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REVIEW

Glycoprotein production in moss bioreactors

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Abstract Complex multimeric recombinant proteins such as therapeutic antibodies require a eukaryotic expression system. Transgenic plants may serve as promising alternatives to the currently favored mammalian cell lines or hybridomas. In contrast to prokaryotic systems, posttranslational modifications of plant and human proteins resemble each other largely, among those, protein N-glycosylation of the complex type. However, a few plant-specific sugar residues may cause immune reactions in humans, representing an obstacle for the broad use of plant-based systems as biopharmaceutical production hosts. The moss *Physcomitrella patens* represents a flexible tissue-culture system for the contained production and secretion of recombinant biopharmaceuticals in photobioreactors. The recent synthesis of therapeutic proteins as a scFv antibody fragment or the large and heavily modified complement regulator factor H demonstrate the versatility of this expression system. A uniquely efficient gene targeting mechanism can be employed to precisely engineer the glycosylation machinery for recombinant products. In this way, *P. patens* lines with non-immunogenic optimized glycan structures were created. Therapeutic antibodies produced in these strains exhibited antibody-dependent cellular cytotoxicity superior to the same molecules synthesized in mammalian cell lines.

Keywords ADCC · Knockout moss · Molecular farming · *Physcomitrella patens* · Plant-made pharmaceuticals · Protein glycosylation

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Introduction

Because of their innate complexity current therapeutic recombinant proteins (biopharmaceuticals) are produced mainly in mammalian expression hosts, especially in Chinese hamster ovary (CHO) cells (Beck et al. 2008). Mammalian cell lines are the workhorse in pharmaceutical glycoprotein production because of their ability for human-like posttranslational glycosylation (Durocher and Butler 2009; Walsh and Jefferis 2006). However, plant-based systems may be considered as alternative production platforms for recombinant glycoproteins with several target proteins already in clinical phases (Karg and Kallio 2009). As higher eukaryotes, plants synthesize complex multimeric proteins and the machinery extent of posttranslational modifications is rather similar between plants and mammals. The establishment of in vitro plant cell or tissue culture offers the possibility for a precise control and standardization of cultivation conditions that allows production under GMP guidelines (Hellwig et al. 2004). On the other hand, a contamination of plant-based production systems by human-pathogenic infectious agents is rather unlikely (Fischer et al. 2004). Meanwhile, several plant-

made pharmaceuticals (PMP) reached late stages in clinical studies (Faye and Gomord 2010). However, for many plant-based systems, large-scale production facilities are difficult to establish and most plant systems cannot be used unobjectionably for the synthesis of glycosylated products. Asparagine-linked posttranslational protein N-glycosylation is a modification found on most human blood proteins and 70% of the biopharmaceutical candidates in preclinical or clinical development are glycoproteins (Durocher and Butler 2009). Glycosylation may be necessary for structural and functional properties of a given protein (Walsh and Jefferis 2006), i.e., antibody-dependent cellular cytotoxicity (ADCC) which means the effectiveness of antibodies used in cancer treatment can be influenced by a specific sugar moiety linked to the *N*-glycans of the antibody (Shields et al. 2002; Shinkawa et al. 2003). N-glycosylation on plant proteins resembles mammalian sugar chains largely. However, plant *N*-glycans bear specific sugar residues, which were proven to be immunogenic (Gomord et al. 2005; Mari 2002). This issue has to be solved before using a plant system as glycoprotein production host. Therefore, RNAi approaches for down-regulation of plant-specific glycosylation were undertaken in the hydrophyte *Lemna minor* as well as in *Nicotiana benthamiana* and *Medicago sativa* (Cox et al. 2006; Sourrouille et al. 2008; Strasser et al. 2008) and glycosylation mutants were established for *Arabidopsis thaliana* (Strasser et al. 2004). A few years ago the moss *Physcomitrella patens* was introduced as an alternative system for biopharmaceutical production that combines low-cost contained cultivation in photobioreactors with a unique feasibility for precise gene targeting (Decker and Reski 2007; Decker and Reski 2008). By the mechanism of homologous recombination in vegetative cells, plant-specific glycosylation pathways have been engineered towards human-like glycans thus deleting any immunogenic potential (Huether et al. 2005; Koprivova et al. 2004). Therapeutic IgG antibodies produced in glyco-optimized moss exhibited superior ADCC compared to the conventionally produced antibody (Schuster et al. 2007).

Here we summarize the progress for use of *P. patens* as an expression system for recombinant therapeutic glycoproteins.

In vitro cultivation

The life cycle of mosses is dominated by the haploid gametophytic stage. Therefore, it starts by germination of a haploid spore rather than with diploid or even polyploid seeds. Under sufficient light and water supply, the filamentous branched protonema tissue grows (Fig. 1a) which proliferates via apical cell divisions and in which every cell is in direct contact with the humid environment (e.g., liquid medium in suspension culture; Fig. 1b). The subsequent development of buds gives rise to the adult gametophore, consisting of shoot-like, leaf-like, and root-like tissues (Fig. 1c). After development of gametangia and fertilization, the diploid sporophyte grows on top of the gametophore (Reski 1998). In vitro cultivation of all stages of the *Physcomitrella* life cycle is routinely performed on agar plates or synthetic meshes where the moss is fed by liquid medium. However, for biotechnological applications, solid medium is mainly chosen for space-saving storage of different transgenic strains, which can be maintained via biannual sub-cultivation (Frank et al. 2005). Cryo-preservation protocols were established for long-term storage (Schulte and Reski 2004) and implemented in the International Moss Stock Center in Freiburg, Germany (IMSC; http://www.moss-stock-center.org), which serves as a Master Cell Bank (MCB).

In addition, scalable vegetative propagation of protonema tissue in suspension cultures has been established (Fig. 1b). *P. patens* grows photo-autotrophic in a simple medium of inorganic salts under relatively low light conditions between 6 and 70 µE. For the cultivation of auxotrophic strains, various additives were tested (Schween et al. 2003). Differentiation into adult tissues can be easily prevented for several months by regular disruption of the filaments without affecting proliferation or productivity of the strains. For higher uniformity and standardization of cultivation different types of photobioreactors were developed, i.e., stirred glass tanks for a volume up to 15 L (Fig. 1d) or modular, glass tubular bioreactors for larger scale (Hohe et al. 2002; Lucumi and Posten 2006; Lucumi et al. 2005; Perner-Nochta et al. 2007). Batch cultivation in a 10 L glass tank bioreactor reached a density of 0.7 g (dry weight)/L after 9 days of cultivation. The growth rate corresponded to a doubling within 1.2 days when the cultures were aerated with 2% CO_2 (Hohe et al. 2002). In a 30 L tubular pilot reactor (Fig. 1e), parameters for high-density and uniform moss cultures from 1 to 3 g dw/L were determined for batch and continuous cultivation (Lucumi and Posten 2006; Lucumi et al. 2005) with a duration of more than 6 weeks. The tubular system was further improved by establishing a modular 100 L GMP-like photobioreactor unit as well as 300 L wave reactors (http://www.greenovation.com). In general, *Physcomitrella* showed itself as robust, fast, and stably growing in bioreactors compared to most other plant cell cultures (Hohe et al. 2002). Because of this robustness, culture conditions like pH or temperature of the medium may be adjusted to the needs of the produced recombinant protein.

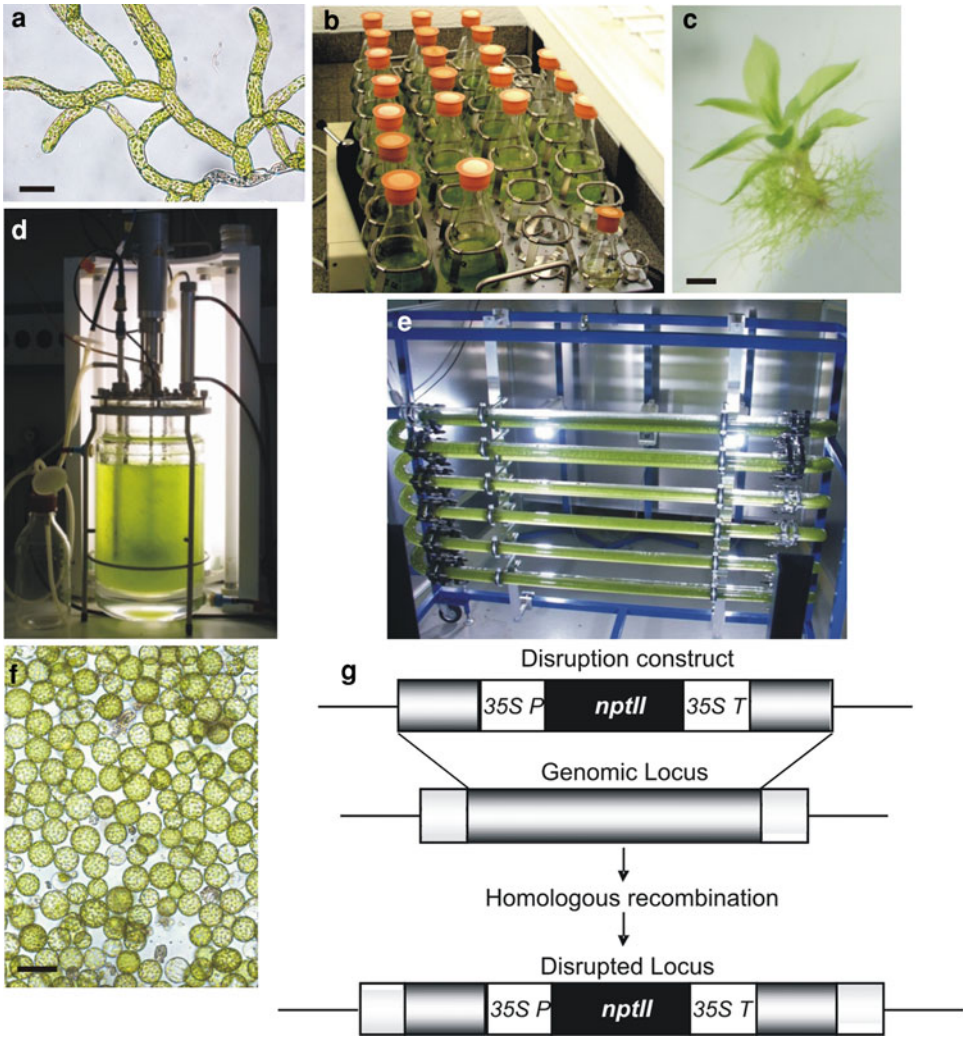

Fig. 1 Moss in vitro cultivation and gene knockout production. **a** Filamentous protonema stage; **b** cultivation of protonema in glass flasks; **c** adult gametophore stage; **d**, **e** propagation of moss in glass tank and tubular photobioreactors, respectively. **f** *P. patens* protoplasts used for transfection and transient secretion of protein products **g** Gene targeting via homologous recombination; a disruption cassette (*white bars* for promoter and terminator sequences, respectively and *black bars* for coding sequence) is flanked by DNA sequences homologous to the gene of interest (*gray bars*). After transfection, the disruption construct is integrated in the homologous genomic locus [modified after (Frank et al. 2005)]. **e** Courtesy of Clemens Posten (KIT, Karlsruhe Institute of Technology). *Scale bars* 50 µm (**a**, **f**), 500 µm (**c**)

The secretory system

Growth as tissue cultures in closed cultivation conditions is advantageous over field production because of improved uniformity as well as safety of production (Hellwig et al. 2004). In addition the setup of a secretory system is enabled which allows easier isolation and purification of the pharmaceutical product. By the addition of the protecting agents polyvinyl pyrrolidone (PVP) and human serum albumin (HSA) to *P. patens* cultures which synthesized the human vascular endothelial growth factor (VEGF), product amounts could be enhanced up to 0.9 mg/g dw (Baur et al. 2005). However, higher concentrations of these additives may result in foam formation or interfere with downstream processing by reduced binding of the product to chromatography columns. Therefore, co-expression of recombinant HSA with the target protein of interest was employed to avoid these effects and was shown to enhance recovery of the target protein up to twofold (Baur et al. 2005).

With the moss secretory system different recombinant therapeutic glycoproteins were successfully expressed, among those human VEGF (Koprivova et al. 2004), erythropoietin (EPO; Weise et al. 2007) as well as the large and heavily modified single-chain protein factor H, a central regulator of the alternative pathway of the human complement system, displaying an important part of the innate immune system of mammals (Buttner-Mainik et al. 2011). At least three different moss-produced recombinant antibodies have been reported so far: two IgG mAb, which were detected in the medium as fully assembled and biologically active proteins (Gorr and Jost 2005; Schuster et al. 2007) and an anti-CD20-scFv antibody fragment (Smidkova et al. 2010).

The genetic toolboxes for improved expression and extracellular targeting were enriched by more than 300,000 *P. patens* expressed sequence tags (ESTs) from different large-scale sequencing projects (Lang et al. 2005; Nishiyama et al. 2003). Based on these data, 5′ regulatory sequences were derived that efficiently drive transgene

expression from plasmid vectors (Horstmann et al. 2004; Jost et al. 2005; Weise et al. 2006). This profound genetic percipience and the excellent amenability of the moss for targeted gene replacements led to the publication of the *P. patens* genome as the first non-vascular plant genome in 2008 (Rensing et al. 2008).

Secretion of the recombinant products can be obtained by the native human signal sequences at the N terminus of the respective proteins. However, in spite of the principle functionality of these human signal peptides in moss, endogenous moss signal sequences turned out to be more efficient for recombinant products (Schaaf et al. 2005). In addition, *P. patens* plastid as well as mitochondria transit peptides and vacuolar signals were identified and characterized by fusion with fluorescent proteins (Gremillon et al. 2007; Kiessling et al. 2000, 2004; Richter et al. 2002; Schaaf et al. 2004). The secretory system has been used for feasibility studies of expression cassettes, transgenic moss strains as well as for expression and purification purposes of new therapeutic target proteins (Gitzinger et al. 2009; Huether et al. 2005; Jost et al. 2005; Koprivova et al. 2004; Schaaf et al. 2005; Weise et al. 2006). For a single-chain antiCD-20-scFv fragment, two moss signal sequences (Schaaf et al. 2005) as well as the rice alpha-amylase and a murine sequence were tested for their effectiveness in secretion of the recombinant protein (Smidkova et al. 2010). Successful secretion of the IgG1 and IgG4 antibodies, however, was facilitated by a signal sequence from another plant species, the *Thuja occidentalis* H1 protein (Gorr and Jost 2005; Schuster et al. 2007). The antibody-producing mosses were created by polyethyleneglycol-mediated co-transfection of protoplasts with two different expression constructs, coding for the light and heavy chains of the immunoglobulins, respectively. With this simple procedure, correct assembly of the secreted IgG molecules was achieved (Gorr and Jost 2005; Schuster et al. 2007).

Added value by engineering of glycosylation

For production of complex secreted glycoproteins, a secretory system is additionally important for navigating the nascent proteins through the endoplasmic reticulum (ER) and the Golgi apparatus, which are responsible for many posttranslational modifications, especially asparagine (N)-linked protein glycosylation. In contrast to the general conservation of protein biosynthesis within the eukaryotic kingdom, N-glycosylation can be species-specific (Rademacher et al. 1988). Therefore, mammalian cell lines are currently preferred for the production of glycoproteins as their glycan patterns are most similar to human sugar structures. Alternative systems have to be adaptable to human glycosylation to contain biological activity or prevent immunogenicity of the recombinant therapeutics. Aside from some differences within sugar residues, plants perform protein N-glycosylation of the complex type which is also common for mammalian glycans (Gomord et al. 2010). The *Physcomitrella* protein N-glycosylation exhibits the same structures as vascular plants (Koprivova et al. 2003; Vietor et al. 2003). Glycosyltransferase genes responsible for key steps of complex-type glycosylation have been identified and isolated from the *P. patens* genome (Koprivova et al. 2003; Parsons, Decker and Reski, unpublished results). The dominant oligosaccharide structures characterized from moss glycoproteins were complex-type bi-antennary glycans with a Man3GlcNAc2 (mannose, *N*-acetylglucosamine) core structure and two terminally attached GlcNAc residues (Fig. 2). This structure is also common on human bi-antennary complex-type glycoproteins (Gomord et al. 2010). In addition, plant glycans may contain two plant-specific residues, a beta1,2-linked xylose as well as a fucose, which is connected to the proximal GlcNAc residue of the glycan core structure with an alpha1,3-linkage (Koprivova et al. 2003; Vietor et al. 2003). These two plant-specific sugar moieties present the most critical differences to human *N*-glycans. While xylose is a sugar not existing in vertebrates, a proximal fucose residue is also present in human glycans. However, here it is connected in a different, alpha1,6-linkage to the glycan core (Lerouge et al. 1998). Both of these sugar residues were shown to confer immunogenicity to a glycoprotein (Bardor et al. 2003; Bencurova et al. 2004; Garcia-Casado et al. 1996; Jin et al. 2008; van Ree et al. 2000). Nearly one-quarter of allergy sufferer exhibited allergy-associated IgE class antibodies which specifically recognized xylose or fucose-containing complex-type glycan structures, even

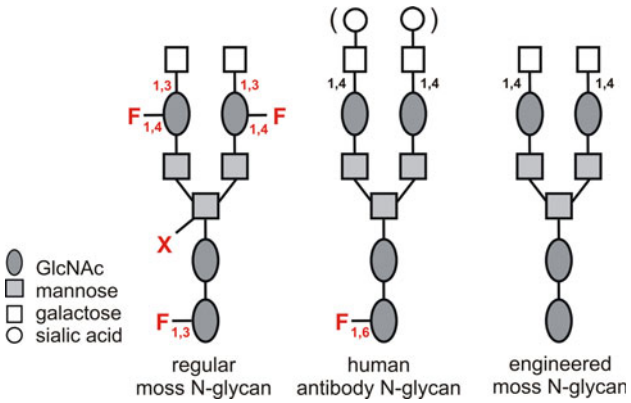

Fig. 2 Typical structures of antibody N-glycosylation. The sugar chains are linked to the protein via the proximal GlcNAc residue. Unmodified moss glycan containing terminal galactose and fucose residues (*left*). Typical bisecting human monoclonal antibody glycan (*middle*). Glyco-engineered moss *N*-glycan providing antibodies with improved ADCC (*right*). *F* fucose; *X* xylose. Sugars or linkages that may result in immunogenicity or low effector function are marked in *red*

if the clinical relevance of carbohydrate-specific antibodies is questioned (Altmann 2007; Mari 2002). However, for therapeutic applications, putatively immunogenic structures have to be omitted (Durocher and Butler 2009; Gomord et al. 2010). In a feasibility study, double mutants deficient of xylose and 1,3 fucose residues at the core glycans were established for *Arabidopsis thaliana* (Strasser et al. 2004) and knockdowns via RNAi were employed to reduce the concentration of the corresponding plant-specific glycosyltransferase mRNAs in *L. minor*, *M. sativa* and *N. benthamiana* (Cox et al. 2006; Sourrouille et al. 2008; Strasser et al. 2008). In contrast to all other plants, *Physcomitrella* offers the unique possibility in that specific genes can be targeted precisely because of the exceptionally high frequency of homologous recombination in the nuclear genomes of vegetative moss cells (e.g., Khraiwesh et al. 2010; Mosquna et al. 2009; Schaefer and Zryd 1997; Strepp et al. 1998). Gene targeting is performed by transfection of moss protoplasts (Fig. 1f) with a disruption cassette flanked by around 500–1,000 bp of moss DNA homologous to the gene of interest (Fig. 1g). By this mechanism, undesirable gene functions can be completely abolished. Moss strains were created which were completely deficient for the mentioned xylose and fucose residues by targeted knockout of the respective glycosyltransferase genes, beta1,2-xylosyltransferase and alpha1,3-fucosyltransferase (Koprivova et al. 2004). With the resulting strains, several products of pharmaceutical value were synthesized, including human VEGF (Koprivova et al. 2004), erythropoietin (Weise et al. 2007), two IgG class antibodies (Gorr and Jost 2005; Schuster et al. 2007) and the complement regulator factor H (Buttner-Mainik et al. 2011).

Further differences of plant *N*-glycans compared to human glycans comprise terminal galactosylation and sialylation. While plant *N*-glycans may contain terminally beta1,3-linked galactose residues, in mammals a galactose is attached via beta1,4-linkage. Human-like galactosylation was partially reached by transformation of tobacco with the human galactosyltransferase gene (Bakker et al. 2001; Palacpac et al. 1999). In *P. patens*, the expression of a human beta1,4 galactosyltransferase gene was reached by "knockin" of this gene into the xylosyltransferase or fucosyltransferase locus, respectively (Huether et al. 2005).

Terminal sialic acids, however, common on mammalian glycoproteins, are generally missing from plant glycans. The presence of terminal sialic acids on human blood proteins enhances their half-life in the circulation (Erbayraktar et al. 2003). By the introduction of five additional mammalian genes encoding the enzymes for biosynthesis, activation, transport and transfer of *N*-acetylneuraminic acid (sialic acid), *N. benthamiana* recently was engineered to efficient sialylation of glycoproteins (Castilho et al. 2010). Recently, a patent (US 7741539) for the production of sialylated proteins in plant cells (e.g., moss) was granted to the company, "greenovation". Therefore, it can be assumed that moss engineering towards glycoprotein sialylation would be feasible in a way comparable to tobacco. However, monoclonal antibodies, comprising the most interesting and largest group of biotech drugs in use and clinical development (Scolnik 2009), contain *N*-glycans, which are only rarely sialylated. Thus, monoclonal antibodies with appropriate glycan patterns could be successfully synthesized in the plant-based protein production hosts *L. minor* and *P. patens* (Cox et al. 2006; Gorr and Jost 2005; Schuster et al. 2007) (Fig. 2).

Glyco-engineering of antibody-producing plant systems turned out to confer an effect to the plant-produced antibodies, which was superior compared to that produced by traditional systems. This could be explained by an increased antibody-dependent cellular cytotoxicity (ADCC), an important effector function of therapeutic antibodies. ADCC means the death of a cancer cell mediated by a killer cell, which has recognized antibodies bound to the surface of the target cell. ADCC is mediated by receptor (especially FcgammaRIIIa; CD16) binding of IgG antibodies. The weak ADCC observed for some anticancer antibodies has to be compensated by applying high concentrations of IgG1 therapeutic antibodies (Preithner et al. 2006). The IgG-receptor-binding affinity was shown to be enhanced when the core fucose residue on human IgG *N*-glycans was missing (Shields et al. 2002; Shinkawa et al. 2003). Increased ADCC of recombinant antibodies produced in glyco-engineered plants was demonstrated for *L. minor* (Cox et al. 2006) as well as for *P. patens* (Nechansky et al. 2007; Schuster et al. 2007). In case of moss, a recombinant monoclonal IgG1 antibody was analyzed which is directed against the tumor-associated glycosylation pattern Lewis Y. This carbohydrate antigen is expressed on the majority of human epithelial carcinomas. When the antibody was produced in glyco-optimized moss strains (devoid of the core fucose residue) it resulted in 40-fold increased lysis capacity compared to the parental antibody which was synthesized in CHO cells (Nechansky et al. 2007; Schuster et al. 2007). These findings impressively demonstrate the suitability of moss bioreactors to produce complex biopharmaceuticals with superior product quality.

Conclusions

The production of therapeutic glycoproteins in the moss bioreactor system offers a safe and efficient alternative to currently used systems. Genome engineering is greatly facilitated by the availability of the *Physcomitrella* genome

sequence. Optimisation of culture conditions and genetic engineering of production lines via precise gene targeting helped to enhance yields and to improve product characteristics/quality. Facilities for production under GMP-standards as well as facilities for long-term storage of Master Cell Banks are in place.

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