark.

Transformation of *Physcomitrella* Protoplasts

For transformation of *Physcomitrella,* polyethylene glycol (PEG)-mediated transformation of protoplasts is almost exclusively applied (Schaefer et al., 1991), even though particle bombardment is also applicable to transform *Physcomitrella* (Sawahel et al., 1992; Cho et al., 1999; Bezanilla et al., 2003). Compared to the PEG-mediated transformation procedure, application of the biolistic method requires less DNA. Furthermore, it was shown that the transformation efficiency obtained by particle bombardment seems to be ten times higher when compared to PEG-mediated transformation of protoplasts (Cho et al., 1999). The transformation using particle bombardment is carried out by shooting the DNA onto protonemal tissue growing on cellophane. After the transformation, transgenic cells can be isolated by blending the tissue and subsequent plating onto selective medium (Cho et al., 1999). For PEG-mediated transformation, protoplasts are obtained by using the enzyme mixture Driselase for cell wall digestion of *Physcomitrella* protonema tissue (Grimsley et al., 1977). Protocols have been described, starting with plant material grown on solid medium using ammonium tartrate as a medium supplement (Ashton and Cove, 1977). The addition of ammonium tartrate to liquid culture medium results in predominant growth of moss protonema in the chloronema stage, which is thought to be the best source for protoplast isolation (Jenkins and Cove, 1983). The cell walls of subsequent developmental stages seem not to be sufficiently susceptible to cell wall degrading enzymes. Reduction of the calcium concentration or lowering the pH of the medium also result in increased protoplast yields (Rother et al., 1994; Hohe and Reski, 2002). In general, for protoplast preparation, *Physcomitrella* liquid cell cultures are commonly used.

The DNA constructs used for generation of transgenic *Physcomitrella* lines should contain a selection marker cassette to allow an easy screening procedure to be used for identification of plants that have undergone integration of the DNA construct. The most commonly used selection marker comprises the *nptII* gene encoding the enzyme neomycin phosphotransferase which confers resistance to geneticin (G418). Alternatively, the *aph* (aminoglycoside phosphotransferase) gene conferring resistance to kanamycin, the *hph* (hygromycin phosphotransferase) gene mediating hygromycin resistance or the *ble* (phleomycin-binding protein) gene conferring resistance to zeomycin may be used (Schaefer et al., 1991; Imaizumi et al., 2002; Kasahara et al., 2004; Mittmann et al., 2004). To drive expression, the resistance genes are usually flanked by the promoter and terminator sequences of cauliflower mosaic virus (CaMV) *35S RNA* or the *nopalin synthase (nos)* gene from *Agrobacterium tumefaciens.* The 35S-driven selection marker cassette can be subcloned from the pRT99 plasmid vector (Topfer et al., 1988). Besides these viral and bacterial promoters, plant-derived promoter regions were shown to be suitable for driving expression of transgenes in *Physcomitrella.* These include heterologous promoters of the rice actin 1 and maize ubiquitin genes, as well as homologous promoters derived from *Physcomitrella* alpha-1,3-fucosyltransferase, beta-1,2-xylosyltransferase, and beta-tubulin genes (Bezanilla et al., 2003; Horstmann et al., 2004; Jost et al., 2005). Before transformation of *Physcomitrella* protoplasts it is recommended to linearize the DNA construct using appropriate restriction enzymes. The transformation of circular plasmids may result in extrachromosomal replication and the generation of unstable transformants (Ashton et al., 2000).

For large-scale protoplast isolation, the use of pH-controlled bioreactor cultivation is most suitable (Hohe and Reski, 2002). Alternatively, plant material grown under standard growth conditions in Erlenmeyer flasks containing Knop medium with reduced calcium concentrations (1/10 $Ca[NO_3]_2$) or reduced pH (4.5) can be used for protoplast isolation. When starting with approximately 200 ml of these cultures, protoplast yield will be sufficient for at least ten independent trans-

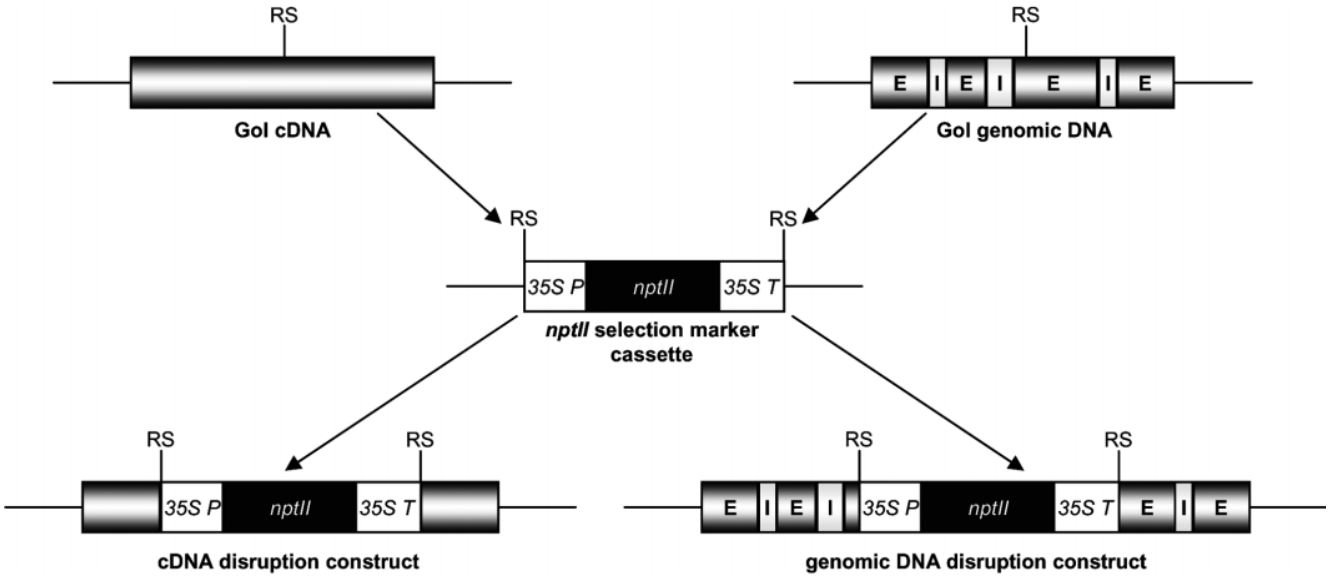

Fig. 3 Generation of targeted knockout constructs. Gene disruption constructs for the generation of targeted knockout plants can either be obtained by insertion of a selection marker cassette (e.g., the *nptII* cassette) into cDNA sequences or by insertion into genomic DNA stretches of the gene of interest (GoI). By choosing a suitable restriction site (RS), the marker cassette is cloned into the cDNA or genomic sequence. The orientation of the *nptII* gene should comply with the orientation of the target gene. As well as using a single restriction site for cloning of the marker cassette, two restriction sites can be chosen for integration of the cassette into the gene of interest. This will result in deletion of a short stretch of cDNA or genomic DNA (not shown). 35S P: CaMV 35S promoter; *nptII:* neomycin phosphotransferase gene; 35S T: CaMV 35S terminator; E: exon; I: intron. The selection marker cassette is not drawn to scale.

formations. The moss material is harvested by filtration through a 100-µm protoplast sieve. Digestion of the cell walls is carried out using 2% Driselase enzyme mixture dissolved in 0.5 M mannitol. The digested moss material is successively passed through sieves with a mesh size of 100 and 45 µm and carefully washed once in 0.5 M mannitol. The transformation procedure can be performed as described by Strepp et al. (1998). After transformation, the protoplasts are cultivated in Knop medium supplemented with 3% mannitol and 5% glucose and grown under reduced light conditions or in the dark for 12 – 16 h followed by cultivation in the same medium for ten days under normal growth conditions. During this time, regeneration of protoplasts is initiated. Afterwards, the regenerating protoplasts are plated onto Petri dishes containing solidified Knop medium. The medium should be covered with a sterile cellophane sheet to facilitate the transfer of regenerating plants at subsequent stages. To select stably transformed plants, the cellophane with the cultures is transferred onto solid medium containing the appropriate antibiotic for two weeks, followed by a two-week release period on medium without antibiotic and a second selection period of two weeks. Plants surviving the second round of selection are considered to be stable transformants. Detailed analysis of those plants by PCR has revealed that more than 98% had stably integrated the transgene into the genome (Schween et al., 2002).

Targeted Knockout in *Physcomitrella*

The high efficiency of homologous recombination found in *Physcomitrella* allows manipulation of the nuclear genome on the basis of single gene knockouts. The targeted knockout of a particular gene is achieved by transformation of *Physcomitrella* protoplasts using DNA fragments harbouring the gene of interest which has been disrupted by the integration of a selection marker cassette. For the generation of these knockout constructs, either cDNA fragments or stretches of genomic DNA can be used. The efficiency of homologous recombination events may, in some cases, be higher using constructs based on genomic sequences because unfavorable exon-intron structures may interfere with the integration of cDNA fragments. As mentioned above, the preparation of knockout constructs can be performed using cDNA or genomic DNA stretches. The approach for the generation of gene disruption constructs is depicted in Fig. **3**. Based on the sequence information, suitable restriction sites have to be identified for the integration of the selection marker cassette. The site of integration should be located approximately in the central region of the gene of interest, creating flanking regions which are roughly of similar size. When using genomic DNA, the selection marker cassette has to be cloned into an exon sequence to ensure the functional deletion of the gene after the mRNA processing events. It is possible to integrate the cassette into a single restriction site present in the sequence, however, the deletion of short stretches using two restriction enzymes for sub-cloning also leads to reliable results. The regions flanking the integrated selection marker cassette should preferably be more than 300 bp in size since increased recombination efficiencies were observed with larger constructs. The orientation of the cassette should conform with the orientation of the respective target gene. Before transformation, it is recommended to release the knockout construct from the vector backbone since transformation with linear constructs results in higher yields of stable transformants compared to transformations using supercoiled DNA (Schaefer et al., 1991; Hohe et al., 2004). The knockout construct can be transferred into *Physcomitrella* protoplasts following the standard transformation protocol described above.

When using multiple knockout constructs for the transformation of *Physcomitrella* protoplasts, it is also possible to generate double and triple targeted knockout lines (Hohe et al., 2004). This may be helpful when redundant genes have to be analyzed.

Analysis of Transformants

After the regeneration of protoplasts and two rounds of selection on antibiotic-containing medium, the transformants can be analyzed for stable integration of the transgene by PCR-based methods. Routinely, a first PCR screen of the transformants is based on detection of stable integration products of the selection marker gene. To verify the integration of constructs containing an *nptII* selection marker cassette, we have developed a one-step protocol to isolate genomic DNA from a large number of transformants which can be used for PCR analyses (Schween et al., 2002). Genomic DNA can be isolated from 1 – 5 mg of regenerated plant tissue by heating the samples at a moderate temperature in a detergent-containing extraction buffer. Aliquots of the resulting extract can be immediately used for PCR analysis without further purification. Beside the detection of the selection marker gene, further analysis of the plants is dependent on the experimental approach. Transformants which have been regenerated from transformations using gene disruption constructs have to be screened for homologous integration of the construct into the gene of interest (Fig. **4**). To verify integration of the disruption construct, primers are derived from sequences of the selection marker cassette and from the gene of interest. The latter have to be derived from a region of the gene which was not part of the disruption construct. When using these primer combinations, PCR products are only obtained when the disruption construct was integrated at the genomic locus of interest by means of homologous recombination. Furthermore, primers derived from the gene of interest flanking the selection marker cassette can be used to screen the transformants for disruption of the genomic wild-type locus. Using these primers, the PCR products will either correspond to the predicted size of the genomic region, indicating the wild-type locus, or the size of the PCR product will be shifted according to the size of the inserted selection marker cassette, indicating a homologous recombination event. For further analyses, e.g., Southern-blot analyses, genomic DNA can be prepared following a detailed protocol described previously (Schlink and Reski, 2002).

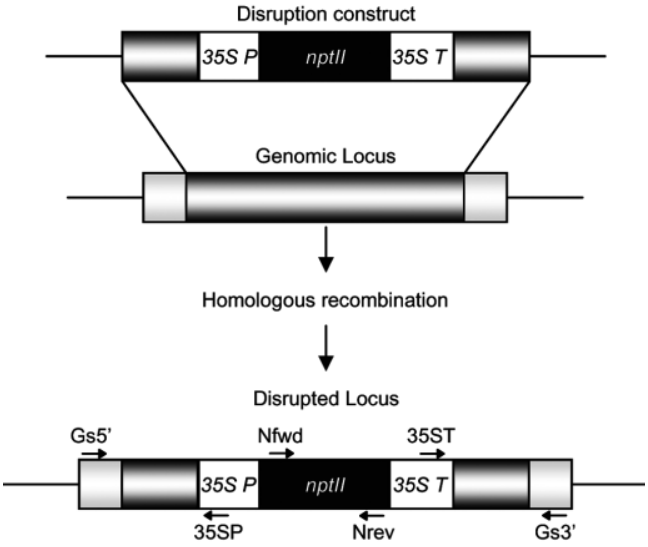

Fig. 4 PCR-based analysis of putative *Physcomitrella* knockout plants. A first PCR is carried out to confirm integration of the selection marker gene, e.g., the *nptII* gene (primers Nfwd and Nrev). To detect homologous recombination and integration at the correct genomic locus, primer combinations are used which consist of primers derived from the selection cassette (35S P and 35S T) and gene-specific primers (Gs5′ and Gs3′) that have not been present in the disruption construct. For detection of correct 5′ integration, the combination Gs5′ and 35S P is used, whereas the combination 35S T and Gs3′ confirms integration of the disruption construct at the 3′ site of the gene. Furthermore, the primer combination Gs5′ and Gs3′ can be used to distinguish between the wild-type and disrupted locus.

Concluding Remarks

The moss *Physcomitrella patens* has become a versatile model system to investigate various questions in plant biology. Beside the high rate of homologous recombination, *Physcomitrella* displays further characteristic features making it a suitable system for plant research. The predominant haploid phase of its life cycle and simple morphological organization simplify analysis of fundamental biological problems. Furthermore, *Physcomitrella* may contribute to improve our knowledge of plant evolution as it belongs to the group of plant species that first colonized dry land. The increasing recognition of *Physcomitrella* as a model plant system is reflected by the initiation of the *Physcomitrella* genome sequencing project in 2004. The first assembly of the genome sequence, which is expected to be released in 2005, will raise a set of challenging questions. The main intention of this manuscript is to provide an overview of present *Physcomitrella* research tools and to encourage researchers not yet working in the field to make use of *Physcomitrella* as an alternative model plant system. The overview given in this manuscript should help to set up transgenic lines for a multitude of scientific questions, as well as biotechnological applications.

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