Chapter 5

Production of Recombinant Glycoproteins in *Nicotiana tabacum* BY-2 Suspension Cells

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Abstract

This protocol describes a robust method to obtain transgenic *Nicotiana tabacum* BY-2 cells that produce glycoproteins of interest via *Agrobacterium tumefaciens* transformation. Compared to biolistics-based transformation, this procedure requires only standard laboratory equipment.

Key words Agrobacterium tumefaciens, Glycoproteins, Nicotiana tabacum BY-2, Recombinant proteins, Transformation

1 Introduction

Plants represent an interesting alternative recombinant protein production platform for several reasons: low cultivation costs, easy scalability, presence of typical eukaryotic post-translational modifications (e.g., N-glycosylation) and absence of human pathogens. Plant cell suspensions grown in bioreactors offer the extra advantages of a short cell cycle and the possibility to easily implement GMP-compatible process conditions [1]. Consequently, several biopharmaceutical proteins have been produced in plant suspension cells. Among them, glucocerebrosidase, produced in carrot cells by Protalix Biotherapeutics for enzyme replacement therapy of Gaucher disease, was approved by FDA in May 2012. Also many other proteins produced in *Nicotiana tabacum* Bright Yellow-2 (BY-2) suspension cells reached clinic trials [2].

BY-2 cell transformation by co-cultivation with *Agrobacterium tumefaciens*, containing a binary vector with a neomycin phosphotransferase expression cassette as selectable marker, was already reported in the eighties [3]. This pioneering study on *N. tabacum* BY-2 cells as well as a later study on *N. tabacum* cv. Xanthi highlighted the physiological state of the tobacco cell suspension as well as the *A. tumefaciens* strain characteristics as two key parameters for

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successful transformation and generation of transgenic tobacco BY-2 cell lines [3, 4]. In addition, it is well known that most virulence genes of *A. tumefaciens* are expressed at low pH and in response to phenolics such as acetosyringone, and that their expression is enhanced in the presence of aldoses (e.g., 10 mM glucose) [5]. It was further reported that the *A. tumefaciens* strain LBA4404 that constitutively expresses a mutant *virGN54D* gene is far more effective than others in generating transformed BY-2 calli [6]. This particular virGN54D LBA4404 *A. tumefaciens* strain was initially generated to enhance stable transformation performance over a wider range of plant species also including the pharmaceutically relevant plant *Catharanthus roseus* [7, 8].

We have been using this strain in our laboratory to generate many different BY-2 transgenic lines that express plasma membrane transporters or proteases of plant origin [9–21] as well as various secreted recombinant glycoproteins of pharmaceutical interest like antibodies [22–24] or glycoproteins from virus envelopes [25, 26]. Anyhow, secreted glycoproteins produced in wild-type BY-2 cells carry N-linked glycans featuring plant residues (beta-1,2-xylose and/or alpha-1,3-fucose) that do not occur in mammalian expression hosts or the human body. When produced in a BY-2 cell line in which the beta-1,2-xylosyltranferases and alpha-1,3-fucolsyltransferases genes were inactivated through CRISPR/Cas9 (XT/FT-KO) [27, 28], the glycoproteins display a glycosylation profile devoid of non-human glyco-epitopes. Here we will describe the methods for transformation of tobacco BY-2 cells and selection of elite transgenic cell lines that are currently used routinely in our laboratory.

2 Materials

2.1 Reagents

1. *Nicotiana tabacum* BY-2 wild-type [29] or XT/FT-KO [27] cells.
2. *Agrobacterium tumefaciens* LBA4404 virG [6, 7].
3. *Escherichia coli* DH5-alpha.
4. Binary plasmid such as pBIN, pPZP, or pCambia backbones containing a kanamycin or hygromycin resistance gene cassette.
5. 2YT: 16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, 1 g/L glucose, 0.2 g/L $MgSO_4$. $7H_2O$. Autoclave and store at room temperature.
6. 2YT RGX: 16 g/L tryptone, 10 g/L yeast extract, 5 g/L NaCl, 1 g/L glucose, 0.2 g/L $MgSO_4.7H_2O$. Autoclave and store at room temperature. Add 20 mg/L rifampicin, 40 mg/L gentamicin, X = 50 mg/L spectinomycin or 50 mg/L kanamycin, before use.

7. SOC: 20 g/L tryptone, 5 g/L yeast extract, 0.5 g/L NaCl, 0.186 g/L KCl, 0.95 g/L $MgCl_2.6H_2O$, 3.6 g/L glucose. Autoclave and store at room temperature.
8. MS medium: 4.4 g/L Murashige and Skoog medium (without sucrose, agar, IAA, and kinetin), 30 g/L sucrose, 0.2 g/L KH_2PO_4, 2.5 mg/L thiamine, 50 mg/L myoinositol, 0.2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), pH 5.8 (adjusted with 1 N KOH). Autoclave and store at room temperature.
9. MS# medium: 4.4 g/L Murashige and Skoog medium (without sucrose, agar, IAA, and kinetin), 30 g/L sucrose, 0.2 g/L KH_2PO_4, 2.5 mg/L thiamine, 50 mg/L myoinositol, 1.8 g/L glucose, 0.2 mg/L 2,4-D, pH 5.3 (adjusted with 1 N KOH). Autoclave and store at room temperature.
10. MSCCX medium: 4.4 g/L Murashige and Skoog medium (without sucrose, agar, IAA, and kinetin), 30 g/L sucrose, 0.2 g/L KH_2PO_4, 2.5 mg/L thiamine, 50 mg/L myoinositol, 0.2 mg/L 2,4-D, pH 5.8 (adjusted with 1 N KOH) (± agar 8 g/L). Autoclave, add 400 mg/L carbenicillin, 500 mg/L cefotaxime, X = 100 mg/L kanamycin or 30 μg/L hygromycin, and pour the plates.
11. Acetosyringone stock solution: 100 mM acetosyringone in ethanol.
12. Grinding buffer: 250 mM sorbitol, 60 mM Tris, 2 mM $Na_2EDTA.2H_2O$, (with or without 10 mM dithiothreitol) pH 8.0 (HCl 1 N).

2.2 Equipments

1. Gene-Pusler Electroporator.
2. Gene-Pulser electroporation cuvette 0.1 cm gap.
3. 6-well plates.
4. Parafilm.
5. Glass Beads (0.75 mm–1 mm).
6. 50-mL Falcon tubes.
7. 15-mL Falcon tubes.
8. 90 mm Petri dishes.
9. 2-mL screw cap tubes.
10. Centrifuge with a 90° swing-out rotor, e.g., Rotafix 32 A (Hettich).
11. Tissue homogenizer Precellys 24 (Bertin Technologies).
12. Orbital shaker for *N. tabacum* BY-2 liquid cultures.

3 Methods

3.1 Obtaining an Agrobacterium tumefaciens with the Binary Plasmid of Interest

1. Prepare electro-competent *A. tumefaciens* LBA4404 virG cells according to [30] and electroporate a 40-μL aliquot with 100 ng binary plasmid in a 0.1 cm cuvette using a commercially available gene pulser and pulse controller. Voltage (1.25 kV), capacitance (25 μF) and resistance (400 Ω) are kept constant. Pulse period should be around 8 ms under these conditions.
2. Add immediately 1 mL of SOC, recover for 4 h in a water bath at 28 °C and spread 200 μL onto a solid 2YT RGX agar plate.
3. Incubate for 3 days at 28 °C.
4. Pick an isolated colony, inoculate 10 mL 2YT RGX and incubate with shaking (120 rpm) for 16 h at 28 °C.
5. Purify plasmid DNA (*see* **Note 1**) and check the integrity of the binary plasmid as well as the presence of the virG plasmid by restriction analysis (*see* **Note 2**).

3.2 Maintenance of Nicotiana tabacum BY-2 Cell Culture

1. Cultivate the wild-type *N. tabacum* BY-2 cell line (or XT/FT-KO) in MS medium in darkness at a temperature of 25 °C with agitation (90 rpm).
2. Subculture by inoculating 50 mL of fresh MS medium in a 250 mL Erlenmeyer with 4 mL of a 7-day old culture.

3.3 Growth of Agrobacterium tumefaciens and Preparation of the Inoculum

1. Streak a line of the recombinant LBA4404 VirG *A. tumefaciens* strain of interest on 2YT RGX agar plate and incubate the plate for 3 days at 28 °C.
2. Transfer half of the line into 10 mL of 2YT RGX medium (*see* **Note 3**), vortex vigorously and incubate the culture for 16 h at 28 °C with agitation (120 rpm).
3. Measure the OD_{600}.
4. Dispense an appropriate culture aliquot into 10 mL of 2YT RGX medium so that the starting OD is 0.6. Incubate the culture at 28 °C with agitation (120 rpm) for 4–5 h until the OD_{600} reaches 1.5.
5. Transfer 1 mL of the culture in an Eppendorf tube and centrifuge at 6000 × *g* for 2 min.
6. Remove the supernatant and wash the cells in 1 mL of MS# by pipetting.
7. Centrifuge at 6000 × *g* for 2 min and remove the supernatant.
8. Resuspend in 1 mL of MS# and mix by pipetting.
9. Use the *A. tumefaciens* suspension for co-cultivation with BY-2 cells.

3.4 Co-cultivation of Nicotiana tabacum BY-2 Cells with Agrobacterium tumefaciens

1. Centrifuge 25 mL of a 4-day old BY-2 cell culture (wild-type or XT/FT-KO) for 5 min at 700 × *g* in a 50-mL Falcon tube, discard the supernatant.
2. Wash the cells once in 40 mL MS#.
3. Centrifuge for 5 min at 700 × *g*.
4. Discard the supernatant and resuspend the cells in 25 mL MS# supplemented with 50 μM acetosyringone (from a 100 mM stock solution prepared in ethanol).
5. In each well of a 6-well plate, mix 4 mL of BY-2 cells in MS# with increasing volume of the *A. tumefaciens* suspension (0, 10, 20, 50, 100, and 200 μL/well).
6. Seal the plate with Parafilm and incubate the plate for 2 days in the dark at 25 °C without agitation (*see* **Note 4**).
7. Collect the cells from each well in a 15-mL Falcon tube, wash the corresponding well with 5 mL MS, mix both suspensions, and centrifuge for 5 min at 700 × *g*.
8. Wash the cells once in 10 mL MS and then once in 10 mL MSCCX by inverting the tubes and centrifuging as before. Remove the supernatant but leave a total volume of 1.8–2.0 mL in each Falcon tube.
9. Resuspend the cells and evenly spread those on a solid MSCCX agar plate (1 plate/co-cultivation) (*see* **Note 5**). Seal each plate with Parafilm and incubate in the dark at 25 °C.
10. Calli should appear after 3–4 weeks.

3.5 Selection of Transgenic BY-2 Cell Lines

1. When the calli are large enough to pick them easily from the non-transformed cell layer, transfer as many calli as necessary with a sterile scalpel onto the surface of a MSCCX plate (9–12 calli/plate). Seal each Petri dish with Parafilm and incubate in the dark at 25 °C.
2. Transfer part of each callus every 2–3 weeks on a new medium. After two passages on MSCCX, use MSX plates since there is no risk anymore of *A. tumefaciens* contamination.
3. Transfer 18 to 24 calli with a sterile scalpel each in a well of a 6-well plate containing 4 mL MSX. Homogenize the cell suspension by pipetting up and down several times with a 5-mL sterile tip. Seal the plates with Parafilm and incubate for 5–7 days at 25 °C in the dark with a 90-rpm rotation.
4. Transfer 400 μL of these starting cultures to 4-mL fresh MSX medium in new 6-well plates and incubate for 7 days at 25 °C in the dark with a 90-rpm rotation.

3.6 Selection of BY-2 Elite Lines

1. Filter cells from each transgenic culture on Miracloth and collect the culture medium (if the protein of interest is secreted) and/or the cells (if the protein of interest is intracellular).
2. To prepare the total soluble protein fraction, transfer 200–300 mg cells into a screw cap 2-mL tube with 300 µL of glass beads (0.75–1 mm). Add 700 µL of grinding buffer (with or without dithiothreitol) (*see* **Note 6**) supplemented with 1 mM phenylmethylsulfonylfluoride and a protease inhibitor cocktail (*see* **Note 7**).
3. Grind the cells (Precellys 24 tissue homogenizer) by three successive runs at 5000 rpm for 40 s with 2 min intervals on ice.
4. Centrifuge the suspension first at 2600 × *g* for 5 min at 4 °C, then at 9300 × *g* for 7 min at 4 °C, and finally at 136,000 × *g* for 15 min at 4 °C. Each time, transfer the supernatant to a new tube. Determine the protein concentration (e.g., by Bradford assay).
5. Quantify the content of the recombinant protein by western blotting and/or ELISA.
6. Select 2–3 positive cell lines to establish the working cell lines.

4 Notes

1. For the preparation of *A. tumefaciens* DNA plasmid, we use a standard purification kit (e.g., High Pure Plasmid Isolation Kit from Roche).
2. The plasmid pBBR1MCS-5 (4768 bp) contains a 1250 bp EcoRI/PstI fragment corresponding to the *virGN54D* gene [7].
3. We use a glass Pasteur pipette, whose tip is closed using a Bunsen burner.
4. We cultivate the 6-well plates without any agitation during the co-cultivation process to ensure the attachment of *A. tumefaciens* to the plant cells.
5. When spreading the cells on the agar plates, be very careful not to touch the lid of the Petri dish because this would seal the lid to the box and thus compromise gas exchanges.
6. If the recombinant protein of interest displays a quaternary structure with disulfide inter-molecular bonds (like IgG or any protein fused to the Fc domain), do not add dithiothreitol in the grinding buffer to preserve the correct quaternary structure.

7. In the lab, we prepare a protease inhibitor cocktail containing 2 μg/mL of leupeptin, aprotinin, antipain, pepstatin, and chymostatin.

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