The moss bioreactor
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The production of recombinant proteins in moss bioreactors provides all of the benefits of molecular farming in plants but avoids many plant-specific disadvantages, such as the genetic instability of de-differentiated cells in suspension culture or the lack of containment during field production. Protein yields are in the same range as those of other cell-culture-based production systems. On top of this, the moss Physcomitrella patens is the only known plant that can be genetically modified by homologous recombination, allowing efficient targeted gene disruption. Thus, the major drawback of producing human proteins in plants, allergic reactions caused by plant-specific glycosylation, can be diminished by targeted knockout of the responsible genes in moss. Unlike all other plants, moss allows straightforward ‘humanisation’ of plant-derived pharmaceuticals.

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Introduction
When compared to microbial (bacteria or yeast and other fungi) or animal (e.g. Chinese Hamster Ovary cells) production systems, plants are superior biofactories for the production of complex recombinant diagnostic and therapeutic proteins. This is because plants can synthesise complex multimeric proteins, at a relatively low cost and short time-to-market, and in the absence of human pathogens [1*2**]. In plants such as tobacco, rice or maize, these benefits are balanced by some disadvantages; for example, when grown in bioreactors, de-differentiated plant cell lines are sensitive to shearing, are genetically instable, and produce comparatively small amounts of recombinant proteins [3]. On the other hand, field production of transgenic plants evokes reservations among consumers, need complex downstream processing and is controlled by complex legislation, especially in European countries. Alternative plant-based protein-production systems, such as the Lemna System™, have therefore been developed (www.biolex.com). The major drawback of therapeutic applications of any plant-derived recombinant protein is plant-specific protein glycosylation, which may cause allergic reactions in patients [4,5]. Recently, the moss Physcomitrella patens, has emerged as an alternative production system that enables us to meet all of the concerns mentioned above ([6]; www.greenovation.com).

In this review, we discuss the recent progress made in establishing moss as a safe and efficient production system for recombinant proteins and describe future perspectives.

Moss development and in-vitro cultivation
The life cycle of mosses begins with the germination of a haploid spore and the subsequent growth of a branched cell filament, the protonema. Bud formation gives rise to the adult plant (i.e. the gametophore), which has stems, leaves and rhizoids. This adult moss plant harbours the sex organs, in which fertilisation takes place (Figure 1). The life cycle is completed by the development of a spore capsule from the embryo. In-vitro cultivation of all stages of the moss life cycle was established years ago. However, the filamentous juvenile tissue (protonema) is the most convenient stage for genetic engineering and biotechnological approaches. The protonema can easily be propagated vegetatively without the production of persistent spores. Adult moss plants are kept on petri dishes mainly as a backup for the maintenance of transgenic strains (Figure 1). For long-term storage, cryo-conservation in liquid nitrogen is feasible ([7]; J Schulte, R Reski, unpublished).

Juvenile tissues are grown in suspension culture in a simple medium of inorganic salts, with light and carbon dioxide as the only energy and carbon sources [8]. Hormones and complex additives are not needed for moss cultivation. Furthermore, regular mechanical disruption of the cell filaments prevents plants from developing adult tissues. Because the cells of protonema are fully differentiated, the genetic instability seen in suspension cultures of de-differentiated higher-plant cells has never been observed with moss. Small-scale cultivation is performed in simple agitated flasks. For medium-scale cultivation, a 10-litre photo-bioreactor comprising a stirred glass tank with external illumination provided by normal fluorescent tubes has been used (Figure 1). Optimal parameters for the success of these bioreactors, such as density of inoculation, vigour of stirring, pH, and aeration,
have been determined [9,10]. Optimal conditions resulted in the growth and differentiation of highly uniform moss cultures, even under semi-continuous cultivation with a daily exchange of about 20% of the cultures [9,10]. Physcomitrella can be grown in a broad pH range in these bioreactors, so the culture conditions can easily be adapted to meet the needs of any recombinant protein that is secreted to the culture medium. In addition, a low-cost 20-litre bioreactor for Physcomitrella cultivation has recently been successfully developed (JS Shin, pers. comm.). As an essential step towards GMP production on an industrial scale, a 30-litre glass tubular moss bioreactor has also been developed. The modular structure of this reactor type will allow easy scaling up by operating several units in parallel [11].

**Genetic toolboxes for the production of recombinant proteins**

Transgenic moss plants are normally created by polyethylene glycol-mediated transfection of protoplasts (e.g. [12]), although alternative transfection protocols that utilise particle bombardment or electroporation have been developed ([13]; EL Decker, A Kinal, R Reski, unpublished). Selection procedures for the generation of stable transgenic plants take about eight weeks. The haploidy of the major moss tissues greatly facilitates the genetic engineering of the relatively small *Physcomitrella* genome (511 Mbp [14]). Thus, recombinant traits are immediately effective in the primary transgenics without the need for complex and time-consuming backcrossing. In addition, expressed sequence tag (EST) data are available from about 25 000 protein-coding genes, providing coverage of more than 95% of the *Physcomitrella* transcriptome [15,C15,16,C15]. Analyses of these datasets reveal that in moss, unlike in higher plants, there is no significant bias for codon usage. Therefore, the recombinant genes do not have to be adapted specifically for protein production in moss. Different widely used heterologous promoters have proven to be functional in *Physcomitrella* [17–20]. A comparative analysis of heterologous promoters from plant and mammalian expression systems, as well as of some *Physcomitrella* promoters, was performed recently (V Horstmann, R Reski, E Decker, unpublished). The assayed promoters included both widely used plant promoters, such as Cauliflower mosaic virus (CaMV)-35S promoters, the nos (nopalinsynthase) promoter, and the rice actin1 promoter, and promoters from mammalian systems, such as the cytomegalo virus (CMV) and the simian virus (SV)40 promoters. The various promoters will facilitate the transcription of multiple foreign genes in engineered mosses. It should be possible to express each transgene — either a gene for a product of interest or a gene encoding an essential modifying enzyme — at the appropriate strength.

A second tool set that is necessary for the production of recombinant proteins comprises signal peptides for the intracellular sorting of proteins to suitable compartments. Moss signal sequences for transport into plastids, mitochondria, vacuoles or the secretory pathway have been identified and characterised ([22]; J Kiessling et al., unpublished; A Schaaf, R Reski, E Decker, unpublished). Targeting of recombinant proteins to the secretory pathway, and thus to the extra-cellular compartment, is favoured for two major reasons. First, the

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**Figure 1**

*Physcomitrella* life cycle and in-vitro cultivation. (a) Gametophytic development starts with the germination of spores and the outgrowth of the filamentous protonema tissue. Bud formation gives rise to the adult moss plant, which carries the sex organs (antheridia and archegonia). After fertilisation of the egg cell, the diploid sporophyte develops. (b) Photobioreactors have been developed for in-vitro propagation of protonema. (c) Gametophores are cultivated on solid medium. Photographs courtesy of Andreas Schaaf.
secretory pathway is ‘the natural’ path for most of the proteins of interest, for example, for secreted human blood serum proteins. Second, secretion of the product greatly facilitates its purification from the simple inorganic medium [P1]. Downstream processing is further simplified because moss naturally secretes a set of fully characterised low-abundance proteins, none of which displays protease activity (D Heintz et al., unpublished). In addition, cultivation with continuous medium exchange is possible with moss bioreactors.

**Targeting of plant-specific glycosylation**

Central characteristics, such as the folding, assembly, function and turnover of proteins, are regulated by their posttranslational modification, especially their glycosylation. All higher eukaryotes, including mosses, share core glycan structures [23,24], the Asn (N)-linked complex glycans [1,2]. By contrast, lower eukaryotes, such as yeast and other fungi, are not able to add complex N-linked glycans to recombinant proteins, although some progress has been made recently in modifying the glycosylation pathway of the yeast *Pichia pastoris* [25,26]. Nevertheless, yeast-specific posttranslational protein modifications such as O-glycosylation still hamper the use of such systems for biotechnology [27,28].

Although humans and plants share a core glycosylation structure in their proteins, two plant-specific sugar residues severely hamper the biotechnological use of plants as biofactories because they might cause immune reactions [4,5,29]. These ‘problematic’ residues are xylose, a sugar that is not present in humans, and fucose, which is differently linked to the respective glycan core in humans and in plants ([30]; Figure 2a). It is widely accepted that the negative effect of foreign glycan structures on plant-derived therapeutic proteins is the major drawback of molecular farming. Different approaches to overcome this problem are being developed. Among these is the retention of the recombinant proteins in the endoplasmic reticulum; this avoids plant-specific glycosylation, which takes place in the Golgi [31]. Other approaches involve the inhibition of Golgi-located glycosyltransferases or the introduction of human glycosyltransferases [4,32]. However, gene disruption by targeted knockouts provides the only reliable method for avoiding the gene products that are responsible for the transfer of allergenic sugar residues to the glycan structure. To date, *P. patens* is the only known plant that can be genetically modified by targeted knockouts via homologous recombination, which can provide efficient and precise gene disruption [12,33–43]. Although mosses and higher plants such as *Lemma, Arabidopsis*, tobacco, maize and rice are separated by approximately 450 million years of evolution, detailed mass spectrometric analyses have provided evidence that the protein glycosylation patterns in these two groups of plants are identical [23,24].

Figure 2

Alteration of plant-specific N-glycosylation. (a) Targeted disruption of the genes for α1,3-fucosyltransferase and β1,2-xylosyltransferase, respectively, leads to a lack of allergenic sugar residues in N-glycan structures in knockout moss (right). These residues are present in the N-glycans of higher plants and of wildtype moss (left). (b) Knockout strategy for targeting a *Physcomitrella* gene by homologous recombination. Upper part: target gene with exons (boxes) and introns (lines). Lower part: knockout construct consisting of homologous stretches (grey boxes) that correspond to parts of the target genes (indicated by dashed lines). The homologous flanks are disrupted by the selection cassette (e.g. an antibiotic resistance gene). GlcNAc, N-acetylglucosamine.

N-acetylglucosaminyl transferase I (GNTI) is the first enzyme in a pathway that produces plant-specific glycosylation of the eukaryotic core structure. The *Arabidopsis* mutant *cgl*, which is deficient in GNTI activity, lacks all Golgi-modified complex N-linked glycans but has normal growth and development [44]. Surprisingly, it seems that moss encodes redundant GNTI functions, as targeted disruption of a single GNTI gene did not alter the overall glycosylation pattern in the knockout plants when compared to non-transgenic wildtype plants [24].

In a more specific approach, the two enzymes responsible for the plant-specific fucosylation and xylosylation, α1,3-fucosyltransferase and β1,2-xylosyltransferase, were directly targeted by homologous recombination (Figure 2). Single and double knockout plants were created and confirmed by techniques such as mass spectrometry. According to matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry analyses, double knockout moss had an unaltered overall N-glycan pattern, except that the putatively allergenic fucose and xylose residues were completely undetectable. Most surprisingly, this dramatic alteration in protein glycosylation had no detectable effect on moss growth and development under *in vitro* conditions (A Koprivova...
et al., unpublished). Thus, these moss strains offer, for the first time, the possibility to produce safe therapeutic proteins with complex-type N-glycosylation in plants without the risk of causing allergies when given to patients.

Characteristics of protein production

Time-to-market with the moss bioreactor is comparable to that of traditional systems. A transient expression system that is based on the transformation of moss protoplasts allows feasibility studies within 10–12 weeks. The development of a stable transgenic production strain takes 4–6 months. A humanised monoclonal antibody for the prevention of deep-vein thrombosis was successfully expressed in moss and is currently in pre-clinical development at AERES Biomedical (www.greenovation.com). The secreted IgG antibody was shown to be correctly assembled and displayed normal activity in binding to its natural ligand. Production of human vascular endothelial growth factor (hVEGF) yielded 30 mg of secreted recombinant protein per litre per day. This corresponds to a typical fedbatch culture over 20 days yielding 600 mg of recombinant protein per litre. In the transient system, moss protoplasts produced and secreted up to 80 pg of hVEGF per day per cell, which is more the quantity of the same protein produced in baculovirus-infected insect cells (www.greenovation.com).

Conclusions

Moss bioreactors promise to provide an efficient system for the production of heterologous therapeutic proteins in safe containment. Genetic engineering of moss strains is established and is facilitated by characteristics such as the prominent haploid stage, the rather small moss genome and the feasibility of targeted knockouts, which will allow further improvement of the glycosylation pathway. This improvement will provide plants with the ability to produce complex-glycosylated proteins in a humanised fashion. A world-wide Physcomitrella genome sequencing project has just begun www.cosmoss.org). This project will further improve the safety of the moss bioreactor by making genome engineering and the characterisation of transgenic strains easier.

Acknowledgements

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


An excellent recent review on the production of biopharmaceuticals in plants.


An outstanding, comprehensive and detailed review of current progress in molecular farming.


domains of different processing enzymes. Various signal sequences for the secretory pathway, ligated to catalytical

The work described in this paper represents a major step in modifying N-glycosylation. The authors identify three key genes for plant-specific glycosylation from the moss Physcomitrella patens and in higher plants. Plant Biol, in press.

The authors identify three key genes for plant-specific glycosylation from moss, enabling the targeted knock-out of these genes.


The work described in this paper represents a major step in modifying N-linked protein glycosylation in yeast. The authors transformed yeast with combinatorial genetic libraries that contained chimeric fusion proteins of various signal sequences for the secretory pathway, ligated to cataytical processes of different processing enzymes.


A functional human antibody is successfully produced in tobacco and retained in the endoplasmic reticulum, thus preventing allergenic glycosylation.