Moss bioreactors producing improved biopharmaceuticals
Eva L Decker and Ralf Reski

Plants may serve as superior production systems for complex recombinant pharmaceuticals. Current strategies for improving plant-based systems include the development of large-scale production facilities as well as the optimisation of protein modifications. While post-translational modifications of plant proteins generally resemble those of mammalian proteins, certain plant-specific protein-linked sugars are immunogenic in humans, a fact that restricts the use of plants in biopharmaceutical production so far. The moss Physcomitrella patens was developed as a contained tissue culture system for recombinant protein production in photo-bioreactors. By targeted gene replacements, moss strains were created with non-immunogenic humanised glycan patterns. These were proven to be superior to currently used mammalian cell lines in producing antibodies with enhanced effectiveness.

Introduction

Current recombinant pharmaceuticals are produced mainly in microbial (e.g. Escherichia coli, Saccharomyces cerevisiae) or mammalian systems (mainly Chinese Hamster Ovary cells). While microbial systems are superior in terms of ease of handling and high product yields, mammalian cell lines are favoured for the production of complex biopharmaceuticals that need proper folding and/or correct post-translational modifications [1,2]. Plant-based systems combine advantages of both production systems: As higher eukaryotes, plants synthesise complex multimetric proteins with post-translational modifications closely resembling mammalian modifications. In addition, production in plants eliminates the risk of product contamination by human pathogens possibly hidden in mammalian cell lines or in their complex organic production media [3]. While agriculture offers easy and nearly unlimited adaptation of production scale combined with low-cost production, non-standardised soil and weather prohibit good manufacturing practice (GMP) conditions – indispensable for pharmaceutical production – for field-grown plants [4]. Some of these limitations can be overcome by contained growth in greenhouses.

In contrast, in vitro plant tissue cultures can be grown in precisely controlled environments suitable for GMP conditions [5]. In addition, such systems offer a cheaper downstream processing, especially when the biopharmaceutical is secreted from the cells. Furthermore, host cells secreting the product to the medium can be grown continuously, minimising the need for batch cultures and huge master cell banks.

Efficient transformation protocols were established for a variety of plants [6]. Subsequently, several were tested as production hosts, leading to plant-derived pharmaceuticals that have been submitted for clinical trials. Only recently the first plant-derived pharmaceutical, a veterinary vaccine produced in plant cell culture, received approval for market release [7]. However, the development of large-scale production facilities is not yet realised in many cases and most plant systems are limited in their product spectrum for glycosylated proteins.

Human blood proteins such as antibodies, growth factors, cytokines, and hormones are prime candidates for complex biopharmaceuticals and most are N-glycosylated at an Asparagine. This glycosylation may be important for activity, stability or immunogenicity [2], for example it may influence affinity and effectiveness of antibodies [8,9]. While plants and mammals glycosilate their proteins in a quite similar fashion, some sugar moieties are plant-specific and thus immunogenic [10,11].

A few years ago, a non-seed plant, the moss Physcomitrella patens, was established and commercialised as a production host for complex biopharmaceuticals as it is exceptionally amenable for precise genetic modifications, for example to modify glycosylation pathways, and allows low-cost cultivation in photo-bioreactors [12]. Here we summarise recent progress in establishing moss as a safe and efficient production system for recombinant biopharmaceuticals.

Moss cultivation

Physcomitrella can be grown throughout its complete life cycle under contained conditions in vitro. Haiploid spores germinate giving rise to a filamentous tissue, the protonema. On these tissues leafy gametophores may develop, closely resembling the morphology of seed plants (Figure 1a). Sex organs occur only under inductive...
conditions, giving rise to the diploid sporophyte with persistent spores. While sexual reproduction occurs predominantly when growing moss on agar plates (Figure 1b) and requires inductive conditions, culture in liquid media such as flasks and bioreactors (Figure 1c,d) favours vegetative growth. Techniques have been established to maintain Physcomitrella predominantly in the actively growing filamentous form. In addition, for long-term storage of moss strains, efficient cryo-protocols were established [13] and realised in the International Moss Stock Centre (http://www.moss-stock-center.org/).

Increasing product yields

Well-established protocols for the generation of stable transgenic moss strains [13,19] were modified to establish a transient expression system that allows proof of principle within days [20]. Recently, the Physcomitrella genome was fully sequenced (http://www.cosmoss.org/) as the fourth plant genome after Arabidopsis, rice and poplar. From that, a comprehensive phylogenetic database for plant transcription factors was established [21], facilitating the design of highly effective Physcomitrella production strains. The strengths of various heterologous promoters from plants and animals were quantified in Physcomitrella and compared to endogenous promoters [22], including moss tubulin and actin promoters [23,24], thus generating a set of vectors activating gene expression in Physcomitrella within three orders of magnitude. In addition, several promoters were identified to be inducible, for example by the plant hormone indole acetic acid (IAA, an auxin) [25,26] or by moderate heat shock [27], in Physcomitrella.

Short N-terminal peptide extensions may navigate proteins from the cytosol to specific organelles. For most post-translational modifications, such as N-glycosylation, a passage through the endoplasmic reticulum and the Golgi-Apparatus is required. At the same time, this is the preferred route for the vast majority of extra-cellular proteins. In various studies such signal peptides were evaluated for functionality in Physcomitrella. Even though signal peptides from human sequences were functional in moss, most plant-derived signal peptides were more efficient in targeting recombinant proteins to the secretory system [24,28]. In addition, signal peptides for intracellular storage of proteins in chloroplasts, mitochondria or the central vacuole were identified and characterised via reporter fusions with fluorescent proteins [29–34].

Protein recovery was markedly increased by addition of stabilising agents (polyvinylpyrrolidone (PVP), human serum albumin (HSA)) to the moss medium [35]. However, at higher concentrations these agents lead to foam formation and thus interfered negatively with the cultivation process as well as with downstream processing. Moreover, adding commercial HSA would introduce a putative source of product contamination. These challenges were elegantly overcome by co-expressing recombinant HSA and the recombinant biopharmaceutical in one production strain [35]. These strains produced only tiny amounts of recombinant HSA, far too low to be beneficial when added to control strains. Although the precise mechanism by which HSA stabilises recombinant proteins in the medium is unknown, it is plausible to assume that the co-production of HSA and a second protein in one plant facilitates their interactions already during their joint passage through the endoplasmic reticulum.

The implementation of cross-flow filtration in the tubular bioreactor module allowed a robust and flexible perfusion of the suspension. Thus, secreted recombinant human VEGF (vascular endothelial growth factor) was harvested and concentrated via continuous product separation [16]. The secretory moss expression system was also used for transient protein production allowing quick feasibility studies of expression cassettes, transgenic moss strains, or new products [23,24,28,33,36–37]. This transient system was optimised for expression of recombinant human VEGF up to 10 μg/mL [20].

Engineering product quality

All non-human expression hosts synthesise recombinant proteins with slight differences to their human counterpart. While folding and assembly of multimeric proteins is essentially the same in all higher eukaryotes (e.g. plants and mammals), post-translational modifications are species-specific. This is one of the reasons why mammalian cell lines such as Chinese Hamster Ovary (CHO) cells are currently the system of choice. Therefore, plant systems should be adaptable to human glycosylation to become a realistic alternative to CHO cells. An appropriate production host is genetically well-characterised and amenable for genetic modifications. The Physcomitrella genome has recently been sequenced and large
amounts of EST data comprise more than 95% of the transcribed moss genes [38–40]. Codon usage in moss allows expression of human genes without previous codon adaptation [39]. The genetically most interesting feature of *Physcomitrella* is the high degree of homologous recombination in its nuclear DNA, greatly facilitating precise and base-specific targeted gene knockouts, a possibility still not given for any other plant.

Parallel to the exploitation of the moss transcriptome, protein N-glycosylation was characterised and shown to exhibit the same structures as in higher plants [41,42]. In addition, the glycosyltransferase genes responsible for key steps of complex-type glycosylation were identified and isolated from the moss genome [41].

The most critical differences between plant and human glycosylation are two plant-specific sugar moieties, a xylose connected to the core mannose residue by beta1,2-linkage and a fucose, alpha1,3-linked to the proximal N-acetylgalactosamine (GlcNAc) residue of the core glycan (Figure 2). While xylose is unknown in human glycans, the proximal fucose residue in human glycans is linked in a different way (alpha1,6). Both plant-specific sugars are immunogenic. About one-quarter of individuals with allergies developed antibodies of the allergy-relevant IgE class which specifically recognise xylose or fucose-containing complex-type glycans. However, the clinical relevance of carbohydrate-specific antibodies is still questionable [10,43]. Moss knockout strains were created which lacked the enzymes responsible for xylosylation and fucosylation, beta1,2-xylosyltransferase and alpha1,3-fucosyltransferase, respectively [37]. The resulting double knockout strains were completely devoid of the allergenic sugar residues and were employed to synthesise several products of pharmaceutical value, including human VEGF [37], IgG class antibodies [44,45], and erythropoietin [46].

Further ‘humanisation’ of plant N-glycans comprised expression of a human beta1,4-galactosyltransferase, responsible for linking terminal galactose to mammalian N-glycans [47]. In addition, the engineering of plant genomes in order to attach sialyl residues to the ends of sugar chains was realised recently [48]. The human-like galactosylation was also realised by ‘knockin’ of the human galactosyltransferase gene in the xylosyltransferase and fucosyltransferase locus, respectively, from double knockout moss strains [36,44].
Sialic acid residues are linked to many human blood proteins and enhance their half-life in the circulation [49]. However, monoclonal antibodies – probably the most interesting and largest group of biotech drugs – bear one conserved glycosylation site with an attached N-glycan, and are only rarely sialylated. Furthermore, these occasionally occurring sialic acid residues seem to have no obvious function. Therefore, plant-based antibody production with appropriate glycosylation is a realisable task which was recently fulfilled in Physcomitrella [44].

Moreover, by glyco-engineering via targeted gene knockouts, the quality of plant-produced antibodies could even be superior to that of CHO cells. The pharmacological efficiency of some antibodies produced in mammalian systems is rather disappointing due to weak antibody-dependent cellular cytotoxicity (ADCC), an important effector function of antibodies. ADCC is mediated by receptor (FcgammaRIII) binding of IgG antibodies. This IgG-receptor binding affinity is increased in antibodies with improved ADCC (bottom). F: fucose; X: xylose. Sugars or linkages that may result in immunogenicity or low effector function are marked in red.

Conclusions
Moss bioreactors offer a safe and efficient scalable system to produce complex modified recombinant pharmaceuticals under GMP conditions. Genome engineering and characterisation of transgenic strains is facilitated by ample genomic resources. Optimisation of culture conditions and genetic engineering of production lines via targeted gene replacement helped to enhance product yields and safety. Improved antibody function via glycan optimisation makes the system advantageous compared to current mammalian-based production systems.

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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


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An excellent review about the importance of protein glycosylation for plant-made pharmaceuticals.


36. Co-expression of HSA offered an elegant way to enhance biopharmaceutical concentration in a secretory production platform.


38. Presentation of plant (moss) production strains with the so far most ‘humanised’ protein glycosylation patterns.


The first reconstruction of the protein glycosylation pathway for sialic acid residues in plant model systems.


Production of an anti-tumor antibody in a plant-based system (Lemna minor) with engineered glycan structures (via RNAi) provided enhanced ADCC compared to the CHO-produced antibody.


Production of an anti-tumor antibody in a glyco-optimised plant (moss)-based system provided enhanced ADCC compared to the CHO-produced antibody.