Introduction

The non-vascular, multicellular land plant *Physcomitrella patens*, a member of the bryophytes, was originally chosen as a model system for the 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 alleviated such analyses and the studies led to valuable contributions in the field. Recently many research groups initiated experimental studies in *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 intensified the use of *Physcomitrella* in plant biology research. *Physcomitrella* combines several characteristic traits making it an advantageous system compared to other plant model systems. A number of these features are also 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 dedifferentiated 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, thus allowing 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 harboring a plasmid containing a resistance marker gene with a second plasmid containing a different selection marker gene. Co-segregation analysis 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 Zryd, 1997). The rate of homologous recombination in *Physcomitrella* is found to be several orders of magnitudes 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 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 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 the 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 gametophore. The monoecious moss species *Physcomitrella* bears both the 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. The 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 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 the 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 genetics 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 lead 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 to access our proprietary EST data are particularly encouraged. The analysis of this data already indicated its value to obtain 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 out of the pool of possible codons. The analysis of gene families in *Physcomitrella* and *Arabidopsis* indicate 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. The analysis of phenotypes of transgenic *Physcomitrella* lines which have been generated in large-scale forward genetics approaches 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 protocols provided we will describe the procedures necessary for the preparation of plant material, *Physcomitrella* transformation, the analyses of transformants and the generation of knockout plants.

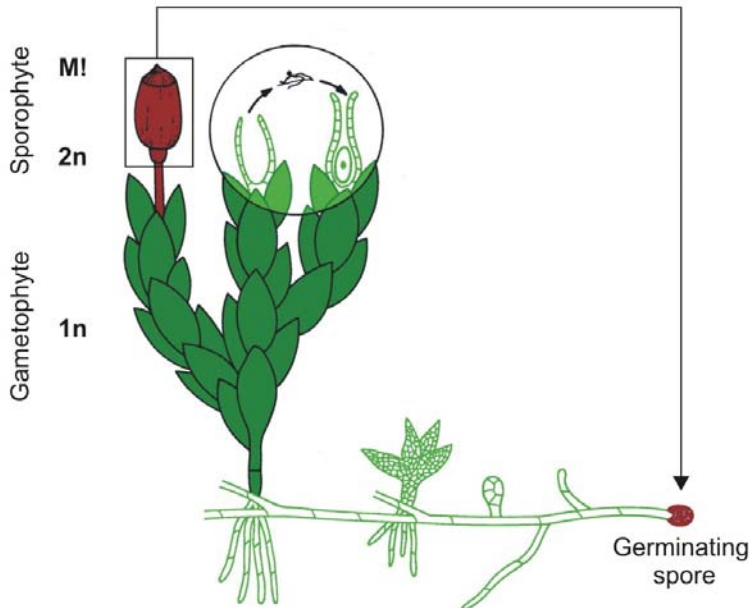

Figure 1

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 archegonia. The diploid sporophyte is highlighted by the surrounding rectangle. Within the spore capsule the cells undergo meiosis and new spores are formed.

References

Bezanilla, M., Pan, A., and Quatrano, R. S. (2003) RNA interference in the moss *Physcomitrella patens*. Plant Physiology 133, 470-474.

Bouche, N., and Bouchez, D. (2001) *Arabidopsis* gene knockout: phenotypes wanted. Current Opinion in Plant Biology 4, 111-117.

Brucker, G., Mittmann, F., Hartmann, E., and Lamparter, T. (2004) Targeted site-directed mutagenesis of a heme oxygenase locus by gene replacement in the moss *Ceratodon purpureus*. Planta published online, DOI:10.1007/s00425-00004-01411-00426.

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

Decker, E. L., and Reski, R. (2004) The moss bioreactor. Current Opinion in Plant Biology 7, 166-170.

Egener, T., Granado, J., Guitton, M. C., Hohe, A., Holtorf, H., Lucht, J. M., Rensing, S. A., Schlink, K., Schulte, J., Schween, G., Zimmermann, S., Duwenig, E., Rak, B., and Reski, R. (2002) High frequency of phenotypic deviations in *Physcomitrella patens* plants transformed with a gene-disruption library. BMC Plant Biology 2, 6.

Girke, T., Schmidt, H., Zahringer, U., Reski, R., and Heinz, E. (1998) Identification of a novel delta 6-acyl-group desaturase by targeted gene disruption in *Physcomitrella patens*. Plant Journal 15, 39-48.

Girod, P. A., Fu, H., Zryd, J. P., and Vierstra, R. D. (1999) Multiubiquitin chain binding subunit MCB1 (RPN10) of the 26S proteasome is essential for developmental progression in *Physcomitrella patens*. Plant Cell 11, 1457-1472.

Heintz, D., Wurtz, V., High, A. A., Van Dorsselaer, A., Reski, R., and Sarnighausen, E. (2004) An efficient protocol for the identification of protein phosphorylation in a seedless plant, sensitive enough to detect members of signalling cascades. Electrophoresis 25, 1149-1159.

Hiwatashi, Y., Nishiyama, T., Fujita, T., and Hasebe, M. (2001) Establishment of gene-trap and enhancer-trap systems in the moss *Physcomitrella patens*. Plant Journal 28, 105-116.

Imaizumi, T., Kadota, A., Hasebe, M., and Wada, M. (2002) Cryptochrome light signals control development to suppress auxin sensitivity in the moss *Physcomitrella patens*. Plant Cell 14, 373-386.

Kammerer, W., and Cove, D. J. (1996) Genetic analysis of the effects of re-transformation of transgenic lines of the moss *Physcomitrella patens*. Molecular and General Genetics 250, 380-382.

Koprivova, A., Meyer, A. J., Schween, G., Herschbach, C., Reski, R., and Kopriva, S. (2002) Functional knockout of the adenosine 5'-phosphosulfate reductase gene in *Physcomitrella patens* revives an old route of sulfate assimilation. Journal of Biological Chemistry 277, 32195-32201.

Koprivova, A., Stemmer, C., Altmann, F., Hoffmann, A., Kopriva, S., Gorr, G., Reski, R., and Decker, E. L. (2004) Targeted knockouts of *Physcomitrella* lacking plant-specific immunogenic N-glycans. Plant Biotechnology Journal 2, 517-523.

Lorenz, S., Tintelnot, S., Reski, R., and Decker, E. L. (2003) Cyclin D-knockout uncouples developmental progression from sugar availability. Plant Molecular Biology 53, 227-236.

Mittmann, F., Brucker, G., Zeidler, M., Repp, A., Abts, T., Hartmann, E., and Hughes, J. (2004) Targeted knockout in *Physcomitrella* reveals direct actions of phytochrome in the cytoplasm. Proceedings of the National Academy of Sciences of the USA 101, 13939-13944.

Nishiyama, T., Fujita, T., Shin, I. T., Seki, M., Nishide, H., Uchiyama, I., Kamiya, A., Carninci, P., Hayashizaki, Y., Shinozaki, K., Kohara, Y., and Hasebe, M. (2003) Comparative genomics of *Physcomitrella patens* gametophytic transcriptome and *Arabidopsis thaliana*: implication for land plant evolution. Proceedings of the National Academy of Sciences of the USA 100, 8007-8012.

Nishiyama, T., Hiwatashi, Y., Sakakibara, I., Kato, M., and Hasebe, M. (2000) Tagged mutagenesis and gene-trap in the moss, *Physcomitrella patens* by shuttle mutagenesis. DNA Research 7, 9-17.

Olsson, T., Thelander, M., and Ronne, H. (2003) A novel type of chloroplast stromal hexokinase is the major glucose-phosphorylating enzyme in the moss *Physcomitrella patens*. Journal of Biological Chemistry 278, 44439-44447.

Rensing, S. A., Rombauts, S., Van de Peer, Y., and Reski, R. (2002) Moss transcriptome and beyond. Trends in Plant Science 7, 535-538.

Repp, A., Mikami, K., Mittmann, F., and Hartmann, E. (2004) Phosphoinositide-specific phospholipase C is involved in cytokinin and gravity responses in the moss *Physcomitrella patens*. Plant Journal 40, 250-259.

Reski, R., and Abel, W. O. (1985) Induction of budding on chloronemata and caulonemata of the moss, *Physcomitrella patens*, using isopentenyladenine. Planta 165, 354-358.

Sakakibara, K., Nishiyama, T., Sumikawa, N., Kofuji, R., Murata, T., and Hasebe, M. (2003) Involvement of auxin and a homeodomain-leucine zipper I gene in rhizoid development of the moss *Physcomitrella patens*. Development 130, 4835-4846.

Sarnighausen, E., Wurtz, V., Heintz, D., Van Dorsselaer, A., and Reski, R. (2004) Mapping of the *Physcomitrella* patens proteome. Phytochemistry 65, 1589-1607.

Schaefer, D. G., and Zryd, J. P. (1997) Efficient gene targeting in the moss *Physcomitrella patens*. Plant Journal 11, 1195-1206.

Scowcroft, W. R., Brettell, R. I. S., Ryan, S. A., Davies, P. A., and Pallotta, M. A. (1987) Somaclonal variation and genomic flux. In Plant Tissue and Cell Culture. (Green, C. E., Somers, D. A., Hackett, W. P. and Biesboer, D. D. ed.), New York: Alan R. Liss Inc., pp. 275-286.

Strepp, R., Scholz, S., Kruse, S., Speth, V., and Reski, R. (1998) Plant nuclear gene knockout reveals a role in plastid division for the homolog of the bacterial cell division protein FtsZ, an ancestral tubulin. Proceedings of the National Academy of Sciences of the USA 95, 4368-4373.