Additional Papers

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Moss (*Physcomitrella patens*) functional genomics — Gene discovery and tool development, with implications for crop plants and human health

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Abstract

Recently, the moss *Physcomitrella patens* was established as a versatile tool in plant functional genomics. Mosses represent the oldest living clade of land plants, separated by approximately 450 million years of evolution from crop plants. Consequently, mosses contain metabolites and genes not known from these seed plants. In *Physcomitrella*, nuclear genes can be targeted by homologous recombination as efficiently as in yeast, allowing reverse genetics approaches in plants at high-throughput levels for the first time. Comprehensive expressed sequence tag databases gave new insights into the levels of diversity in land plants which are now ready to be exploited in plant biotechnology. In forward genetics screens, saturated tagged mutant collections help to unravel novel gene – function relationships. Additionally, proteomics tools are at hand to analyse subcellular proteomes, as well as the phosphoproteome, as the core of eukaryotic signal transduction. Moreover, specifically designed *Physcomitrella* strains can produce human therapeutic proteins safely and cost-effectively in bioreactors.

Keywords: *bioinformatics, bioreactor, EST database, gene knockout, homologous recombination, molecular farming, mutant collection, plant functional genomics, proteomics, systems biology*

INTRODUCTION

Modern plant biotechnology faces three major public demands:

(1) A growing human population requires more food, while arable land is decreasing in amount and quality. Here, drought, salt and heavy metals in particular are major constraints.
(2) An ageing human population requires higher quality food, to be able to prevent typical 'diseases of civilisation', like cardiovascular diseases and cancer.
(3) A paradigm shift in medicine from unspecific blockbusters to a personalised treatment requires the safe and cost-effective production of complex biopharmaceuticals for diagnosis and treatment.

Recent work on mosses, namely the moss *Physcomitrella patens*, may contribute to all of these fields of plant biotechnology.

Mosses are small land plants and are quite different from crop plants. In fact, the last common ancestor of mosses and crop plants lived about 450 million years ago.[1] Every plant exhibits an alternation between two generations. In all 'higher' plants, the dominating generation is the

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diploid sporophyte: because they are dispersed by seeds, they are described as 'seed plants'. In mosses, however, the dominating generation is the haploid gametophyte and they are distributed by spores.[2] Haploidy of the major moss tissues makes life a bit different for plant geneticists: there are no dominant/recessive traits in haploids and consequently no need for back-crossings to obtain isogenic lines. Yet, gene analyses via traditional Mendelian genetics are more complicated in mosses. Most important, however, is the highly efficient homologous recombination machinery in the moss genome.[3,4] This feature facilitates straightforward reverse genetics approaches in moss at a speed and efficiency so far only known from fungi such as yeast. Virtually every gene can be knocked out by targeted,[5–7] as well as by unbiased, approaches in attempts to establish saturated mutant collections.[8] With either method, the resulting phenotype can be screened within weeks after transformation.

The high rate of homologous recombination in *Physcomitrella* enables high-throughput reserve genetics approaches

Gene targeting in moss is in fact about five orders of magnitude more efficient than in any seed plant, and still about two orders of magnitude more efficient than in the embryonic stem cells of mice.[9] Consequently, several independent knockout plants can be generated for one single construct, allowing fast and reliable gene function annotations.

This paper focuses on recent advantages in high-throughput methodology applied to the moss *Physcomitrella patens* and highlights the potential benefits of this research for human health. Because of space constraints, only a selection of results can be presented and the authors apologise for not citing all publications in this field.

MOSS GENOME AND TRANSCRIPTOME

To date, the genomes of two seed-plant species have been fully sequenced, the 125 megabase pair (Mbp) genome of the dicotyledonous model plant *Arabidopsis thaliana*, a weed,[10] and the 420 Mbp and 466 Mbp genomes of two varieties of the monocotyledonous rice crop *Oryza sativa*.[11,12] In the near future, the whole 550 Mbp genome of a poplar tree, *Populus trichocarpa*, will be released.[13] Interestingly, in plants there is no strict correlation between the morphological complexity of a species and the complexity of its genome. As an example, *Physcomitrella* has less complex tissues, and a lower variety of cell types and organs, compared with seed plants, but it has a genome of approximately 511 Mbp, divided between 27 chromosomes.[14] An international Moss Genome Sequencing Consortium was established in 2004 (www.plant-biotech.net/moss2004, Freiburg University, cited 28th October, 2004) aiming at deciphering the complete *Physcomitrella* genome. This was initiated by the award of a grant from the US Department of Energy to its Joint Genome Institute for an eightfold shotgun sequencing (www.jgi.doe.gov/sequencing/why/CSP2005/physcomitrella.html, Joint Genome Institute, University of California, cited 28th October, 2004). Completing work, such as assembly, filling the gaps and annotation of the genome, will need to be secured by additional national or international grants. This will be the first sequenced haploid genome from any higher eukaryote. The haploidy of the genome will make genome assembly very straightforward. Moreover, comparison with sequenced seed plant genomes may unravel some fundamental questions: how can haploid genomes maintain their integrity during evolution? What are the core characters maintained during evolution of land plants? And, finally, what is the molecular basis for the highly efficient homologous recombination in the moss genome? The answers may help to bring about substantial improvement in the quality and stability of transgenic crop plants in the future.[15]

Assembly and annotation of the *Physcomitrella* genome will greatly benefit from the wealth of expressed sequence tag (EST) data that have been collected in the

past few years.[16,17] Including both public and proprietary resources, well over 200,000 *Physcomitrella* ESTs are now available. Analysis of this dataset indicates that there are about 30,000 different protein-encoding moss genes, about 6,000 of which do not have clear homologues in public databases and are thought to be 'novel genes'. About 100 genes have no homologues in plants, but do have homologues in other organisms, from yeast to human, and are thought to be 'retained genes' (unpublished). Moss-specific DNA repeats have been identified, and hidden Markov models (HMM), as well as support vector machines (SVM) for splice-site predictions, have been trained for *Physcomitrella* datasets (www.cosmoss.org, Freiburg University, cited 28th October, 2004).

The launched *Physcomitrella* genome will help to elucidate the function of a large number of novel genes

MOSS EXPRESSION PROFILING

One important method in the whole functional genomics toolbox[18] is transcriptomics, ideally the genome-wide analysis of gene expression under various conditions. Co-regulation of sets of genes upon given stimuli points to their putative function. *Physcomitrella* is highly tolerant to a variety of abiotic stresses like drought, salinity, low temperature and heavy metal stress. Consequently, the expression of several genes is regulated by these environmental stimuli.[19–23] The functional characterisation of stress-related genes by reverse genetics approaches is straightforward in moss, as targeted knockouts display elevated sensitivity to such environmental stresses.[19] Furthermore, the analyses of stress-responsive gene expression in *Physcomitrella* will help to unravel the underlying regulatory networks that mediate abiotic stress tolerance. One such mediator is the plant hormone absasic acid (ABA). In seed plants, as well as in moss, many stress-responsive genes seem to be regulated by ABA-dependent signalling pathways. In contrast to seed plants, however, *Physcomitrella* internal ABA levels do not rise during cold acclimation,[23] and regulation of conserved stress-associated genes may be more complex in moss than in seed plants.[20]

About 500 moss genes without clear functional annotation ('novel genes') have been spotted upon a macroarray to assay their regulation upon stimuli such as drought and/or ABA. From these 500 genes, 9 per cent (46) reacted to these stimuli, identifying themselves as putatively being involved in stress tolerance (unpublished). In a wider approach, a moss microarray was produced that is an approximately 20,000 feature array of 60-mer oligonucleotides synthesised *in situ* on glass slides, using Agilent Technologies' 'SurePrint' inkjet technology. The sequences represented on the chip correspond to all of the predicted transcripts derived by cluster analysis of the publicly accessible *P. patens* EST sets. The chips are analysed by hybridisation with Cy-3/Cy-5-labelled cDNA for pairwise comparisons of transcript abundance in messenger RNA samples.[24] The chips are available through MOgene LC, an Agilent certified facility based in St Louis, MO, USA (www.mogene.com, MOGene LC, St. Louis, cited 28th October, 2004). MOgene LC also offers, as a service, a complete analysis of RNA (total) samples, as well as more detailed statistical examination of the data. Proof of principle was gained by examination of ABA-regulated gene expression (www.biology.wustl.edu/faculty/quatrano/fig13.html, Washington University, St. Louis, MO, USA, cited 28th October).

MOSS PROTEOMICS

Given that most biological reactions are not mediated by RNAs but by the encoded proteins, and that protein activity is often regulated by post-translational modifications unpredictable from the primary nucleic acids sequence, attempts to examine the protein complement of a genome (proteomics) have proceeded

A set of proteomic tools is available to study diverse biological aspects in *Physcomitrella*

apace over the past few decades.[25] Prerequisites for large-scale protein identification, however, are comprehensive nucleic acid sequence databases, preferably from the species under investigation. Furthermore, like all sensitive functional genomics approaches, proteomics relies on the reproducibility of the biological samples, often a severe problem for biologists working with plants grown in soil and climate chambers. As an additional asset in this respect, *Physcomitrella* can be grown highly reproducibly in bioreactors (Figure 1).[26]

Standard proteomics approaches rely on protein separation in two-dimensional gels and subsequent identification utilising mass spectrometry. With this method, more than 300 *Physcomitrella* proteins have been identified, about 15 per cent of them with as yet unclear functions.[27] Subsequent approaches will rely on analyses of subcellular proteomes following available protocols for isolation of moss organelles.[28] Such approaches, however, preferentially identify abundant proteins, while the majority of regulatory proteins are only present in minute amounts and, therefore, mainly escape detection. Almost all aspects of life, both in prokaryotes and in eukaryotes, are regulated by signal transduction chains, with the signals being transmitted via reversible phosphorylation/dephosphorylation. Therefore, highly reproducible and sensitive methods to assay this type of post-translational modification on a genome-wide scale, termed 'phosphoproteomics', are highly desirable. Such a protocol has recently been established for *Physcomitrella*.[29] By combining C18 reverse-phase chromatography (RP-C18), immobilised Fe^{3+} metal affinity chromatography (IMAC), capillary zone electrophoresis (CZE), liquid chromatography-tandem mass spectrometry (LC-MS/MS) and matrix-assisted laser desorption/ionisation-time of flight-mass spectrometry (MALDI-TOF-MS), Heintz *et al*.[29] were able to identify more than 250 moss phosphopeptides, some of which are known members of signal transduction chains. This protocol will surely serve as the basis for unravelling several signal networks in the moss. As quantification of phosphorylation events is possible at the femtomol level, such analyses are the prerequisite for transition from functional genomics approaches into the holistic systems biology approaches for any given organism.

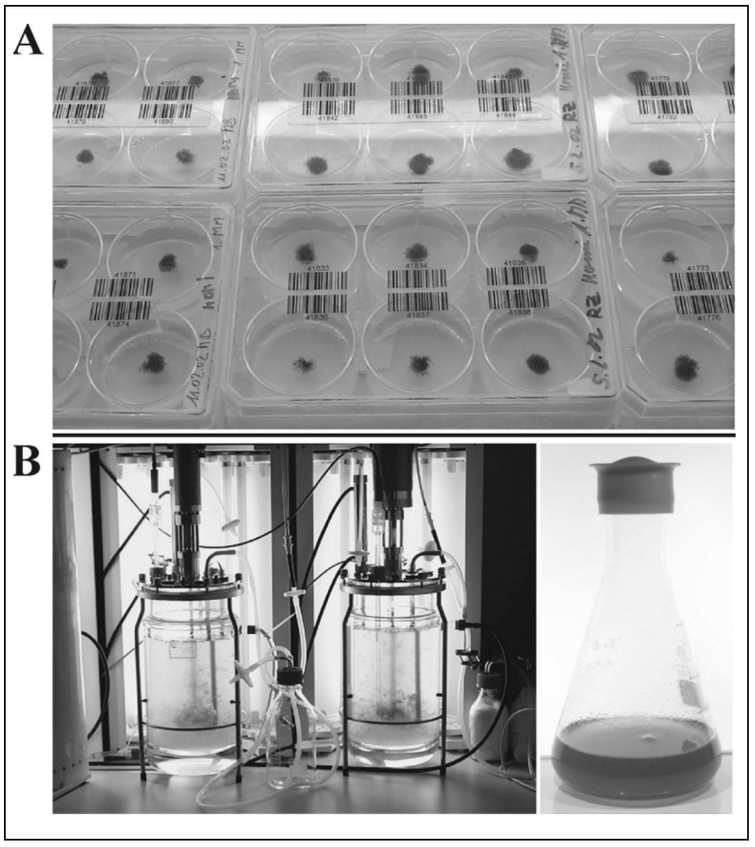

Figure 1: Cultivation of *Physcomitrella* plants
Physcomitrella plants can be axenically cultivated under highly standardised conditions. (A) *Physcomitrella* grown on solid medium. When plants are grown on solid medium they undergo normal development. Sexual reproduction can be initiated by particular external stimuli, resulting in the completion of the life cycle. (B) Cultivation of *Physcomitrella* plants in liquid cultures. The plants are grown in small-scale cultures in Erlenmeyer flasks or in large volume bioreactors. Subculturing and mechanical disruption of the plants causes predominant growth at the protonemal stage.

MOSS METABOLITES

When compared with animals, plants have an amazingly high number of complex secondary metabolites. Indeed, current estimates point to a total of more

than 2,000,000 different metabolites synthesised by the plant kingdom, most of which are as yet unidentified. Several of them, however, have long been known and are used intensively by humans both therapeutically and abusively.[30] An emerging field in plant functional genomics — plant metabolomics — is devoted to these substances.[31]

Polyunsaturated fatty acids (PUFAs), which are synthesised by a variety of plants, are important for the human diet. PUFAs like arachidonic acid (AA) and eicosapentaenoic acid (EPA), however, are only produced by non-seed plants like the moss *Physcomitrella*. While the precise function of these highly abundant PUFAs in plants still awaits elucidation, it is well documented that in humans they play a key role in eicosanoid metabolism.[32] Likewise, very-long-chain fatty acids (VLCFAs) are widespread in the plant kingdom, although polyunsaturated VLCFAs are most abundant in non-seed plants, like mosses. These polyunsaturated VLCFAs have gained increasing attention, as they are beneficial for human growth and health when included in the diet.[33–35] At present, most of these fatty acids are supplied by consumption of fish. In order to provide alternative and more sustainable sources of these substances, several approaches have been undertaken to isolate relevant genes and, by genetic engineering, modify oilseed crops such that they produce these beneficial polyunsaturated VLCFAs. In this context, several genes encoding as yet unknown desaturases and elongases have been isolated from *Physcomitrella*, and their function established by targeted gene disruption.[6,36] Proof of principle has recently been achieved by tissue-specific expression of these and other genes in transgenic tobacco (*Nicotiana tabacum*) and linseed (*Linum usitatissimum*).[37]

***Physcomitrella* produces specific metabolities not known from higher plants**

Besides its importance for human health, AA can be further metabolised in plants, for example by a class of enzymes, the lipoxygenases (LOXes), which generate volatile compounds called oxylipins. Recently, a detailed analysis of these pathways uncovered previously unknown LOX pathways that generate a multitude of volatile oxylipins in *Physcomitrella*. Indeed, this moss produces not only typical plant metabolites, but also typical animal, algal and mushroom metabolites, thus combining, in a unique way, metabolic themes from different kingdoms.[38] Part of this metabolic complexity was subsequently pinpointed to the activity of a novel multifunctional LOX with fatty acid hydroperoxide-cleaving activity (unpublished). In addition to the oxylipin cocktail, *Physcomitrella* obviously offers more odours, as it also releases, so far unknown in other plants, tetracyclic diterpenes into the atmosphere.[39] These are only the tip of the iceberg, as plenty of moss secondary metabolites lie, as yet, undiscovered. As some of them clearly have antifungal activity, biotechnological use of these metabolites is obvious,[40] although still in its infancy.

MOSS PHENOMICS

Whereas reverse genetics annotates function to a given gene by its targeted disruption and subsequent analysis of the loss of function mutant, forward genetics starts from desired mutant phenotypes with subsequent analysis of the altered gene. Both approaches can be combined with transcriptomics, proteomics and metabolomics. Ideally, forward genetics employs an unbiased, tagged and saturated mutant collection. In order to detect unexpected gene function relationships, an unbiased — that is, non-selected — mutation is pivotal. To ease gene isolation, the mutated genes should be tagged by a known, artificially introduced nucleotide sequence, which serves as the starting point for gene cloning. Finally, to exploit this approach fully, the mutant collection should be sufficiently large that, statistically, every single gene of the organism under study has been mutated, displaying the highest possible variation of that organism in the collection — that is, it should be saturated. Functional

genomics approaches starting from such a collection are called 'phenomics'.

Whereas such approaches in seed plants rely either on the introduction of a bacterial vector (Ti-plasmid) or on the activity of transposable elements,[41–43] *Physcomitrella* offers the benefit of homologous recombination. A shuttle mutagenesis system was established on the basis of a bacterial transposon, which, in turn, was used to mutate a low-redundant *Physcomitrella* cDNA collection in *Escherichia coli*. These plasmids were isolated in an unbiased manner and used to target the respective genomic loci via homologous recombination (Figure 2).[44] Using pools of such knockout constructs, not only a single locus, but occasionally two or even three independent loci can be targeted in *Physcomitrella* via one single transformation event.[45] Mutant production was performed in a high-throughput manner, resulting in 160 transgenics a day, 800 a week, every week up to the final number of approximately 75,000 plants. For cryopreservation and long-term storage, these plants are stored in liquid nitrogen.[46] Interestingly, from the first 16,000 plants, around 16 per cent displayed altered morphological, developmental or physiological phenotypes[8] (Figure 3). This figure extends beyond similar approaches in seed plants, for example *Arabidopsis*, where phenotypic variations are only found in a small number of mutant lines.[47]

A saturated *Physcomitrella* mutant collection displays a high degree of deviating phenotypes

MOLECULAR FARMING IN MOSS BIOREACTORS

While there is a rapidly increasing demand for complex biopharmaceuticals, recent production capacities might limit an adequate supply of these products. Beside the expansion of already-existing production facilities based on bacteria, yeasts or mammalian cells, plant-based

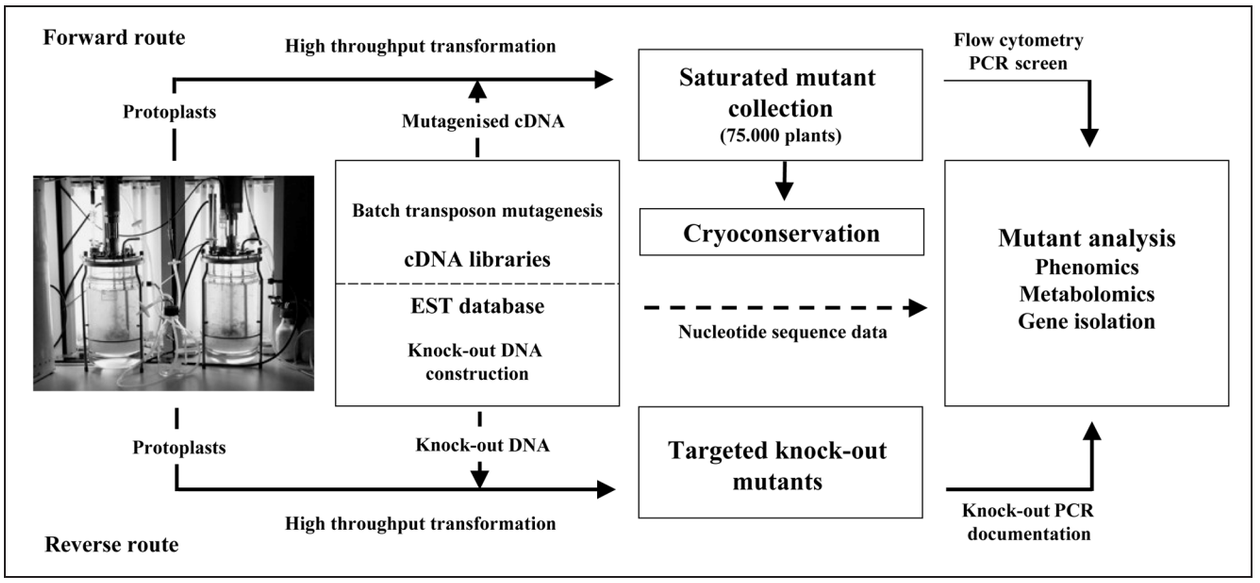

Figure 2: Schematic representation of the high-throughput platform for production of tagged *Physcomitrella patens* mutants. This scheme outlines the processes that underlie the production of tagged *Physcomitrella* mutants. Two strategies of mutant production are distinguished: a forward and a reverse route. The forward route uses anonymous cDNA clones to produce a saturated mutant collection of around 75,000 plants. The cDNAs are batch-mutagenised using a transposon shuttle mutagenesis system to disrupt the DNA coding sequence with a selection cassette. Mutagenised plasmid DNA is transferred into *Physcomitrella* protoplasts by way of a high-throughput transformation platform. Mutants generated by the forward route are subjected to phenotypic and metabolic profiling. The reverse route is based on known moss candidate genes, the function of which is investigated by direct gene knockout. Disruption constructs are transfected into protoplasts and resistant plants screened by polymerase chain reaction (PCR) for targeted transgene insertion. All mutants are subjected to further molecular analyses. EST = expressed sequence tag (adapted from Holtorf *et al*., in 'New Frontiers in Bryology: Physiology, Molecular Biology and Applied Genomics', Kluwer Academic Press, Dordrecht, The Netherlands)

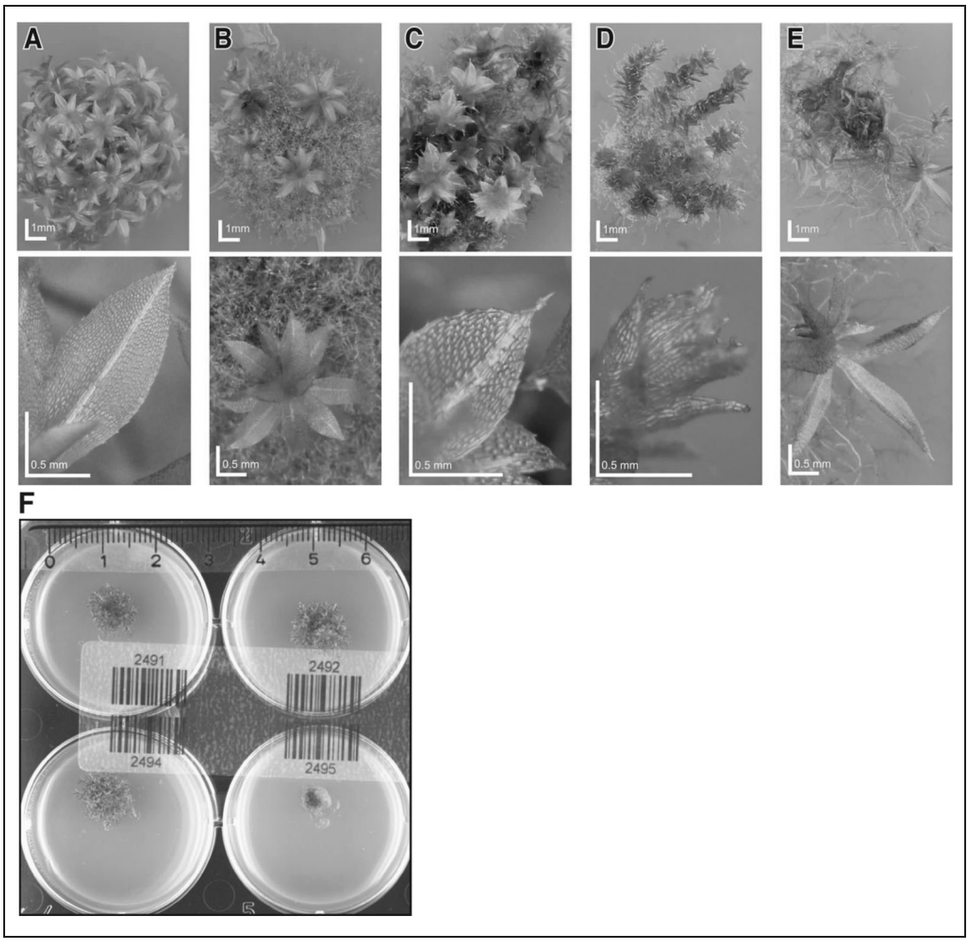

Figure 3: Phenotypic and metabolic mutants of *Physcomitrella* transformants generated using a gene disruption library. Panels A to E show *Physcomitrella* plants with deviating developmental phenotypes. *Physcomitrella* wild-type and transformed plants were grown on minimal medium to induce differentiation and development of gametophores. For each plant, an overview (upper row, scale bar corresponds to 1 mm) and a close-up (bottom row, scale bar corresponds to 0.5 mm) is shown. (A) Haploid wild-type moss plant completely covered with leafy gametophores and close-up of wild-type leaf. (B) Transformant affected in differentiation, mostly comprising filamentous protonema with a reduced number of gametophores, but normal leaf morphology (haploid). (C) Transformant showing retarded growth, a reduced number of gametophores per moss plant and altered leaf morphology (haploid). (D) Transformant displaying altered growth phenotype (polyploid). (E) Transformant showing retarded growth and elongated, narrow leaves (polyploid). (F) Isolation of metabolic mutants. Physcomitrella plants derived from transformation with the gene-disruption library and regenerated on supplemented medium were split into two parts, which were transferred to minimal medium with and without supplements and cultured for 8 weeks. The picture shows four independent *Physcomitrella* transformants cultured on minimal medium, one of which (bottom right) displays a clear growth defect. All four plants grew equally well on supplemented medium (data not shown). The scale bar at the top indicates size in centimetres. (Adapted from Egener *et al.*, *BMC Plant Biol.*, Vol. 2, p. 6.)

expression systems are a promising alternative for the production of such therapeutic and diagnostic proteins.[48] The field production of transgenic plants is restricted by legal and public constraints, particularly in European countries, whereas plant bioreactors meet the requirements of biological

containment and production to good manufacturing practice (GMP) standards. Many plant cell culture systems are subject to genetic instability;[49] the moss bioreactor, however, contains well-differentiated, genetically stable cell types.[50] Moreover, *Physcomitrella* grows in a simple mineral medium, with light and carbon dioxide as the only energy and carbon source, allowing cheap production of large quantities of plant material when compared with the complex culture conditions for mammalian cells. Several tools have been developed to optimise *Physcomitrella* strains according to particular demands.[51] Different heterologous, as well as homologous, promoters have been characterised, enabling the production of recombinant proteins at different expression levels.[52] A severe constraint for plant-derived pharmaceuticals is the post-translational attachment of plant-specific sugar residues to proteins, as these residues may cause allergic reactions in humans.[53,54] Notably, xylose — a sugar not present in humans — and fucose — which is differently linked to the glycan core in humans compared with plants — are the allergenic residues of plant glycoproteins.[55] The two enzymes α1,3-fucosyltransferase and β1,2-xylosyltransferase, which catalyse the transfer of these sugar residues to the glycan backbone, were subjected to a targeted double knockout approach, resulting in moss plants without any fucose or xylose residues attached to their proteins, while the overall N-glycan pattern remained unaffected in these transgenic plants.[56] Surprisingly, this 'humanisation' of moss protein glycosylation did not affect plant growth and development, nor secretion of a recombinant human growth factor under *in vitro* conditions. This *Physcomitrella* line, avoiding allergenic plant-specific *N*-glycosylation patterns and further optimised mutant lines, may serve as a valuable production platform to tap the full potential of plant-derived therapeutic and diagnostic proteins.

***Physcomitrella* can be grown in bioreactors to produce biopharmaceuticals used in therapy and diagnosis**

CONCLUSIONS

So far, the majority of plant researchers have focused on several seed plants. Due to their unique position in the evolution of land plants, mosses, like *Physcomitrella patens*, offer additional advantages and insights. Relevant tools for gene discovery have been developed, and an international genome sequencing project will further broaden the use of *Physcomitrella* in applied, as well as in fundamental, biology. Proof of principle has already been established for several moss genes with unique properties and implications for crop plants and for human health. The production of human proteins in moss bioreactors presents an elegant alternative to established systems such as Chinese hamster ovary cells.

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