Chapter 15

Gene Targeting for Precision Glyco-Engineering: Production of Biopharmaceuticals Devoid of Plant-Typical Glycosylation in Moss Bioreactors

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Abstract

One of the main challenges for the production of biopharmaceuticals in plant-based systems is the modulation of plant-specific glycosylation patterns towards a humanized form. Posttranslational modifications in plants are similar to those in humans, but several differences affect product quality and efficacy and can also cause immune responses in patients. In the moss *Physcomitrella patens* highly efficient gene targeting via homologous recombination enables glyco-engineering to obtain suitable platform lines for the production of recombinant proteins and biopharmaceuticals. Here we describe the methods which are effective for creating gene targeting constructs and transgenic moss lines as well as confirming successful homologous integration of the constructs and modification of target gene expression.

Key words *Physcomitrella patens*, Gene targeting, Homologous recombination, Knockout construct, Protoplast transformation, Glyco-engineering, Biopharmaceutical production, Plant-made pharmaceuticals, Molecular farming

1 Introduction

Several features of the moss *Physcomitrella patens* make it a superior platform for the production of recombinant proteins in a plant-based system. The dominant, gametophytic phase in the life cycle is haploid and represented by filamentous protonema and leafy gametophores. Protonema, the fast-growing juvenile tissue type [1, 2] used for production purposes, is maintained by regular disruption of the moss material in suspension cultures, either in flasks or different types of photobioreactors [1, 3–8]. The adult gametophore tissue can be grown on solid medium in Petri dishes or multiwell plates [9, 10]. The diploid phase within the moss life cycle is restricted to the sporophyte, which develops on the apices of the gametophores and produces haploid spores upon maturation and after meiosis. The photoautotrophic growth in controlled conditions and contained systems [5, 10–12] and the possibility

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of secretion of products into the surrounding simple mineral medium [13, 14] are advantageous for the production of recombinant proteins and facilitate downstream processing. The *Physcomitrella* genome has been sequenced [15], a prerequisite for targeted alterations of the DNA sequence, and multiple tools to access this information are available on www.cosmoss.org [16]. Additionally, a collection of gene expression data covering different developmental stages and growth conditions is available at www.genevestigator.com [17]. Until now, *Physcomitrella* is the only plant system in which the generation of targeted gene knockouts or insertions of transgenes are feasible [18–22] with efficiencies similar to yeast [23, 24]. For gene targeting the mechanism of homologous recombination (HR) is utilized, which enables the repair of DNA double-strand breaks in somatic cells and the mixture of genetic material during meiosis [25].

A classical gene-targeting construct for the polyethylene glycol (PEG)-mediated transfection of *Physcomitrella* protoplasts consists of a selection marker, including promoter and terminator, flanked by regions homologous to the moss genome at the insertion site (Fig. 1a). In case of a knockout construct, the selection marker will

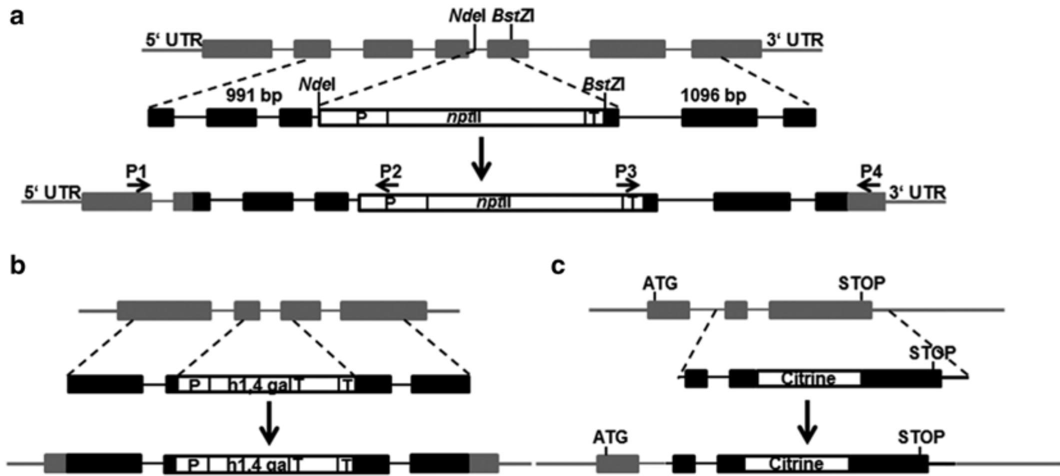

Fig. 1 Gene structure, targeting constructs, and genomic *loci* after the integration of a knockout construct (**a**), knockout-knockin construct (**b**), and targeted insertion of a reporter (**c**) into the genome of *Physcomitrella*. Exons are presented as *rectangles*, introns as *thin lines*, and UTRs as *bold lines*. *Grey color* represents the genomic *locus*, *black* the homologous regions of the targeting construct, and *white* the selection marker or transgene inserted via the constructs. *P* Promoter, *T* Terminator. (**a**) Δ*fuc-t* knockout (modified after [26]) in which the α1,3-fucosyltransferase from moss is disrupted by replacement of a central part of the gene by the selection marker *nptII* using the restriction sites for *NdeI* and *BstZI*. PCR-based screening of the plants can be done using the primer pairs P1 and P2 for correct 5′-integration, and P3 and P4 for correct 3′-integration. To prove the loss of transcript using cDNA as template the primers P1 and P4 are used for RT-PCR. (**b**) Δ *galt1-h1,4galT* knockout-knockin in which the β1,3-galactosyltransferase 1 from moss was replaced by the human β1,4-galactosyltransferase (h1,4 galT) (modified after [28]). (**c**) Internal tagging of a target gene via targeted insertion of the fluorescent marker citrine under the control of the respective moss promoter (modified after [32])

disrupt or replace the respective gene partially or completely. Gene targeting can also be used for targeted insertion of a transgene, either by replacing the native moss gene, e.g., a gene responsible for unwanted glycosylation [26–29] (Fig. 1b), by inserting into a neutral locus [30], or by fusing a reporter gene to the gene of interest [31, 32] (Fig. 1c). The flanking regions homologous to the corresponding sequence in the moss genome are necessary for the targeted integration, as they enable the sequence specificity of the HR mechanism, leading to the recombination event exchanging the native locus in the moss genome by the transgene. Due to the mechanism of HR it is advisable to deliver the DNA for stable integration of the targeting construct into the genome in a linearized way [23, 33], while for transient expression circular DNA is delivered [13]. The efficiency of gene targeting is drastically influenced by different features of the construct: For high efficiencies the length of the homologous regions should be 800 base pairs and longer, with approximately the same size for both sides. In addition it is advisable to release the gene-targeting construct from the vector backbone by restriction digest in such a way that ends homologous to the moss genome without foreign bases originating from the vector backbone are generated [23]. The delivery of the DNA to the moss genome used for the transformation of protoplasts is facilitated by $Ca(NO_3)_2$ providing divalent cations necessary for its uptake [34]. After transfection, complete plants directly regenerate from the protoplasts without a callus phase or the need for the addition of phytohormones [35, 36]. For selection of transgenic plants the appropriate antibiotic has to be added to the growth medium. For the generation of multiple knockouts several selection markers are established for the use in *Physcomitrella*: G418/neomycin (nptII), hygromycin (hpt), zeocin (zeo), and sulfadiazine (sul) resistance cassettes [37]. After an initial screening with direct genomic PCR from single moss shoots [38], the lines will be validated via RT-PCR or qPCR for the loss of the transcript in the case of a knockout or presence of the transgene mRNA, respectively. In case of insertion of a heterologous gene product, subsequently Western blotting or ELISA will be used for proving integrity and yields of the protein product [14, 39]. The number of transgene copies inserted into the genome may be tested via Southern blotting or a qPCR-based method. Gene targeting has been employed for glyco-engineering approaches of *Physcomitrella*, resulting in the removal of putatively immunogenic N-glycosylation patterns consisting of the core xylose and fucose [26] as well as Lewis-A epitopes [28] and additionally O-glycosylation by prolyl-hydroxylation [29]. The progress of glycoprotein production in glyco-engineered moss was reviewed recently [12, 40].

2 Materials

2.1 Plant Material and Cultivation

1. The *P. patens* (Hedw.) Bruch & Schimp wild-type strain which was used for genome sequencing, different accessions and transgenic lines are available from the International Moss Stock Center (IMSC Freiburg, http://www.moss-stock-center.org) which provides long-term storage and master cell banks via cryopreservation [41].
2. *Physcomitrella* is cultured photoautotrophically in mineral Knop medium [42]: 250 mg/L KH_2PO_4, 250 mg/L KCl, 250 mg/L $MgSO_4$, 1,000 mg/L $Ca(NO_3)_2$, 12.5 mg/L $FeSO_4$, including microelements (50 µM H_3BO_3, 50 µM $MnSO_4 \times H_2O$, 15 µM $ZnSO_4 \times 7\ H_2O$, 2.5 µM KJ, 0.5 µM $Na_2MoO_4 \times 2\ H_2O$, 0.05 µM $CuSO_4 \times 5H_2O$, 0.05 µM $CoCl_2 \times 6H_2O$) [43]. Adjust pH to 5.8 with KOH. Solid medium contains 12 g/L purified agar. Sterilize by autoclaving (*see* **Note 1**).
3. Two kinds of sterile controls are used to check the sterility of cultures on solid medium (in 6 cm Petri dishes): Knop medium supplemented with 1 % (*w*/*v*) glucose and Lysogeny Broth (LB: 10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, adjust pH to 7.0 with NaOH, add 15 g/L Bacto Agar) [44]. Sterilize by autoclaving.

2.2 Protoplast Isolation, Transfection, and Regeneration

1. 100–200 mL protonema suspension culture grown in Knop medium pH 4.5, either from the bioreactor or flasks as starting material (around 250 mg/L of dry weight). Last disruption of the material taken from flasks is 7 days and additional change to fresh medium 1 day prior to isolation of protoplasts (*see* **Note 2**).
2. Mannitol solution: 0.5 M mannitol, adjust to pH 5.8 with KOH and the osmolarity to 560 mOs using mannitol. Sterilize by autoclaving.
3. 4 % (*w*/*v*) Driselase solution: Dissolve 0.4 g of Driselase in 10 mL of 0.5 M mannitol and vortex briefly (*see* **Note 3**). Keep the solution protected from light by covering and incubate for complete dissolution of the enzyme for at least 45 min at room temperature on a rotating table. Centrifuge at $2{,}300 \times g$ for 10 min and sterilize the supernatant by filtration.
4. 3 M medium: 5 mM $MgCl_2$, 0.1 % (*w*/*v*) 2-(N-morpholino) ethanesulfonic acid (MES), 0.48 M mannitol. Adjust the pH to 5.6 with KOH and the osmolarity to 580 mOs. Sterilize by autoclaving.
5. Regeneration medium: Knop medium supplemented with 5 % (*w*/*v*) glucose and 3 % (*w*/*v*) mannitol. Adjust the pH to 5.8

with KOH and the osmolarity to 540 mOs using mannitol. Sterilize by filtration.

6. Polyethylene glycol (PEG): 40 % (*w*/*v*) PEG_{4000} in 3 M medium, sterilize by filtration.
7. For targeted integration the DNA is linearized prior to transformation to release the gene-targeting construct from the vector backbone: In this case 10–25 μg of DNA is used per transformation (*see* **Note 4**), for transient approaches up to 50 μg of the circular plasmid. The DNA is sterilized by standard ethanol precipitation. Solve the pellet completely in 0.1 M $Ca(NO_3)_2$ in a total volume of 100 μL per transformation.
8. Special equipment: Two syringes (10 or 20 mL) and sterile filters (0.22 μm mesh size), glass tubes (15 mL volume with screw caps and round bottoms, two for isolation of protoplasts and one further for each transfection), 10 mL glass or plastic pipettes (normal and wide opening), plastic tips (normal + cut tips) for 1 mL micropipette, protoplast sieves (Wilson, UK): 2 × 100 μm and 1 × 50 μm mesh size, Fuchs-Rosenthal or comparable chamber. Autoclaved cellophane sheets.
9. Selection medium: Prepare standard solid growth medium, which needs to be supplemented with 1 g/L MES in case of zeocin selection, and sterilize by autoclaving. Let the medium cool to 50 °C and add the appropriate amount of antibiotic (G418: 12.5 mg/L, hygromycin 25 mg/L, zeocin: 100 mg/L, sulfadiazin 100 mg/L).

2.3 Screening Procedure

1. Buffer for direct PCR: 20 mM $(NH_4)_2SO_4$ and 75 mM Tris–HCl pH 8.8 mixed with 1 mL/L Tween20.
2. 3 mM spermidine solution: Dissolve 38.2 mg of spermidine trihydrochloride in 50 mL of H_2O, and sterilize by filtration.

3 Methods

3.1 Design and Cloning of the Knockout Construct

1. Identify the appropriate genomic region for your knockout (www.cosmoss.org). A central part of the gene, coding for a part important for the protein function, or the whole coding sequence should be deleted upon insertion of the targeting construct (*see* **Note 5**).
2. Pick the 5′- and 3′-regions necessary for homologous integration of the transgene into the moss genome, neighboring the region you wish to delete. For high efficiency of gene targeting both homologous flanks should be about the same size; an optimum is reached with 800 bp in length [23]. The homologous regions will be isolated from moss genomic DNA by PCR. The selection marker is either released from a suitable

plasmid by restriction digest or also amplified via PCR. Established selection markers are nptII, hpt, zeo, or sul. Dependent on the cloning strategy it might be necessary to insert restriction sites or overlapping regions to the next fragment via the primers used for amplification of the different fragments.

3. If the selection marker is not included in the gene-targeting construct (Fig. 1b, c) co-transformation with a circular plasmid carrying the antibiotics resistance may be performed (*see* **Note 6**).
4. To facilitate the release of the gene-targeting construct from the vector backbone in such a way that ends homologous to the moss genome are created, it is advisable to include endogenous restriction sites from the moss genomic sequence at the ends of the homologous flanks. Otherwise it is possible to use Type IIS restriction enzymes (e.g., *Lgu*I) which cleave DNA outside the recognition site.
5. Cloning of the constructs can be done either in a classical way using restriction digest and ligation [44] or by PCR-based methods, e.g., Gibson cloning, a one-step isothermal in vitro recombination technology to join several DNA fragments [45].

3.2 Culture of Moss Material

1. The standard growth conditions are 22 °C with a 16/8-h light/dark photoperiod and light intensity of 50–70 μmol/m^2/s^1. Flasks are placed on rotating shakers with 125 rpm speed.
2. Protonema is maintained by regular cutting of the material. For cultures growing in flasks (500 mL Erlenmeyer flasks with 180 mL Knop medium, covered with a lid permeable to air), the moss is disrupted weekly using an Ultra-Turrax (Ika, Staufen, Germany) with a speed of 18,000 rpm for 1 min. Subsequently the medium is filtered through a sieve with 100 μm mesh size and the moss transferred to fresh medium.
3. Axenic growth of the cultures is checked regularly with material taken during subculture of the moss. The material is transferred to Knop with 1 % (*w/v*) glucose as well as to LB plates. The sterile controls are incubated at 22 °C for 3 weeks (for LB) and 2 months (for Knop with glucose) and checked for growth of contaminants.

3.3 Isolation of Physcomitrella Protoplasts

1. Prepare the Driselase solution as described in Subheading 2.
2. Filtrate 100–200 mL of protonema culture from bioreactor or liquid culture in flasks with a 100 μm protoplast sieve. Transfer the material with tweezers to a Petri dish (9 cm diameter) and add 8 mL of 0.5 M mannitol solution. Add 8 mL of the

Driselase stock solution, resulting in a final concentration of Driselase of 2 % (*w/v*) (*see* **Note 3**). Seal the Petri dish, protect from light, and incubate for 45 min at room temperature on a rotary shaker with slow agitation.

3. Using a pipette with wide opening (*see* **Note 7**), pass the moss material slowly and successively through a protoplast sieve with a mesh size of 100 μm. Rinse the Petri dish with additional 3 mL of 0.5 M mannitol and pass it through the same sieve. The protoplasts released from the filaments by cell wall digestion are in the filtrate.
4. Pass the filtrate through a second protoplast sieve with a mesh size of 50 μm. Rinse the sieve box again with 3 mL of 0.5 M mannitol and pass it through the filter; transfer the filtrate containing the protoplasts in equal parts to two glass tubes.
5. Centrifuge the filtrate in the glass tubes for 10 min at $45 \times g$ (set slow speed for acceleration and brake). Carefully discard the supernatant by pipetting and wash the protoplasts by resuspending each pellet in 10 mL of 0.5 M mannitol by gentle rolling of the tubes between your hands.
6. Centrifuge again for 10 min at $45 \times g$, discard the supernatant, and resuspend each pellet in 5 mL of 0.5 M mannitol. Combine both samples in one of the glass tubes and mix well by gentle rolling.
7. Take a 100 μL aliquot with a cut pipette tip and determine the protoplast number using a counting chamber. Meanwhile centrifuge protoplasts (combined in one tube) again for 10 min at $45 \times g$. Discard the supernatant and resuspend the pellet in 3 M medium adjusting a density of 1.2×10^6 protoplasts/mL (*see* **Note 8**).

3.4 Transformation of *Physcomitrella* Protoplasts

1. For each transfection, transfer 100 μL of DNA solution in 0.1 M $Ca(NO_3)_2$ into a glass tube and carefully add 250 μL of the protoplast solution (300,000 protoplasts) using a cut pipette tip. Add 350 μL of the PEG solution and mix well by gentle rolling. Incubate the mixture for 30 min at room temperature, and mix again every 5 min by gentle rolling.
2. Dilute the mixture with 3 M medium every 5 min, successively adding 1, 2, 3, and 4 mL, and carefully mix the solution after each step by rolling the tube.
3. Centrifuge for 10 min at $45 \times g$, discard the supernatant, and resuspend the protoplasts in 4 mL regeneration medium by gentle rolling. Transfer the protoplast solution of each transfection reaction into two wells of a 6-well culture plate using cut pipette tips.

3.5 Regeneration and Selection of Transgenic Plants

1. Seal the plate (*see* **Note 9**) and incubate overnight in the dark at 22 °C followed by incubation under standard growth conditions.
2. If selection is carried out with G418 or hygromycin, the protoplasts are transferred to solid Knop medium covered with cellophane sheets after 10 days of regeneration in liquid medium (1–1.5 mL per plate). After further 3 days of growth on solid standard medium, transfer the cellophane sheets on which the protoplasts adhere to selective medium for 2 weeks (*see* **Note 6**).
3. Selection using zeocin or sulfadiazine will be started after the first cell division in the majority of protoplasts (around 1 week after transformation). Add the antibiotic to a final concentration of 50 μg/mL and incubate for further 3 days. Transfer the protoplasts to solid selective medium covered with cellophane sheets (1–1.5 mL per plate). Duration of the first phase of selection is 3 weeks; change the selective medium once after 1.5 weeks (*see* **Note 6**).
4. For release of selection, the cellophane sheets will be transferred to medium without selection for 2 weeks, followed by at least one other cycle of selection of 2-week length for all antibiotics.
5. Once the plants are big enough to be picked from the cellophane with a needle or tweezers, isolate single clones and put them to the medium directly.

3.6 Screening Procedure

1. Direct PCR (modified from [38]) can be carried out as soon as it is possible to pick a part of a young plantlet. Transfer the material with tweezers to a tube with 40 μL of $(NH_4)_2SO_4$ buffer and crush with either tweezers or small glass beads. After incubation for 30 min at 45 °C, freeze the samples and reheat again for 30 min at 45 °C before the first PCR reaction (*see* **Note 10**). If using Taq DNA polymerase, a standard PCR reaction will be set up using 3 μL of this crude extract and 2 μL of 3 mM spermidine solution in a total volume of 25 μL. Use one primer pair amplifying the selection marker or a housekeeping gene like TBP, coding for a TATA-binding protein, or C45, coding for the ribosomal protein L21 [20, 29] to check for successful DNA extraction.
2. Primer design: To check for the correct integration of the transgene into the genome at the 5′- and 3′-location, two primer pairs are designed (Fig. 1a): each of them with one primer binding upstream (5′-region, P1 in Fig. 1a) or downstream (3′-region, P4 in Fig. 1a) of the homologous flanks of the knockout construct oriented towards the direction of the selection marker. The other primer of each pair is located in the

5′-part (e.g., promoter, P2 in Fig. 1a) or the 3′-part (e.g., terminator, P3 in Fig. 1a), respectively, of the selection marker oriented towards the genomic region outside the construct (*see* **Note 11**). Additionally it is also possible to check for the loss of the wild-type *locus* or in case of the insertion of heterologous DNA for the presence of the transgene (e.g., with primers P1 and P4 in Fig. 1a).

3. To confirm the loss of transcript in a knockout (or the gain of a transcript in an overexpression line), RT-PCR (e.g., with primers P1 and P4 in Fig. 1a) or qPCR is done. For RNA isolation use a phenol/chloroform extraction method or a commercially available product for guanidinium thiocyanate-phenol-chloroform extraction, followed by cDNA synthesis as required for the further PCR application.

4 Notes

1. For the generation of transgenic plants, all steps starting from the cell culture and protoplast isolation must be carried out in sterile conditions: work in a laminar airflow cabinet and make sure that all solutions and equipment are sterilized in the appropriate way.
2. For pre-culture of protonema in flasks the pH of the Knop medium needs to be shifted from 5.8 to 4.5 1 week prior to protoplast isolation, with an additional change to fresh Knop medium pH 4.5 1 or 2 days before the experiment. Sterilize this medium by filtration, as autoclaving would result in a shift of pH. If there is no possibility to set up liquid cultures, protonema also can be grown on solid medium covered with cellophane sheets. For subculture the protonema from these plates is harvested weekly with tweezers, and the material transferred to an appropriate vial in some liquid medium for cutting, filtered, and subsequently spread on new plates.
3. It is possible to use half the amount of Driselase resulting in a final concentration of 1 % (*w*/*v*) for cell wall digest, if the incubation time of the moss material will be increased to 2 h.
4. When preparing the DNA for transformation, there are two possibilities: a mixture of insert and vector backbone; in this case always check a small sample on an agarose gel to ensure that the digestion is complete. This option is quick and cheap, but it is possible that illegitimate integrations of the vector backbone into the moss genome occur. Additionally keep in mind that the amount of targeting construct is dependent on the molar ratio of the whole plasmid to the targeting construct. The procedure of purifying the targeting construct using a

column- or silica gel-based method from the vector backbone is more time consuming and expensive and there might be the risk of losing a certain proportion of the DNA during the process. Usually 10–15 μg of purified insert is used for one transformation.

5. Always design the knockout construct in a way that at least a part of the coding sequence is deleted. At least one side of the deleted part needs to be located in an exon.
6. If the gene-targeting construct does not include a selection marker (Fig. 1b, c), co-transfection of the respective circular plasmid carrying the antibiotic resistance and the linearized targeting construct will be done in a molar ratio of up to 1:10. Once the regenerating protoplasts are transferred to the selective medium there will be no release of the selection. Selection will last for 3–6 weeks with a change to fresh plates every 1.5 weeks.
7. Protoplasts are very sensitive to shear stress: perform all steps after the cell wall digestion very carefully, using pipettes with wide openings and cut plastic tips (cut with scissors prior to autoclaving), mixing by gentle rolling of the tube, and perform all centrifugation steps at slow speed with slow acceleration and braking.
8. Calculation of the amount of protoplast after counting in the Fuchs-Rosenthal chamber: number of protoplasts per large square $\times$ 5,000 = number of protoplasts/mL (example: 20 protoplasts/large square correlate to 1×10^5 protoplasts/mL).
9. To allow aeration, the plates are usually sealed with Parafilm and a small proportion of Micropore (1/8 of the length). Plates with regenerating protoplasts on cellophane sheets or for long-term storage are sealed with Parafilm only.
10. If the protocol using the crude DNA extraction for direct PCR is not successful, try one of the commercially available kits for direct PCR from plant material or do a proper extraction of genomic DNA using one of the various plant protocols (e.g., using CTAB) or kits for the isolation of genomic DNA from plants. In this case a higher amount of moss material will be needed.
11. For the screening procedures never forget to include the proper controls. Always include a wild-type sample and a negative control. If a multiple transgenic plant based on a different genotype other than the wild type was generated, then the parental line needs to be included. In some cases a positive control using a dilution of the plasmid containing the knockout construct might be helpful.

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