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Jacqueline MacDonald
Igor Kolotilin
Rima Menassa *Editors*

# Recombinant Proteins from Plants

## Methods and Protocols

*Second Edition*

Humana Press

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Edited by

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# Preface

Recombinant protein production in plants is becoming an increasingly attractive alternative to conventional production platforms due to promising cost and safety benefits.

Up-to-date scientific achievements from the world's top researchers are conveniently arranged in the new addition of *Recombinant Proteins from Plants* as a collection of protocols for use with a variety of plant expression systems. Various aspects of production are covered including vector selection and cloning; product improvements for stability, glycosylation, and antibiotic-free selection; extraction and scale-up; and analysis of transgenic plants and their recombinant proteins.

This new edition of *Recombinant Proteins from Plants* is an ideal reference for those who are interested in plant molecular biology and molecular farming.

***London, ON, Canada***

***Jacqueline MacDonald***
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# Part I

## Vectors and Transformation

# Chapter 1

# Chloroplast-Based Expression of Recombinant Proteins by Gateway® Cloning Technology

**Johanna Gottschamel and Andreas Lössl**

## Abstract

Plastid transformation for the expression of recombinant proteins and entire enzymatic pathways has become a promising tool for plant biotechnology in the past decade. Several improvements of the technology have turned plant plastids into robust and dependable expression platforms for multiple high value compounds. In this chapter, we describe our current methodology based on Gateway® recombinant cloning, which we have adapted for plastid transformation. We describe the steps required for cloning, biolistic transformation, identification, and regeneration of transplastomic plant lines and Western blot analysis.

**Key words** Plastid transformation, Gateway® recombinant cloning technology, Biolistic transformation, Southern blot analysis, Western blot analysis

## 1 Introduction

To transform plastids with an expression cassette, it takes a transformation vector which is optimally adapted to the requirements of chloroplasts. We observe a considerably quick growth of knowledge on plastid genetics, as well as a speedy development of novel expression cassettes which are most decisive for successful protein expression. To keep pace with this progress it is necessary to have at hand a spectrum of quick and efficient methods for vector cloning, plant transformation, and subsequent analysis of novel transplastomic plant lines.

The construction of new plastid transformation vectors usually requires the insertion of an expression cassette between two homologous flanks. The conventional method for this step is enzymatic restriction digestion and ligation which strongly depends on the availability of compatible cutting sites. A limited choice of enzymes often makes vector construction a lengthy process, with several

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intermediate steps required for a single cassette. To efficiently circumvent this problem and to increase the versatility of plastid transformation vectors, the Gateway® cloning technology can be adapted to the requirements of the plastidial expression system [1]. While the compatibility of Gateway® recombination sites with plastid specific 5′ regulatory sequences was previously unknown [2, 3], recently the feasibility and convenience of placing one of the two recombination sites between a plastid promoter (P*rrn*PEP + NEP) and the T7g10 5′UTR [4] has been proven [1]. Gateway® cloning employs the bacteriophage λ derived *att* recombination sites and Clonase® enzyme mixes from Invitrogen™, which contain the necessary enzymes for each recombination reaction [5]. In a first step, the gene of interest (goi) needs to be flanked with the recombination sites *att*B1 and *att*B2 which can be synthesized by PCR. The *att*B1-goi-*att*B2 fragment is then transferred into the pDONR™ carrying the *att*P sites, yielding the entry clone pENTR™-goi [5]. This Integrase-mediated recombination reaction is referred to as the BP reaction since the *att*B and *att*P sites are involved [5]. In the last step, mediated by Integrase and Excisionase, the goi is integrated into the destination vector (pDEST™-CP). This LR reaction, so called because *att*L and *att*R sites are involved, results in the final plastid-specific expression vector (pEXP™-goi) used for chloroplast transformation and protein expression.

Here, we demonstrate the complete procedure of chloroplast-based expression of recombinant proteins. We give detailed advice on every necessary step covering vector construction, plant transformation by the biolistic method, DNA analysis of transplastomic plant lines and recombinant protein expression level analysis.

## 2 Materials

All solutions are prepared with double-distilled water, autoclaved and stored at room temperature and procedures are carried out at room temperature, unless otherwise indicated. Stock solutions of antibiotics and hormones are filter-sterilized and stored in aliquots at −20 °C.

### 2.1 Vector Construction: Gateway® Cloning

1. PCR Cloning System with Gateway® Technology with pDONR™221 and OmniMAX™2 Competent Cells (Invitrogen™, USA), store cells and enzyme mix at −80 °C and store all other components at −20 °C.
2. Gateway® LR Clonase® Enzyme Mix (Invitrogen™, USA), store at −80 °C.
3. Gateway® Vector Conversion System with One Shot® ccdB Survival Cells (Invitrogen™, USA), store cells at −80 °C and the rest of the Kit at −20 °C.
4. DNA ligase.

5. DNA gel extraction kit.
6. Plasmid isolation kit.
7. DNA ladder.
8. 6× loading dye.
9. Primers: pM13(-20)F 5′-GTAAAACGACGGCCAG-3′
   pM13(-29)R 5′-CAGGAAACAGCTATGACC-3′.
10. PCR reagents (DNA polymerase, dNTP mix, buffer).
11. Suitable restriction enzyme.
12. Ethidium bromide, 1 % in water.
13. 1 % agarose gel: 1 g agarose dissolved in 100 ml 1× TAE Buffer.
14. Gel-electrophoresis system for agarose gels: gel casting equipment, horizontal electrophoresis cell and power supply.
15. TE Buffer: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
16. 10× TAE Buffer: 2 M Tris, 0.7 % acetic acid, 0.05 M EDTA.
17. Antibiotic stock solutions: 25 mg/ml ampicillin; 3 mg/ml chloramphenicol; 25 mg/ml kanamycin; 100 mg/ml spectinomycin (*see* **Note 1**).
18. Transgene (*goi*) sequence flanked by Gateway® recombination sites *att*B1 and *att*B2 (*see* **Note 2**).
19. Plastid transformation vector containing the *aad*A expression cassette and a promoter for transgene expression flanked by target sequences that are homologous to the integration site in the chloroplast genome (INSL and INSR) (*see* **Note 3**).
20. LB medium: 10 g/l bacto tryptone, 5 g/l yeast extract, 10 g/l NaCl (10 g/l agar for solid medium).
21. LB medium containing 30 mg/l chloramphenicol and 100 mg/l ampicillin for selection and growth of *E. coli* colonies transformed with plastid destination vector pDEST-CP.
22. LB medium containing 100 mg/l kanamycin for selection and growth of *E. coli* colonies transformed with the pENTR-*goi* vector.
23. LB medium containing 500 mg/l spectinomycin and 100 mg/l ampicillin for selection and growth of *E. coli* colonies transformed with the final plastid transformation vector pEXP-*goi*.

### 2.2 Plant Transformation and Regeneration

1. Seeds of *Nicotiana tabacum* cv. Petit Havana.
2. Culture boxes (e.g. Magenta GA-7 with lid, Magenta Corp., USA).
3. PDS-1000/He Particle Delivery System (Bio-Rad, USA).
4. Helium tank.
5. Gold microcarriers, 0.6 μm (Bio-Rad, USA).
6. Macrocarriers (Bio-Rad, USA).

7. Macrocarrier holders (Bio-Rad, USA).
8. Rupture disks, 1100 psi (Bio-Rad, USA).
9. $CaCl_2$ 2.5 M.
10. Spermidine 0.1 M.
11. Sodium acetate, 3 M (NaAc).
12. 6-Benzylaminopurine, 1 mg/ml (BAP).
13. Naphthalene acetic acid, 1 mg/ml (NAA).
14. Spectinomycin, 100 mg/ml.
15. MS medium, pH 5.8: 4.4 g/l MS-Salt including vitamins, 10 g/l sucrose, pH to 5.8 using NaOH, 3.1 g/l Gelzan™ (CP Kelco, USA).
16. RMOP medium, pH 5.8: 4.4 g/l MS-Salt including vitamins, 30 g/l Sucrose, pH to 5.8 using NaOH, 3.1 g/l Gelzan™ , 1 mg/l BAP, 100 µg/l NAA.
17. RMOP medium with 500 mg/l spectinomycin.
18. Parafilm (Pechiney Plastic Packaging Co., USA).
19. DNA extraction kit.
20. Components for PCR, including appropriate primers (*see* Fig. 4).

### 2.3 Southern Blot Analysis

1. DIG high prime DNA labeling and detection starter kit II, random primed DNA labeling with digoxigenin-dUTP, alkali-labile and chemiluminescence detection with CSPD (Roche Applied Science, USA).
2. Roti®-Nylon plus, pore size 0.45 µm (Carl Roth GmbH, Germany).
3. 1× CTAB-Buffer: 2 % hexadecyl-trimethyl-ammonium bromide (CTAB), 20 mM EDTA, 1.4 M NaCl, 1 % PVP (40), 200 mM Tris–HCl, pH 8.
4. Suitable restriction enzyme (*see* **Note 4**).
5. 1 % agarose gel: 1 g agarose dissolved in 100 ml 1× TAE Buffer.
6. 10× TAE Buffer: 2 M Tris, 0.7 % acetic acid, 0.05 M EDTA.
7. 6× Loading dye.
8. DNA ladder.
9. Suitable primer pair for probe amplification, and wild-type plant DNA or plasmid vector as a template for amplification.
10. Gel extraction kit.
11. 0.2 M EDTA, pH to 8.0 with NaOH.
12. 20× SSC Buffer, pH 7: 3 M NaCl, 300 mM sodium citrate, adjust pH with 1 M HCl.
13. 1 M maleic acid, pH 7.5 (*see* **Note 5**).
14. 1.5 M NaCl.

15. Maleic acid Buffer, pH 7.5: 0.1 M Maleic acid, pH 7.5, 0.1 M NaCl.
16. Wash Buffer, pH 7.5: Maleic acid Buffer with 0.3 % Tween-20.
17. 1× Blocking solution: 10× blocking solution (supplied with the labeling and detection starter kit, **item 1**) diluted with maleic acid buffer.
18. Antibody solution: anti-digoxigenin-AP antibody (75 mU/ml) diluted 1:10,000 in 1× blocking solution.
19. Detection buffer, pH 9.5: 0.1 M Tris–HCl, 0.1 M NaCl.
20. Hybridization oven and hybridization tubes.
21. Development folder, film, developer, fixer, dark room.

### 2.4 Western Blot Analysis

1. AP-Conjugate Substrate Kit (Bio-Rad, USA) or Sigmafast™ BCIP®/NBT (Sigma) (*see* **Note 6**).
2. Pierce™ BCA Protein Assay Kit (Thermo Scientific, USA).
3. Quick Start™ Bradford Protein Assay Kit 1 (Bio-Rad, USA).
4. Primary antibody (*see* **Note 7**).
5. Secondary antibody: Anti-Rabbit IgG (Fc), Alkaline Phosphatase conjugated (Promega, USA) (*see* **Note 8**).
6. Pre-stained protein marker.
7. 1× Blotting Buffer, pH 8.3: 25 mM Tris, 192 mM Glycine, 20 % Methanol (*see* **Note 9**).
8. 5× Laemmli Buffer (LB), pH 6.8: 10 % SDS, 10 % 13 M β-Mercaptoethanol, 50 % Glycerol, 0,09 % Bromophenol Blue, 312.5 mM Tris–HCl, pH 6.8.
9. Plant Extraction Buffer for total soluble protein (PEB I): 100 mM NaCl, 10 mM EDTA, 200 mM Tris–HCl pH 8, 0.05 % Tween-20, 0.1 % SDS, 14 mM β-Mercaptoethanol, 200 mM Sucrose, 1 cOmplete Protease Inhibitor Cocktail Tablet/50 ml buffer.
10. Plant Extraction Buffer for total protein (PEB II): 0.7 M sucrose, 0.5 M Tris, 50 mM EDTA, 0.1 M KCl, 0.2 % β-Mercaptoethanol, 1 cOmplete Protease Inhibitor Cocktail Tablet/50 ml buffer.
11. 10× running buffer, pH 8.3: 0.25 M Tris, 1.92 M Glycine, 1 % SDS (*see* **Note 10**).
12. 4× separating gel buffer, pH 8.8: 1.5 M Tris–HCl, 0.2 % SDS.
13. 4× stacking gel buffer, pH 6.8: 0.5 M Tris–HCl, 0.2 % SDS.
14. 10× tris-buffered saline (TBS), pH 7.6: 1.37 M NaCl, 200 mM Tris–HCl.
15. 1× TBS-T: 1× TBS, 1 ml Tween-20.

16. Nitrocellulose or polyvinyl difluoride (PVDF) membrane.
17. Blocking solution: 0.5 % BSA in 1× TBS-T.
18. 30 % acrylamide solution: dilute 40 % acrylamide/bis-acrylamide (19:1) with water (*see* **Note 11**).
19. 10 % APS (ammonium persulfate) (*see* **Note 12**).
20. TEMED (*N*,*N*,*N*′,*N*′-Tetramethylethylenediamine).
21. 0.1 M $NH_4OAc$ in methanol.
22. Equipment for Polyacrylamide-gel-electrophoresis: gel casting equipment (short plates, spacer plates, combs, casting frame, and casting stand), electrophoresis tank, and power supply.
23. Electroblotting System: tank, electrodes, gel holder cassettes, foam pads, filter paper.

## 3 Methods

All procedures are carried out at room temperature (RT) unless otherwise indicated.

### 3.1 Vector Construction: Gateway® Cloning

#### 3.1.1 General Considerations

1. *E. coli* overnight growth: use either LB plates with 10 g/l agar and appropriate antibiotics at 37 °C, or 5 ml liquid LB medium containing appropriate antibiotics at 37 °C and 200 rpm shacking.
2. Prepare glycerol stocks with a final glycerol concentration of 33 % from each overnight *E. coli* culture, shock freeze in liquid $N_2$ and store at −80 °C.
3. All plasmids and ligation products are transformed into either chemically competent One Shot® OmniMAX™ 2 T1R *E. coli* or chemically competent One Shot® ccdB Survival™ 2 T1R *E. coli* by heat shock method according to the manufacturer's protocol (Invitrogen™) (*see* **Note 13**).
4. Restriction digest, DNA purification from agarose gel, plasmid isolation and ligation reactions are carried out according to the protocol provided by the Kits' manufacturer.
5. The main steps of the cloning procedure are depicted in Fig. 1.

#### 3.1.2 Conversion of a Plastid Transformation Vector into a Gateway® Compatible Plastid Destination Vector

1. Linearize the plastid transformation vector with the INSL-aadA-INSR sequence with a suitable restriction enzyme to create blunt ends (Fig. 1a) (*see* **Note 14**).
2. Separate the restriction fragments by electrophoreses in a 1 % agarose gel containing 0.5 μg/ml Ethidium bromide, visualize under UV light, cut out the correct sized backbone fragment, and purify from the gel using a Gel Extraction Kit.

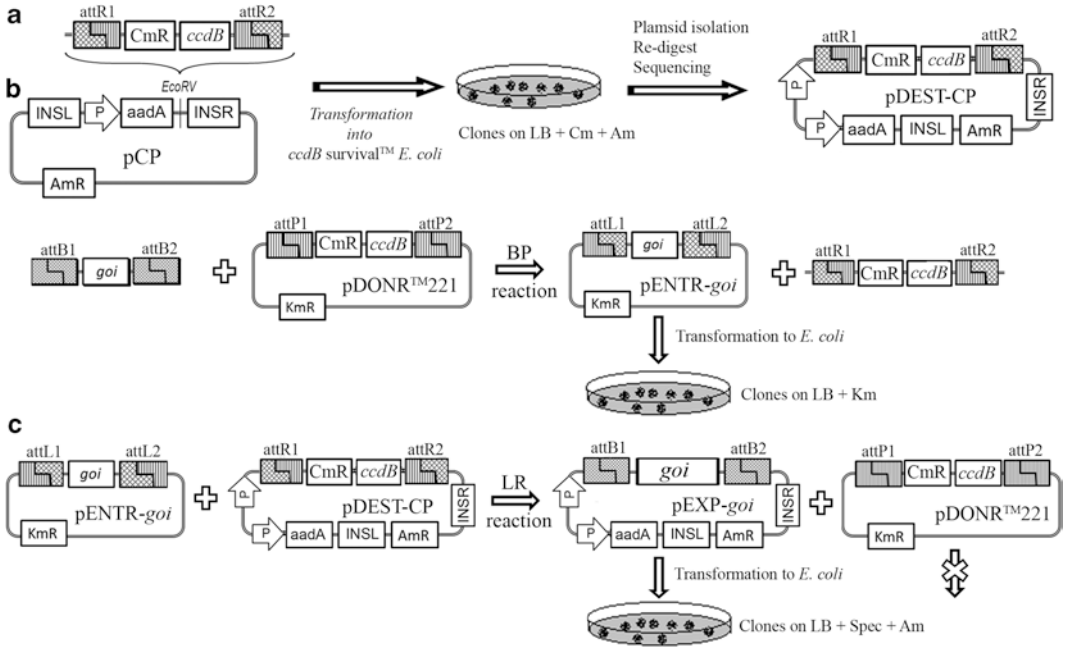


**Fig. 1** Schematic representation of the construction of the plastid transformation vector. (**a**) The destination vector pDEST-CP is created by blunt end ligation of the Gateway® reading frame cassette (*att*R1-*ccd*B-CmR-*att*R2) into a suitable backbone vector containing the homologous regions necessary for integration into the chloroplast genome (INSL/INSR). (**b**) The BP reaction (*att*B × *att*P → *att*L + *att*R) is performed between the *att*B sites flanking the goi sequence and the *att*P sites of pDONR™221 resulting in the Entry vector pENTR-goi. (**c**) The LR reaction (*att*L × *att*R → *att*B + *att*P) between the pENTR-goi and the pDEST-CP yields the final plastid transformation vector pEXP-goi. The pDONR™221 is recreated as a by-product of the LR reaction. However, the OmniMax™ *E. coli* cells that are transformed with this plasmid will not survive due to the lethal effects of the *ccd*B gene product [6]. Therefore, colonies should contain the pEXP-goi plasmid only. *aad*A spectinomycin resistance gene, *Spec* spectinomycin, *INSL/INSR* left/right insertion site, *Am* ampicillin, *AmR* ampicillin resistance gene, *ccd*B control of cell death gene, *Cm* chloramphenicol, *CmR* chloramphenicol resistance gene, *Km* kanamycin, *KmR* kanamycin resistance gene, *att*B1/B2/P1/P2/L1/L2/R1/R2 Gateway® recombination sites

3. Set up the ligation reaction according to the supplier's protocol using 10 ng of the Gateway® reading frame cassette (Rf, supplied with the Gateway® Vector Conversion System Kit) and 50 ng of the purified backbone vector.
4. Transform 5 µl of the ligation product into 50 µl chemically competent One Shot® *ccdB* survival™ *E. coli* and plate on LB + agar plates containing both ampicillin and chloramphenicol (*see* **Note 15**).
5. Pick three to five single colonies and cultivate them in liquid LB medium containing ampicillin and chloramphenicol overnight at 37 °C with shaking.

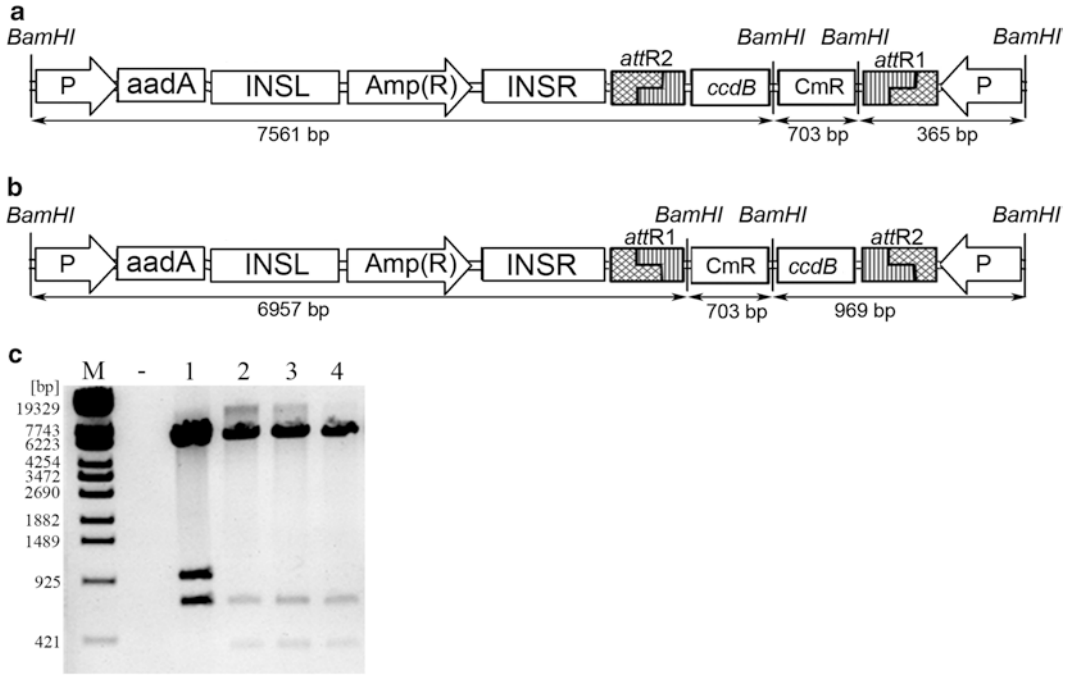


**Fig. 2** Example of restriction digest pattern of putative pDEST-CP. DNA isolated from colonies grown on LB medium containing both ampicillin and chloramphenicol is digested with *BamHI*. (**a**) Schematic representation of pDEST-CP with proper orientated Gateway® Rf; (**b**) Schematic representation of pDEST-CP with reverse oriented Gateway® Rf; (**c**) *BamHI*-digested putative pDEST-CP: Lane 2, 3 and 4 show the expected restriction pattern for correct insertion of the Rf into the vector backbone. Lane 1 shows the expected bands for reverse insertion of the Rf into the vector backbone. *M* Lambda DNA/Eco130I (*StyI*) Marker 16, Fermentas. *aadA* spectinomycin resistance gene, *INSL/INSR* left/right insertion site, *AmR* ampicillin resistance gene, *ccdB* control of cell death gene, *CmR* chloramphenicol resistance gene, *KmR* kanamycin resistance gene, *att*B1/B2/P1/P2/L1/L2/R1/R2 Gateway® recombination sites, *P* promoter

6. Use 1 ml of the cell suspension to prepare a glycerol stock and isolate plasmid DNA from the remaining 4 ml overnight culture.
7. Check for the presence of the Rf by restriction digest with a suitable enzyme as depicted in Fig. 2 (*see* **Note 16**).
8. DNA showing the correct restriction digest pattern is sent for sequencing to verify the correct orientation of the Rf in the plastid targeted pDEST-CP vector.

### *3.1.3 Creation of the Entry Clone*

1. The BP-reaction (Fig. 1b) is set up containing 50 fmol pDONR™221, 50 fmol goi, 2 µl BP enzyme mix and TE buffer up to a total reaction volume of 20 µl and incubated at 25 °C for 1 h (*see* **Note 17**).
2. Stop the BP-reaction by adding 1 µL Proteinase K (2 µg/µl) and incubating at 37 °C for 10 min.

3. Transform 1 μl of the BP-reaction into chemically competent One Shot® OmniMax™ *E. coli* by the heat shock method and plate on LB + agar plates containing kanamycin.
4. Identify positive clones using colony PCR with primers pM13(-20)F and pM13(-29)R (*see* **Note 18**).
5. Cultivate the remaining portions of three to five positive colonies overnight in liquid LB medium containing kanamycin at 37 °C with shaking.
6. Use 1 ml of the cell suspension to prepare the glycerol stock and isolate plasmid DNA from the remaining 4 ml overnight culture.
7. Send plasmid DNA for sequencing to verify the correct insertion of the *goi* sequence in the pENTR-*goi* vector.

#### 3.1.4 Creation of the Final Plastid Transformation Vector

1. The LR-reaction (Fig. 1c) is set up containing 150 ng pENTR-goi, 150 ng pDEST-CP, 2 μl LR enzyme mix and TE buffer up to a total reaction volume of 20 μl and incubated at 25 °C for 1 h.
2. Stop the LR- reaction by adding 1 μl Proteinase K (2 μg/μl) and incubating at 37 °C for 10 min.
3. Transform 1 μl of the LR reaction into chemically competent One Shot® OmniMax™ *E. coli* and plate on LB + agar plates containing spectinomycin and ampicillin.
4. Pick three to five single colonies and cultivate them in liquid LB medium containing spectinomycin and ampicillin overnight at 37 °C with shaking.
5. Isolate plasmid DNA from the overnight cultures and send for sequencing to verify the correct sequence of the final plastid transformation vector pEXP-*goi*.

### 3.2 Plant Transformation by Biolistic Method and Regeneration of Transplastomic Plants

#### 3.2.1 Growth of Plant Material

1. Sterilize seeds by washing three times in 70 % ethanol, soaking for 1 min in 6 % household bleach, followed by four washes with sterile water and air drying at room temperature on the clean bench. Store the seeds at +4 °C.
2. Germinate wild-type tobacco seeds on MS medium and transfer plantlets into sterile culture boxes. Harvest leaves from 6 weeks old plants grown under sterile conditions, place on RMOP-medium with the abaxial side facing up and incubate at 25 °C in the dark overnight before bombardment (*see* **Note 19**).

#### 3.2.2 Preparation of Microcarrier Solution

1. Weigh the required amount of gold microcarriers in a 1.5 ml tube. 500 μg of gold particles, coated with 1 μg of DNA, are used for each bombardment (*see* **Note 20**).
2. Add 1 ml of 70 % ethanol, vortex for 2 min and centrifuged at 10,000 × *g* for 3 min.

3. Discard the supernatant and resuspend the pellet in 70 % ethanol, vortex for 1 min and then incubate at 4 °C for 15 min with shaking at 100 rpm.
4. Pellet the particles by centrifugation at 10,000 × *g* for 2 min, discard the supernatant and resuspend the gold particles in sterile 50 % glycerol for a final concentration of 50 mg/ml (*see* **Note 21**).

### 3.2.3 DNA Coating of Microcarriers

1. Add 50 µl gold suspension into a 1.5 ml tube (*see* **Note 22**).
2. Add 5 µg transformation vector, 50 µl 2.5 M $CaCl_2$ and 20 µl 0.1 M spermidine to the gold particles and vortex (*see* **Notes 23** and **24**).
3. Incubate for 10 min on ice and then centrifuge for 1 min at 10,000 × *g*.
4. Carefully remove the supernatant and wash the pellet first in 70 % ethanol and then with 140 µl of 100 % ethanol. For each wash, centrifuge for 1 min at 10,000 × *g*.
5. Remove the supernatant and resuspend the DNA-coated microcarriers in 50 µl of 100 % ethanol. Keep on ice until use (*see* **Note 25**).

### 3.2.4 Bombardment

1. All procedures are performed in a clean bench and transformation of chloroplasts is achieved with the biolistic transformation method using a PDS-1000/He Particle Delivery System (Bio-Rad, USA).
2. Sterilize all equipment and the bombardment chamber with 70 % ethanol.
3. Open the valve of the helium tank until the manometer indicates a pressure of around 1300 psi. Switch on the vacuum pump and the PDS-1000 System.
4. Pipet 10 µl of DNA-coated gold particles onto the macrocarrier in the macrocarrier holder. Allow the ethanol to evaporate.
5. Place a sterile rupture disk of 1100 psi into the retaining cap of the PDS-1000 and secure it to the gas acceleration tube.
6. Assemble, secure, and place the stopping screen and macrocarrier holder in the first slot from the top of the bombardment chamber as explained in the manual provided with the Particle Delivery System.
7. Place the open Petri dish containing the leaf on RMOP medium in the target holder of the PDS-1000 and put into the third slot from the top of the bombardment chamber (*see* **Note 26**).
8. Close the bombardment chamber and apply a vacuum pressure of 28 in. Hg, as displayed on the vacuum gauge of the PSD-1000.

9. Press the "Fire" button on the PDS-1000 and hold until the rupture disk bursts. This burst is associated with a loud popping sound. Immediately release the "Fire" button.
10. Release the vacuum. Place a lid over the Petri dish containing the bombarded tissue, and seal with Parafilm.
11. Replace the rupture disk with a new one, insert the next macrocarrier into the PDS-1000, and repeat the bombardment steps (*see* **Note 27**).
12. After the last bombardment, close the valve of the helium tank and release the pressure from the system by performing a mock bombardment without a rupture disk.
13. Turn off the Particle Bombardment system and the vacuum pump after use.

*3.2.5 Regeneration of Transformed Plants (Fig. 3)*

1. Place the bombarded leaf discs on RMOP medium and incubate in the dark at 25 °C for 2 days.
2. Cut the leaf discs into small pieces (~5 $mm^2$), transfer to RMOP medium containing 500 mg/l spectinomycin and keep at 25 °C under standard light conditions (*see* **Note 28**).
3. Three to 4 weeks after the transformation, the resistant shoots start to regenerate. Transfer them to fresh medium (*see* **Note 29**).

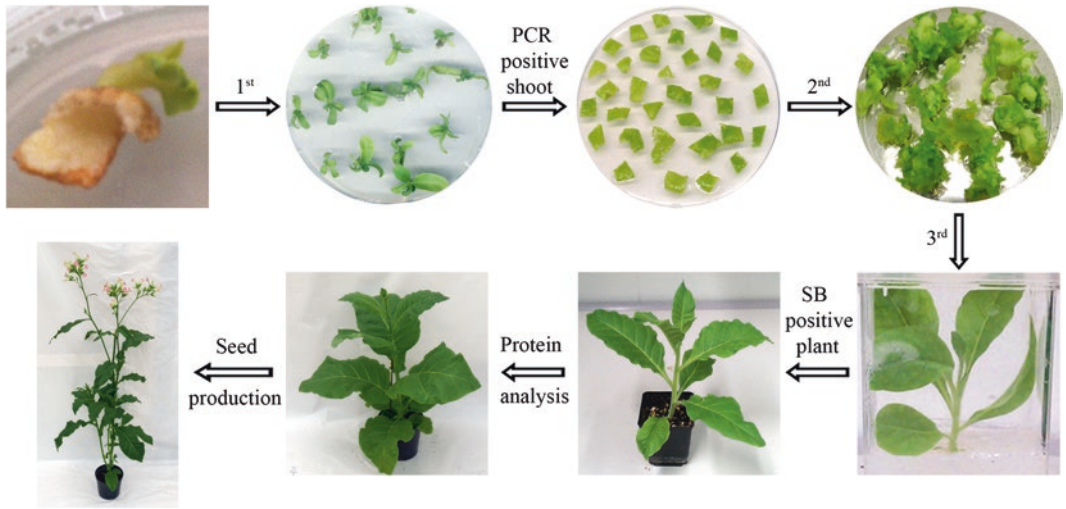


**Fig. 3** Regeneration of transplastomic plant. In the first round of regeneration the shoots emerging from the bombarded tissue cultivated on spectinomycin-containing RMOP medium are transferred to fresh medium and tested for transgene integration by PCR. PCR-positive shoots are subjected to a second round of regeneration by cutting them into small pieces and placing them on new spectinomycin-containing RMOP medium. Newly emerging shoots are rooted as a third round of regeneration and the homoplastomic state of the plant is verified by Southern blot (SB). Homoplastomic plants are transferred to soil, protein analyses are carried out and seeds are produced

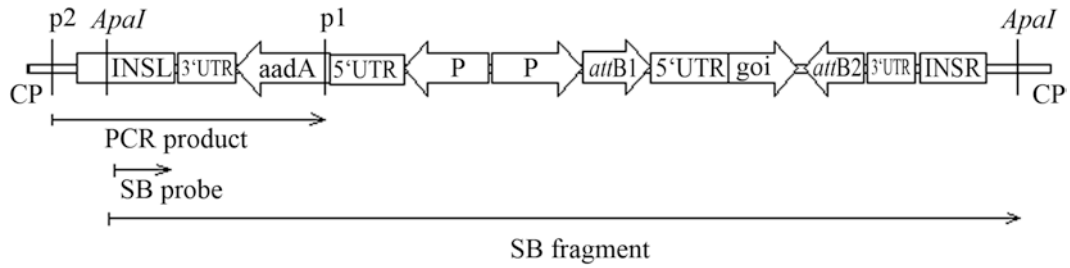


**Fig. 4** Schematic representation of the transgene expression cassette integrated into the chloroplast genome (CP). Primers p1 and p2 are used to verify transgene integration after the first round of regeneration. After the third round of regeneration, total plant DNA is isolated and digested with a suitable restriction enzyme (*Apa*I in this figure) and subjected to Southern blot analysis using a SB probe that binds inside the transgene expression cassette. *INSL/INSR* left/right insertion site, *3′UTR* 3′ untranslated region, *5′UTR* 5′ untranslated region, *aad*A spectinomycin resistance gene, *P* promoter, *att*B1/*att*B2 gateway® recombination sites, *goi* gene of interest

4. Isolate plant DNA from regenerating shoots (*see* **Note 30**).
5. Verify integration of the transgene expression cassette into the tobacco plastid genome by PCR, using primer p1 that binds inside the transgene expression cassette and primer p2 binding inside the plastid genome next to the integration site (Fig. 4).
6. Cut PCR-positive shoots into small pieces and subject them to a second round of regeneration on RMOP medium containing spectinomycin.
7. Transfer shoots emerging from the second round of regeneration to culture boxes with MS medium for rooting (*see* **Note 31**).

### *3.3 Southern Blot Analysis*

All procedures are carried out at room temperature unless otherwise indicated.

#### *3.3.1 Plant DNA Preparation*

1. Freeze 100 mg of plant material in liquid $N_2$ and grind into a homogenous powder using the Retsch-mill (2 min at frequency 3000/s).
2. Add 0.5 ml of pre-warmed CTAB Buffer, vortex and incubate at 65 °C for 90 min (*see* **Note 32**).
3. Allow the samples to cool for 5 min at room temperature and then add 500 µl chloroform, vortex, and centrifuge for 10 min at 16,200 × *g* at RT (*see* **Note 33**).
4. Collect the upper, aqueous phase and transfer it into a new, clean tube (*see* **Note 34**).
5. Add 1 ml of chilled 100 % isopropanol to precipitate the DNA, and invert the tubes several times prior to centrifugation at 16,200 × *g* for 10 min at +4 °C.

6. Discard the supernatant and wash the pellet in 70 % ethanol, and centrifuge at 16,200 × *g* for 10 min at +4 °C. Repeat the washing step twice.
7. Discard the supernatant and air dry the pellet (*see* **Note 35**).
8. Resuspend the dried pellet in 50 µl TE buffer containing RNAse, and measure the DNA concentration (*see* **Note 36**).
9. Digest 10 µg of plant DNA from the transplastomic and the wild-type plants in suitable restriction enzyme reactions according to the conditions recommended by the manufacturer.

### 3.3.2 Probe Preparation

1. Suitable primers for the amplification of probe fragment from wild-type DNA should be chosen such that the probe is about 700–1000 bp long (Fig. 4).
2. Perform the PCR reaction, load the PCR reaction on a 1 % agarose gel, separate by electrophoresis and cut out and purify the probe from the gel.
3. Determine the concentration of the purified probe.
4. Denature the probe and DNA ladder by boiling for 10 min and immediately chill on ice for further use in the labeling reaction.
5. Set up the labeling reaction for the probe containing 1 µg of probe, 4 µl of Dig High Prime and water up to a total reaction volume of 20 µl.
6. Set up a second labeling reaction containing 2 µl DNA ladder, 4 µl of Dig High Prime and water up to a total reaction volume of 20 µl.
7. The labeling reactions are incubated overnight at 37 °C, stopped by adding 1 µl of 0.5 M EDTA and store at –20 °C until used.

### 3.3.3 Gel Electrophoresis and Semi-dry Transfer of DNA

1. The 10 µg restriction-digested DNA is slowly separated by gel electrophoresis using a 1 % agarose gel without ethidium bromide, using a low voltage of around 40 V (*see* **Notes 37** and **38**).
2. After the separation, stain the gel by incubating in 1× TAE buffer with ethidium bromide for 30 min (*see* **Note 39**).
3. Visualize the DNA by exposure to UV light and cut off the parts of the gel that are not needed for transfer (*see* **Note 40**).
4. Place the gel in 0.25 M HCl, rock gently for 5 min, and repeat.
5. Place the gel in 0.4 M NaOH, rock gently for 15 min, and repeat.
6. Cut a piece of membrane to the same size as the gel.
7. Adhere plastic wrap to the lab bench, making sure that it is smooth. The gel is placed on top of the wrap and strips of

Parafilm are taped around the gel to prevent it from moving. Place the membrane on top of the gel followed by two layers of Whatman filter paper and a stack of paper towels. Place a glass plate on top of the paper stack and about 1–2 kg of weight (*see* **Note 41**).

8. Make sure that the blot is stable, leveled and that the weight cannot fall off. Allow the DNA to transfer from the gel to the membrane by capillary force overnight.
9. Disassemble the blot and rinse the membrane briefly in 2× SSC buffer.
10. Fix the DNA to the membrane by baking at 80 °C for 2 h (*see* **Note 42**).

### *3.3.4 Hybridization and Detection*

1. Carefully place the membrane in a hybridization tube so that the DNA containing side does not touch the glass.
2. Add 20 ml of pre-warmed DIG Easy Hyb-solution (from the DIG high prime DNA labeling and detection starter kit) to the membrane and incubate in the hybridization oven for at least 1 h at 45 °C.
3. Denature the labeled marker and probe at 120 °C for 10 min and immediately chill on ice.
4. Combine 30 µl labeled probe and 2 µl marker with the DIG Easy Hyb-solution, add to the tube containing the membrane, and hybridize overnight at 45 °C (*see* **Note 43**).
5. Transfer the membrane to a sandwich box and store the Hyb + probe + marker solution at −20 °C (*see* **Note 44**).
6. Wash the membrane twice for 5 min in 100 ml 2× SSC + 0.1 % SDS at room temperature (RT), then twice for 15 min in 100 ml 0.5× SSC + 0.1 % SDS at 65 °C, followed by 5 min in 100 ml wash buffer at RT.
7. Incubate the membrane in 100 ml 1× B\blocking solution for 30 min at RT.
8. Incubate the membrane with 20 ml antibody solution for 30 min at RT.
9. Wash the membrane twice for 15 min in 100 ml wash buffer at RT and equilibrate in 30 ml detection buffer for 5 min at RT before detection.
10. Place the membrane with the DNA side facing up on a development folder and apply 1 ml CSPD ready-to-use solution (from the DIG high prime DNA labeling and detection starter kit) (*see* **Note 45**).
11. Incubate the membrane for 5 min at RT and then for 10 min at 37 °C.
12. Squeeze out excess liquid and seal the development folder.

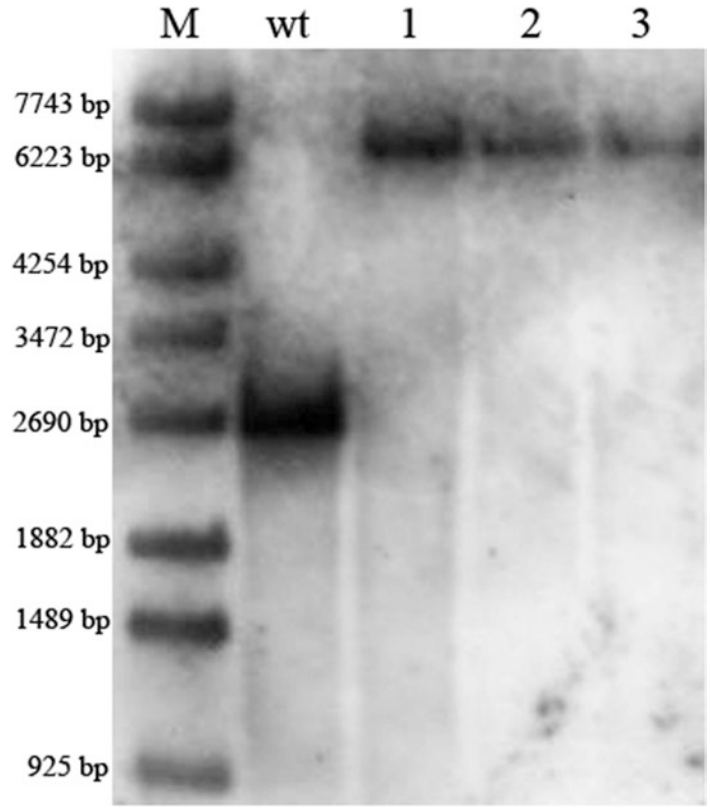


**Fig. 5** Southern blot performed with *ApaI* digested DNA of three independently regenerated plants (lanes 1–3) and one wild-type (wt) plant. 10 μg of DNA were loaded for each sample. Integration of the transgene expression cassette into the plastid genome results in a 6568 bp fragment. The absence of signal corresponding to the wild-type fragment (2649 bp) confirms the homoplastomic state of the regenerated plants. *M* DNA ladder, molecular weight is indicated next to the marker band

13. Expose the membrane to X-ray film for 10–30 min at room temperature in the dark room and develop the films. Figure 5 shows an example of a Southern blot performed with three transgenic and a wild-type sample (*see* **Notes 46** and **47**).

### *3.4 Western Blot Analysis*

All procedures are carried out on the bench, except for steps involving toxic fume producing substances such as TEMED, phenol and β-Mercaptoethanol which require a fume hood. When handling liquid acrylamide during the gel casting, nitrile gloves should be used. Protein samples should always be kept on ice. Always include protein extract from a wild-type sample as a negative control on the Immunoblot. If the expression level of the recombinant protein has to be quantified, a purified standard protein with known concentration needs to be included in the Western blot (*see* **Note 48**).

### 3.4.1 Protein Isolation and Sample Preparation (See **Note 49**)

Protein can be isolated either as total soluble protein (TSP) or total protein (TP). Both methods are described below. **Steps 7–9** apply to both methods.

1. *For* TSP *isolation*: grind 200 mg of frozen leaf sample into fine powder using liquid nitrogen and homogenize in 500 µl PEB I by vortexing for 3 min at RT.
2. Centrifuge for 10 min at 16,200 × *g* at +4 °C, collect the supernatant, and keep on ice until further use (*see* **Note 50**).
3. Quantify the concentration of the isolated TSP by Bradford assay using the Bradford assay kit, following the instructions of the manufacturer. Proceed to **step 7**.
4. *For* TP *isolation*: grind 200 mg of frozen leaf sample into fine powder using liquid nitrogen and homogenize in 500 µl PEB II by vortexing for 1 min at RT.
5. Add 500 µl phenol, vortex briefly and centrifuge at 16,200 × *g* for 10 min at +4 °C (*see* **Note 51**). Transfer 200 µl of the upper green supernatant into a new tube, add 1 ml of 0.1 M $NH_4OAc$ in methanol, and precipitate the proteins for 3 h at −20 °C. After centrifugation at 16,200 × *g* at +4 °C for 10 min, wash the pellet twice with 500 µl 0.1 M $NH_4OAc$ in methanol and then air dry at RT. Finally, dissolve the protein pellet in 100 µl 1 % SDS and store at −20 °C (*see* **Note 50**).
6. Quantify the concentration of the isolated TP by BCA assay using the BCA Protein Assay kit, following the instructions of the manufacturer (*see* **Note 52**).
7. Based on quantification of the extracted protein by Bradford or BCA, dilute the samples to achieve equal loading amounts of TSP or TP, respectively, for each sample (*see* **Note 53**).
8. Add 4 µl of 5× LB buffer to 16 µl of sample and denature the samples at 95 °C for 10 min in a heating block (*see* **Note 54**).
9. Briefly centrifuge each sample to collect the entire sample at the bottom of the tube, and keep the sample at RT until it is loaded onto the gel.

### 3.4.2 SDS-PAGE and Transfer

1. Assemble the short plate and the spacer plate in the casting frame and placed in the casting stand (*see* **Note 55**).
2. Prepare 10 ml of 12 % separating gel by mixing 3.4 ml distilled water, 4 ml 30 % acrylamide solution, 2.5 ml 4× separating gel buffer, 40 µl 10 % APS, and 26 µl TEMED (*see* **Note 56**).
3. Pour the gel between the plates, leaving around 1 cm of space at the top end of the short glass plate.
4. Immediately overlay the separating gel with 0.5 ml isopropanol and keep it at RT for polymerization (*see* **Note 57**).

5. When the leftover gel in the tube has solidified, pour out the isopropanol and prepare 3 ml of stacking gel by mixing: 1.8 ml distilled water, 0.49 ml 30 % Acrylamide solution, 0.75 ml 4× stacking gel buffer, 8 µl 10 % APS, and 15 µl TEMED.
6. Pour the stacking gel on top of the separating gel, gently press the comb into place and keep the gel at RT until the stacking gel is polymerized (*see* **Note 58**).
7. Remove the comb. Take the casted polyacrylamide gels out of the casting device and assemble them into the electrophoresis chamber (*see* **Note 59**).
8. Add 1× running buffer between the gels and up to the filling mark on the electrophoresis chamber.
9. Load 1–5 µl pre-stained protein marker and 20 µl of each sample into the wells and set the power supply at constant voltage of 200 V. The gels are run until the blue dye reaches the bottom of the glass plates.
10. Stain one gel is stained with Coomassie Brilliant Blue for 1 h at RT and de-stain overnight in 10 % acetic acid at RT. Subject the second gel to immunoblot detection (*see* **Note 60**).
11. Cut the membrane and filter paper to the same size as the gel. Quickly pre-wet the membrane with distilled water and then equilibrate for 10 min in 1× blotting buffer (*see* **Note 61**).
12. Place the blotting cassette with the black side down in a plastic tray filled with 1× blotting buffer and assemble the layers in the following order from bottom to top: fiber pad, three pieces of filter paper, gel, membrane, three pieces of filter paper, fiber pad (*see* **Note 62**).
13. Transfer proteins by applying a constant current of 125 mA/gel for 90 min (*see* **Note 63**).

*3.4.3 Detection and Quantification (Fig. 6)*

1. Disable the transfer and rinse the membranes with distilled water for 5 min at RT to remove the methanol (*see* **Note 64**).
2. Block the membrane with 0.5 % BSA in 1× TBS-T for 1 h at RT and then wash three times with 1× TBS-T for 10 min at RT (*see* **Note 65**).
3. Incubate the membrane with primary antibody diluted in TBS-T (1:500 to 1:1000, depending on the antibody) for 1 h at RT with gentle shaking (*see* **Note 65**).
4. Wash the membrane three times in 1× TBS-T for 10 min at RT.
5. Incubate the membrane with AP conjugated secondary antibody diluted in TBS-T (1:10,000) for 1 h at RT with gentle shaking (*see* **Note 66**).
6. Wash the membrane three times in 1× TBS-T for 10 min at RT.

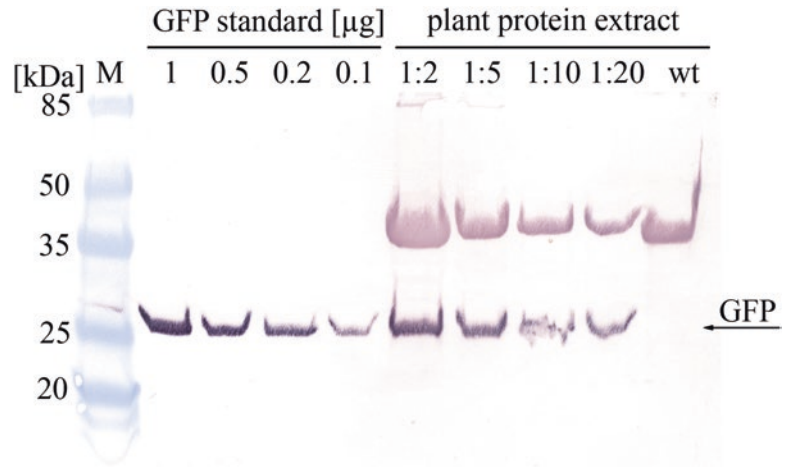


**Fig. 6** Western blot analysis. Total soluble protein extracts from a homoplastomic tobacco plant expressing GFP in the chloroplast, and from a wild-type plant, were loaded onto a 12 % polyacrylamide gel, separated by gel electrophoreses, blotted by tank blot onto a nitrocellulose membrane and detected with a rabbit-anti-GFP primary antibody and an AP-conjugated anti-rabbit-IgG secondary antibody. Bands were visualized by colorimetric detection using Sigmafast™ BCIP®/NBT. The GFP-specific band is visible at 28 kDa, while the band migrating at ~35 kDa is due to nonspecific binding of the antibody to a plant protein. In order to quantify the expression level of GFP, the protein extract was loaded in a dilution series (1:2, 1:5, 1:10 and 1:20). *M* protein marker III, Peqlab

7. The protein bands are visualized by incubating the membrane with the alkaline phosphatase substrate solution (e.g. Sigmafast™ BCIP®/NBT) according to the supplier's recommendation (*see* **Note 67**).
8. The recombinant protein expression level is determined by comparing the signal intensity of the bands of the standard protein with those of the sample (*see* **Note 68**).
9. The yield of recombinant protein is commonly reported as the percentage of recombinant protein of total soluble protein: *X*% of TSP (*see* **Notes 69** and **70**).

## 4 Notes

1. Chloramphenicol is dissolved in 100 % ethanol.
2. The *att*B1-*goi*-*att*B2 sequence can either be generated by adding the *att*B1 (5′-ACAAGTTTGTACAAAAAAGCAGGCT-3′) and *att*B2 (5′-ACCACTTTGTACAAGAAAGCTGGGT-3′) sequence to the *goi* by PCR or by linearizing a plasmid already containing the complete sequence with a suitable restriction enzyme.

3. The homologous INSL/INSR sequence should be species-specific. Although transformation of potato with tobacco INSL/INSR has been reported [7], the efficiency is considerably higher when using species-specific sequences. They can be obtained by PCR amplification with suitable primers from the corresponding wild-type genome. Nucleotide sequence of the *aad*A cassette is provided, e.g. on the EMBL EBI database under accession number FN396960.
4. Choose a restriction enzyme that cuts in the plastid genome close to the homologous flanks INSL/INSR. Fragments that are too large are time-consuming to separate during electrophoresis.
5. Set pH with solid NaOH.
6. There are several options to visualize the protein bands either with chemiluminescent or with colorimetric reaction. Chemiluminescent labeling is the more sensitive method, but colorimetric assays do not require a dark room. Choose the method of signal visualization based on the available equipment and infrastructure in your laboratory.
7. The primary antibody can either be one specific for the recombinant protein or if the protein has a His tag, a wide variety of anti-His-antibodies are available.
8. Choose the secondary antibody according to the primary antibody, e.g. if your primary antibody is produced in rabbit, then the secondary antibody must be an anti-rabbit-antibody produced in a different animal.
9. The pH equilibrates on its own to 8.3, do not adjust! Blotting buffer can be stored at +4 °C and reused several times.
10. The pH settles automatically between 8.1 and 8.5, do not adjust.
11. Dilute with distilled water to 30 % and store at +4 °C in a light-impermeable container.
12. APS is stable at +4 °C for 1 week. Store aliquots at −20 °C, as repeated thawing and freezing is not a problem.
13. We split the competent cells into aliquots of 10 µl and add 1 µl of plasmid DNA (~200 ng/µl)/BP reaction or LR reaction for the transformation. Keep the cells on ice for 30 min, heat shock at 42 °C for 30 s, and return to ice for 2 min. Add 100 µl of SOC medium and incubate at 37 °C and 250 rpm for 1 h. The cells are then spun down and resuspended in 40 µl SOC medium, and 20 µl of each transformation are spread on a solid LB plate with appropriate antibiotic.
14. If it is not possible to use an enzyme that creates blunt ends, you have to convert the ends of the vector to blunt double-stranded DNA after digestion using T4 DNA polymerase or Klenow fragment.

15. Propagation of the destination vector is only possible in *ccdB* survival™ *E. coli* that contain a gyrA462 mutation providing resistance to the lethal effects of *ccd*B.
16. Choose a restriction enzyme that will yield a clearly distinguishable pattern for right and wrong orientated insertion of the Rf into the backbone vector (for an example *see* Fig. 1).
17. 50 femtomol (fmol) of pDONR™221 are approximately 150 ng with $N = 4700$ bp. Use the formula given below to calculate the amount in nanograms (ng) corresponding to 50 fmol of goi with $N$ being the size in bp of the goi sequence.

$$\mathrm{ng}[\mathrm{goi}] = (50\,\mathrm{fmol}) \times N \times \left(660\left[\frac{\mathrm{fg}}{\mathrm{fmol}}\right]\right) \times 1\mathrm{ng}/10^6\,\mathrm{fg}$$

18. Pick half the colony with a sterile toothpick and suspend it in 10 µl PCR reaction containing the primers, DNA polymerase, buffer and water and run a standard PCR program with annealing temperature around 55 °C and an elongation time suitable for the expected PCR product. Choose colonies that are nicely scattered, number them clearly on the bottom of the plate and store the plate upside down at +4 °C. This allows easy identification and sampling of the colonies with a positive PCR result for further cultivation.
19. Rooted plants are transferred to soil and grown in the greenhouse with additional light for 16 h and light intensity of 300 µE/s/m$^2$, at 25 °C and relative humidity of 60 %.
20. The gold particles suspended in glycerol can be stored at –20 °C nearly infinitely long, if kept sterile. It is convenient to prepare a 50 mg/ml gold stock solution and store it in aliquots of 500 µl.
21. It is crucial to keep the gold suspension sterile, otherwise you take the risk of contaminating every single bombarded leave.
22. 10 µl of gold suspension is required for each bombardment. Five shots for each transformation vector are considered a suitable number of repetitions to generate a reasonable number of regenerating plants.
23. Fix the tube containing the gold particles to the vortex and add DNA, $CaCl_2$ and spermidine in this order while vortexing.
24. The concentration of the transformation vector should be around 1 µg/µl so as not to over-dilute the coating reaction. Plasmid DNA can be concentrated by ethanol-precipitation prior to coating: plasmid DNA is incubated with 1/10 volume of 3 M NaAc and 3 volumes of 96 % ethanol overnight at –20 °C. The solution is centrifuged at 16,200 × *g* at +4 °C for 15 min, and the pellet-washed twice with 70 % ethanol, air-dried

for 20 min at room temperature and dissolved in 1× TE Buffer and stored at –20 °C.

25. The gold will stick to the walls of the tube. Use a 200 µl pipette tip to scrape it down and suspend the particles well by pipetting up and down repeatedly. Alternatively, the particles can be suspended by holding the tube briefly in an ultrasonic bath.
26. The abaxial side of the leave is bombarded.
27. If successive bombardments with different plasmids are carried out, it is very important to clean the bombardment chamber and all the equipment with 70 % ethanol between bombardments to avoid DNA cross-contaminations.
28. Turn the leaf pieces upside down so that the bombarded side touches the medium and gently press the edges into the medium to secure maximum contact with the antibiotic-containing medium. This helps to avoid regeneration of shoots from untransformed cells.
29. Spectinomycin resistance can also be caused by a spontaneous point mutation in the plastid 16S rRNA [8]. In order to eliminate false-positive shoots, one leaf piece from each newly regenerating shoot can be cultivated on RMOP medium containing 500 mg/l streptomycin. Tissue from plants transformed with the *aad*A gene will remain green, while shoots able to grow on spectinomycin due to the point mutation will turn white. However, due to its highly mutagenic properties, streptomycin is not suitable for the regeneration of homoplastomic plants.
30. Plant DNA can be isolated using a commercially available Kit, but the CTAB-based method [9] offers an efficient and economical alternative.
31. All in vitro cultures are incubated at 25 °C, with a 16 h light to 8 h dark cycle in growth chambers equipped with Universal lamps with white fluorescence, light intensity; 0.5–1 W/m$^2$ Osram L85 W/25.
32. Incubation is best done in a water-bath and samples are inverted occasionally during the incubation to mix the extract.
33. Chloroform is heavier than water and will drag proteins and cell debris to the bottom of the tube.
34. In order to get a very clean DNA repeat the chloroform step.
35. Remove as much of the ethanol as possible using a fine pipette tip, then carefully turn the tube upside down and place it on a paper towel. Make sure that the pellet stays inside the tube.
36. Store DNA at +4 °C overnight or at –20 °C for a longer time. If the DNA concentration is not high enough, scale up the

DNA isolation procedure and subsequently concentrate the DNA by ethanol precipitation.

37. Run a fast test gel with 1–2 µl of digested DNA at 80 V to determine the efficiency of the digest and to make sure that the concentration of DNA is the same in all samples.
38. Load equal amounts of DNA for each sample to obtain similar signal intensities and run the DNA ladder on both sides of the gel to make it easier to cut out the correct range of the gel before the transfer.
39. Running the gel without ethidium bromide results in a better separation of the fragments, since the intercalation of ethidium bromide with the DNA varies, depending on the DNA's GC content.
40. Only the gel part that contains the restriction fragments detectable by the probe needs to be transferred. Cut the gel above and below that size, but not too close to your fragment size. For example if you are detecting a 3 kb fragment for the wild-type and a 5 kb fragment for transplastomic samples, cut the gel to include fragments ranging from 2 to 8 kb.
41. Any other smooth plastic surface can be used instead of the plastic wrap, like a small plastic bag or a transparent folder. The Whatman filter paper should be the size of the gel while the paper towels should be slightly bigger. Use 1 l bottles filled with water as weight.
42. A UV cross-linker can also be used if available.
43. The optimal hybridization temperature $T_{hyb}$ of probe to target is 20–25 °C lower than the melting point of the probe-target hybrid.
    $T_m = 49.82 + 0.41\ (\%\ G+C) - 600/L$, where $L$ = length of the hybrid in base pairs, and (% G + C) = percentage of G and C residues in probe sequence.
44. The solution can be reused after denaturation at 68 °C for 10 min.
45. Immediately cover the membrane with the second sheet of the folder to spread the substrate evenly and without air bubbles over the membrane.
46. In order to obtain a digital image, the developed films can be scanned with a flatbed scanner having a transparency feature. Or alternatively, a charge coupled device (CCD) camera can be used to capture the chemiluminescence image directly from the membrane.
47. After exposure, the membrane can be saved in the same sealed folder at –20 °C, stripped and used for re-probing if necessary. Stripping off probe and antibody from the membrane is achieved by first washing the membrane thoroughly in distilled

water, washing it two times in 0.2 M NaOH + 0.1 % SDS for 15 min at 37 °C and then washing it two times in 2× SSC for 5 min at RT. Store the membrane in 2× SSC until usage and proceed with pre-hybridization and hybridization.

48. If your recombinant protein is not commercially available, an alternative option to obtain this standard protein is to express it in *E. coli* and purify it from the cell lysate. Since the chloroplast's transcription and translation machinery are related to the prokaryote's, in most cases the promoter in the plastid transformation vector will also drive expression in *E. coli*.
49. Many plastid expressed proteins are soluble and easily extractable by the TSP method, but several recombinant proteins are more challenging and need to be extracted using the TP method.
50. Aliquot the extract into several tubes, keep one tube on ice for the quantification and immediate Western blot, but store the others at −20 °C. Since repeated thawing and freezing can degrade the recombinant protein, take a new aliquot for every new set-up of the experiment.
51. Phenol is commonly delivered with an overlay of buffer solution that prevents oxidation of the phenol. Make sure not to take the buffer solution instead of the phenol and check for the color of the phenol: it should be colorless. A red/pinkish color indicates oxidation.
52. The Bradford assay is sensitive to SDS. The BCA assay is sensitive to Imidazole, so proteins that have been purified via IMAC can be quantified by BCA assay.
53. Use PEB I for dilution of TSP extracts and 1 % SDS for dilution of TP extracts.
54. Make sure that the tubes cannot burst open during the denaturation by either using safe-lock tubes or by putting a small weight, like a tube rack, on top of the tubes.
55. Clean the glass plates carefully and wipe them with ethanol to remove all traces of lipids and other contaminations.
56. Calculate the amount of Separating gel and Stacking gel that is needed for the size of your gel. 10 ml Separating gel and 3 ml Stacking gel are enough to prepare two gels with 8.3 × 7.3 cm dimensions and a 0.75 mm gel thickness.
57. The isopropanol smoothens the upper border of the gel and excludes oxygen which would prevent polymerization of the gel. Keep the excess gel in the tube to check when the polymerization is finished.
58. Add so much gel that it spills over the glass plate, and then insert the comb. Once inserted, do not pull the comb back

out, as this will result in air bubbles in the stacking gel and poorly separated wells.

59. Casted gels can be wrapped in wet paper towels, sealed in a plastic bag and stored for up to 3 days at +4 °C. Leave the comb in the gel for storage.
60. Coomassie staining is performed mainly to verify uniform sample loading. If the recombinant protein is small, then the upper part of the gel containing the Rubisco large subunit (~55 kDa) can be cut off and stained, while the lower part is blotted and used for Immunoblot analysis.
61. Follow the manufacturer's procedure, since this step varies depending on the membrane.
62. During the transfer the direction of protein transport is from cathode to anode. So the final set-up in the tank blot always has to be: cathode → gel → membrane → anode.
63. Since the performance of the blotting is improved at lower temperatures, most tank blot devices offer the option of adding a cooling cartridge within the transfer chamber. This allows you to perform the blotting at around +4 °C while keeping the set-up in the normal laboratory and furthermore reduces the volume of necessary buffer.
64. Collect the blotting buffer and store it at +4 °C. It can be re-used several times. When pre-stained protein marker is used, the success of the transfer is immediately visible and the orientation of the membrane is clear, but in addition one edge of the membrane can be cut off to indicate orientation.
65. This step can also be done by overnight incubation at +4 °C.
66. Antibody solution can be stored at +4 °C and re-used several times. If necessary the membrane can be stored for several days in TBS-T at +4 °C at this point.
67. We use both, the AP-Conjugate Substrate Kit (Bio-Rad, USA) and the Sigmafast™ BCIP®/NBT (Sigma) in our experiments with equally good results for colorimetric detection.
68. There are several free softwares available that allow you to quantify the signal intensities on your blot from digital images, e.g. ImageJ software.
69. Example: The TSP is quantified by Bradford as 3000 µg/µl, 10 µg TSP were loaded in each well onto the gel and visual comparison of the intensity of the bands on the Western blot band results in 0.5 µg recombinant protein. So the yield can be calculated as 5 % of TSP.
70. The same procedure as in **Note 69** is valid if the protein was extracted by total protein extraction. Then the result is reported as *X*% of TP.

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# Chapter 2

# Multigene Engineering in Rice Using High-Capacity *Agrobacterium tumefaciens* BIBAC Vectors

**Ruifeng He**

## Abstract

The high-capacity binary bacterial artificial chromosome (BIBAC) vector system permits the insertion of large fragments of DNA, up to 150 kb, into plants via *Agrobacterium*-mediated transformation. Here, we describe an optimized protocol for transformation of japonica rice (*Oryza sativa* L.) using this system. Calli derived from mature embryos are transformed using *Agrobacterium* strain LBA4404 that carries the BIBAC vector and the super-virulent helper plasmid pCH32. Transformed calli are then regenerated using optimized media and tested for transgene integration by PCR, GUS assay, and Southern blot analyses.

**Key words** Binary bacterial artificial chromosome (BIBAC), Transformation of large DNA fragment, *Agrobacterium tumefaciens*, Rice (*Oryza sativa* L.)

## 1 Introduction

Binary vector systems such as the pGreen series and the pCAMBIA series have been wildly used in *Agrobacterium*-mediated transformation of plants [1]. Whereas plant transformation with DNA fragments below 20 kb is routine, success in stable plant transformation with DNA fragments larger than 50 kb has been limited [2].

A reliable system for transforming large fragments of DNA (>50 kb) into plants makes it feasible to introduce a natural gene cluster or multiple genes into a single locus. Thus, several disease resistance and/or pest resistance genes, or a gene cluster encoding the enzymes of a metabolic pathway, could be simultaneously introduced in one transformation step. Large insert transformation would make it feasible to study the expression of plant genes or gene clusters in their native genomic context and might eliminate integration site-dependent gene expression, which can be a serious problem in plant transformation experiments.

The high-capacity binary bacterial artificial chromosome (BIBAC) [3] can replicate in both *Escherichia coli* and *Agrobacterium tumefaciens* and permit the insertion of large fragments of DNA

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(up to 150 kb) in tobacco [4] and tomato [5]. BIBAC libraries can not only be used for large-insert DNA library construction [6–8], but also facilitate gene discovery and functional studies by direct transformation of BIBACs carrying multiple genes or gene clusters into plants [9]. This transformation system opens the way for metabolic engineering in plants [10, 11].

Rice (*Oryza sativa* L.), a monocotyledonous plant, is a staple food for almost two-thirds of the world's population. In addition, its relatively small genome makes it an important model cereal for genome research and breeding. While *Agrobacterium*-mediated transformation of rice has been widely reported [12], the introduced DNA fragments have generally not been larger than 20 kb. In this chapter, we described a BIBAC-based protocol for transforming large fragments of DNA into rice.

## 2 Materials

### 2.1 Plants, Bacteria, and Vectors

1. Mature seeds of the *japonica* rice cultivar. There are various *japonica* rice cultivars available, but the seeds that we routinely use are from H1493, an early growing *japonica* variety initially bred in China [13].
2. *Agrobacterium tumefaciens* strain LBA4404 (Invitrogen).
3. BIBAC2 vector [3] carrying the gene(s) of interest. BIBAC library construction was described previously [7]. The vector is available from Cornell University (http://www.cctec.cornell.edu/express%20licensing/materials/BIBAC/).
4. pCH32 virulence helper plasmid with *virG* and *virE* loci, containing *virE1* and *virE2* [3], also available from Cornell University.

### 2.2 Antibiotics and Selective Agents

1. Rifampicin.
2. Streptomycin.
3. Tetracycline.
4. Cefotaxime.
5. Kanamycin.
6. Hygromycin.

### 2.3 Media

1. Induction medium N6I: 4 g/L Chu (N6) medium with vitamins (Plantmedia, Dublin, OH, USA), 1.0 g/L proline, 0.4 g/L Casein Enzymatic Hydrolysate (CH) (Sigma), 2.0 g/L 2,4-Dichlorophenoxyacetic acid (2,4-D), 45 g/L sucrose, 3 g/L phytagel (Sigma), pH 5.9.
2. Pre-cultivation medium N6P: 4 g/L N6 medium with vitamins, 0.6 g/L proline, 0.6 g/L CH, 2.0 g/L 2,4-D, 30 g/L maltose, 3 g/L phytagel, pH 5.6 (*see* **Note 1**).

3. *Agrobacterium* resuspend and infection medium N6A: 4 g/L N6 medium with vitamins, 0.6 g/L proline, 0.6 g/L CH, 2.0 g/L 2,4-D, 30 g/L maltose, pH 5.6. Add 100 μM acetosyringone before using.
4. Co-cultivation medium N6C: N6A supplemented with 3 g/L phytagel.
5. Resting medium N6R: 4 g/L N6 medium with vitamins, 1.2 g/L proline, 0.4 g/L CH, 2.5 g/L 2,4-D, 30 g/L sucrose, 3 g/L phytagel, pH 5.8. Add 400 mg/L cefotaxime before using.
6. Selection medium N6S: 4 g/L N6 medium with vitamins, 1.2 g/L proline, 0.4 g/L CH, 2.5 g/L 2,4-D, 30 g/L sucrose, 3 g/L phytagel, pH 5.9. Add 250 mg/L cefotaxime, 50 mg/L hygromycin before using.
7. Pre-regeneration medium MSP: 4.2 g/L Murashige and Skoog (MS) medium with vitamins, 2 g/L CH, 2 mg/L kinetin, 0.2 mg/L naphthalene acetic acid (NAA), 30 g/L maltose, 3 g/L phytagel, pH 5.8.
8. Regeneration medium MSR: 4.2 g/L MS medium with vitamins, 3 mg/L kinetin, 0.5 mg/L NAA, 30 g/L maltose, 3 g/L phytagel, pH 5.8.
9. Root-growing medium MSG: 2.1 g/L MS medium with vitamins, 0.2 mg/L NAA, 10 g/L sucrose, 2.5 g/L phytagel, pH 5.8. Add 50 mg/L hygromycin before using. Solidify in 30 × 200 mm tubes.
10. Solid LB medium supplemented with 25 mg/L rifampicin, 25 mg/L streptomycin, 50 mg/L kanamycin, and 5 mg/L tetracycline.

### 2.4 Other Materials for Transformation

1. Sterile filter paper.
2. 70 % ethanol.
3. 20 % bleach with 0.1 % Tween 20.
4. Parafilm.
5. Spectrophotometer.
6. Electroporator.
7. Laminar flow hood.
8. Unlit growth chamber.
9. Greenhouse.
10. Pots.

### 2.5 GUS Assay

1. X-gluc buffer: 100 mM sodium phosphate buffer ($NaH_2PO_4$-Na2HPO4), pH 7.0, 1 mM X-gluc, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, and 0.1 % Triton X-100.
2. Methanol.

### 2.6 PCR

1. DNA isolation kit.
2. Thermocycler.
3. *Taq* DNA polymerase and appropriate buffer.
4. Primers: *gus* sequence primers (5′-TCGCGAAAACTGTGGA ATTGATC-3′ and 5′-AGCCGACAGCAGCAGTTTCAT-3′), *hpt* sequence primers (5′-GATGTAGGAGGGCGTGGATATG TC-3′ and 5′-CTTCTACACAGCCATCGGTCCAGA-3′), and *nptII* sequence primers (5′-TCGGCTATGACTGGGCACA ACAGA-3′ and 5′-AAGAAGGCGATAGAAGGCGATGCG-3′).
5. Agarose gel and equipment for DNA electrophoresis.

### 2.7 Southern Blot

1. $^{32}$P-dCTP labeling kit.
2. Primers for *hpt*, *nptII*, or the gene of interest.
3. *EcoR*V and *Hind*III restriction enzymes, with appropriate buffer for double digest.
4. Agarose gel and equipment for DNA electrophoresis.
5. Hybond-N$^+$ nylon membrane filters (Amersham, England).
6. 0.2 M HCl.
7. 0.4 M NaOH.
8. Pre-hybridization buffer: 5× saline-sodium citrate (SSC), 5× Denhardt's regent, 0.5 % sodium dodecyl sulfate (SDS) and 100 μg/ml denatured, fragmented, salmon sperm DNA.
9. Wash buffer A: 1× SSC, 0.1 % SDS. A 20× stock solution of SSC contains 3 M sodium chloride and 0.3 M sodium citrate, adjusted to pH 7.0 with HCl.
10. Wash buffer B: 0.5× SSC, 0.1 % SDS.
11. Shaking incubator.
12. X-ray film and equipment for autoradiography.

## 3 Methods

### 3.1 Agrobacterium Transformations

1. Transfer the virulence helper plasmid pCH32 to *Agrobacterium* strain LBA4404 by electroporation using an electroporator at 1.8 kV/0.1 cm.
2. Introduce the BIBAC plasmid with a large insert fragment containing the gene(s) of interest to *Agrobacterium* strain LBA4404 harboring pCH32 by electroporation (*see* **Note 2**).

### 3.2 Rice Transformation and Regeneration

#### 3.2.1 Callus Induction

1. Dehusk 100–120 mature, fresh rice seeds (sufficient for one transformation).
2. Soak the seeds in 70 % ethyl alcohol for 1 min, then surface-sterilize in 20 % bleach with 0.1 % Tween-20 for 30 min with shaking.

3. Rinse the seeds four to five times with sterile water in a laminar flow hood.
4. Dry the seeds on sterile filter paper.
5. Partially submerge 10–12 seeds on a single plate containing solid N6I medium with the embryo facing up.
6. Seal the plate with Parafilm and place it inside an unlit growth chamber at 26 ± 2 °C for 3–4 weeks. Calli are formed from the scutella after 3–4 weeks in culture.

*3.2.2 Callus Subculture and Pre-cultivation*

1. Dissect out the light yellow, compact, and relatively dry calli and subculture on the same N6I medium for another 2–3 weeks.
2. Select the actively growing, healthy looking, embryogenic calli and inoculate onto pre-cultivation medium N6P for 4 days.

*3.2.3 Agrobacterium Preparation, Infection, and Co-cultivation*

1. Grow *Agrobacterium* strain LBA4404 with pCH32 (harboring the BIBAC plasmid with a large insert fragment) for 2–3 days at 28 °C on solid LB medium supplemented with 25 mg/L rifampicin, 25 mg/L streptomycin, 50 mg/L kanamycin, and 5 mg/L tetracycline.
2. Resuspend agrobacterial cells in 25 ml liquid medium N6A with shaking (150 rpm) at 28 °C for 1.5–2 h to an OD600 = 1.0 (*see* **Note 3**).
3. Immerse the embryonic calli from the 4-day culture in agrobacterial suspension for 15 min with gently shaking (80 rpm).
4. Remove the excess bacteria by decanting the liquid.
5. Transfer the calli onto sterile filter paper for blot-drying, and then place them on co-cultivation medium N6C in the dark at 24 °C for 3 days (*see* **Note 4**).

*3.2.4 Resting and Selection*

1. Wash the infected calli in sterile water several times, until the water becomes clear.
2. Wash twice (15 min each time) with sterile water containing 400 mg/L of cefotaxime with gently shaking (80 rpm).
3. Dry the calli on filter paper and transfer to resting medium N6R at 26 ± 2 °C for 1 week (*see* **Note 5**).
4. Transfer the calli to selection medium N6S and sub-culture them every 2 weeks. Keep the cultures in the dark at 26 ± 2 °C for 6–8 weeks until resistant calli proliferate (*see* **Note 6**).

*3.2.5 Regeneration and Rooting*

1. Move the hygromycin-resistant calli to pre-regeneration medium MSP for 1 week at a temperature of 26 ± 2 °C in a culture room at photoperiodic regime of 16/8 (light/dark) cycle.
2. Culture the growing calli on regeneration medium MSR for 2–4 weeks (*see* **Note 7**).

3. When the shoots develop into 2–4 cm plantlets, transfer them to magenta boxes containing 30 mL root-growing medium MSG (*see* **Note 8**).
4. Grow the plantlets under the same conditions stated above for 2–3 weeks.
5. Transfer well-rooted plants to soil in pots and grow them in a greenhouse.

### 3.3 GUS Assay

T-DNA delivery into calli and plantlets can be confirmed by histochemical assays for GUS.

1. Incubate tissue segments in X-gluc buffer at 37 °C overnight.
2. Wash leaf segments twice in 99 % methanol for 2 h before visual examination.

### 3.4 PCR Analysis

T-DNA delivery can also be confirmed by PCR amplification of the inserted sequences.

1. Isolate genomic DNA from young leaves of the control (untransformed plant), T0, T1 (from selfed seeds of T0 transformants), and T2 (from selfed seeds of T1 transformants) transgenic rice plants [14], using a DNA isolation kit or standard protocol.
2. Set up a 25 μl PCR reaction for each sample containing 20 ng of genomic DNA, 1 μM each of forward and reverse primer (for *gus*, *nptII*, or *htp*), 1× PCR buffer, and 0.5 units of *Taq* DNA polymerase.
3. Run the following program for PCR: 94 °C for 5 min for 1 cycle; 94 °C for 50 s, 55 °C for 50 s, 72 °C for 80 s for 34 cycles; 72 °C for 10 min for 1 cycle.
4. Analyze PCR products by gel electrophoresis on 1 % agarose gels. The predicted specific sequences should be 998 bp for *gus*, 722 bp for *nptII*, and 852 bp for *hpt*.

### 3.5 Southern Analysis

1. To create labeled probe, the PCR-amplified specific fragments of *hpt*/*nptII* gene and insert genes are labeled with $^{32}$P-dCTP by random priming according to the manufacturer's recommendation (Promega, USA).
2. Digest 5 μg of genomic DNA with 20 U *Eco*RV and 20 U *Hind*III at 37 °C overnight.
3. Run the digest on 1 % agarose gel at 4 V/cm for 16 h.
4. Soak the gel in 0.2 M HCl for 8 min, then in water for 1 min.
5. Transfer the DNA fragments onto a Hybond-N+ nylon membrane filter under alkaline conditions (0.4 M NaOH) for 24 h.
6. Add labeled probe to the pre-hybridization buffer (see above).

7. Hybridize overnight at 65 °C in an incubator with gentle shaking.
8. Wash the membrane at 65 °C, with wash buffer A for 20 min, then with wash buffer B for 15 min.
9. Blot dry, wrap in plastic wrap and autoradiograph for 3–5 days depending on the strength of the hybridization signals.

## 4 Notes

1. It has been reported that acidic pH during co-cultivation enhances the expression of *vir* genes [12]. Therefore, the same acidic condition (pH 5.6) during pre-cultivation would be beneficial to the transformation of large DNA fragments.
2. Additional *vir* genes as well as the *Agrobacterium* genetic background are very important for the transformation of large DNA fragments in rice. Among the various strains of *Agrobacterium* tested, only the strains with additional pCH32 could successfully produce transformants. A significant enhancement of transformation efficiency was observed when the strain LBA4404 with pCH32 contains additional *vir* genes [15].
3. The concentration of *Agrobacterium* cells is considered to be a critical factor for the efficiency of transformation. Low concentrations can reduce the frequency of T-DNA transfer. With the BIBAC system, a high concentration (OD600 = 1.0–1.2) of *Agrobacterium* cells improves the efficiency of transformation.
4. The addition of acetosyringone (100 μM) during co-cultivation is essential for transformation. Other factors, such as an acidic pH, relatively lower culture temperature (24 °C) and the duration of co-cultivation (2–4 days) were optimized to develop an efficient transformation procedure using the BIBAC system. It was observed that 2–4 days (depending on the bacterial density used for infection) of co-cultivation resulted in the highest GUS activity. Longer co-cultivation periods (>4 days) resulted in an abundant proliferation of bacteria and consequently decreased the regeneration frequency of the selected calli.
5. Excessive bacteria may put serious stress on plant cells and affect their regeneration potential. Calli are often completely colonized by the *Agrobacterium* making elimination of bacteria in subsequent stages more difficult. Controlling *Agrobacterium* overgrowth after co-cultivation is crucial, but prolonged exposure to the antibiotic cefotaxime or timentin, when combined with hygromycin selection, has a detrimental effect to infected calli. To overcome this problem, the co-cultivated calli are first washed with cefotaxime, then placed on

resting medium containing cefotaxime without hygromycin, before being transferred to selective plates. Using this method, *Agrobacterium* growth can be fully restrained without exposing the calli to the combined stresses of *Agrobacterium* and selection agent (hygromycin).

6. The duration of selection is very important. In our experiments, most regenerated shoots obtained after only 2–3 weeks selection were shown not to be transgenic. Therefore, a longer selection time of 6–8 weeks on 50 mg/L hygromycin was needed.
7. In our experience, shoot regeneration was delayed or absent when regeneration medium contained hygromycin. Hence, calli selected by hygromycin for several cycles were regenerated without hygromycin, thereby maintaining a balance between stringency of selection and regeneration efficiency.
8. Transformation efficiency is significantly improved using this procedure, involving selection of calli first on regeneration medium MSR and then on root-growing medium MSG. A low sucrose content of 10 g/L in MSG was found to be optimal for improving selection efficiency and reducing false positives. Sucrose is required for shoot growth, but is not critical once the shoots are fully developed.

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# Chapter 3

# Virus-Derived Vectors for the Expression of Multiple Proteins in Plants

**Pooja Saxena, Eva C. Thuenemann, Frank Sainsbury, and George P. Lomonossoff**

## Abstract

This chapter constitutes a practical guide to using the "pEAQ" vector series for transient or stable expression of one or more protein(s) in *Nicotiana benthamiana* plants. The pEAQ vectors are a series of small binary vectors designed for controlled expression of multiple proteins in plants. To achieve high levels of expression, an expression system based on translational enhancement by the untranslated regions of RNA-2 from cowpea mosaic virus (CPMV), named CPMV-*HT*, is used. The expression vector pEAQ-*HT* combines the user-friendly pEAQ plasmid with CPMV-*HT* to provide a system for high-level expression of proteins in plants.

**Key words** Transient expression, Agro-infiltration, Viral vectors, pEAQ, CPMV-*HT*

## 1 Introduction

The efficient co-expression of multiple proteins within the same cell represents a major challenge to plant expression methods, especially if the various polypeptides need to be present in differing amounts. This has proved very difficult to achieve, especially with transient expression approaches using replicating virus vectors due to virus exclusion: the phenomenon whereby the presence of a replicating RNA within a cell effectively excludes the replication of a second construct. Thus expression systems based on monopartite, replication-competent viruses, are essentially limited to the production of a single protein within a given cell unless multiple proteins can be expressed from a single viral construct or a second, non-competing virus is used for the expression of the second protein [1]. However, the former generally results in low yields with inserts often deleted due to pressure on replication and the latter can be rather complex to implement. Alternatively, a naturally multipartite virus, in which the genome is divided into multiple nucleic

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acid segments, can be used to express multiple proteins. The replication mechanism of such viruses requires that multiple RNA molecules have to be replicated within the same cell, implying that virus exclusion will not occur [2]. However, even if co-expression can be achieved, it is very difficult to control the relative levels of the different proteins as the size of the inserted sequence has a major effect on the rate of replication of a given construct.

The use of non-replicating transient expression systems, such as the cowpea mosaic virus-based *HyperTrans* (CPMV-*HT*) system [3], offers considerable potential benefits for the production of multiple proteins: it is possible to control the level of expression of the individual components and multiple constructs can be expressed in the same cell without the problem of virus exclusion found with replicating systems. Expression using the CPMV-*HT* system involves inserting the sequence(s) to be expressed between a modified 5′ UTR and the 3′ UTR from CPMV RNA-2 to create a sequence encoding an efficient mRNA molecule. This sequence, in turn, is placed between a CaMV 35S promoter and a *nos* terminator within the T-DNA region of a binary vector to create an "*HT* cassette" for plant-based expression. The level of expression from the *HT* cassette can be modulated by altering the sequence of the 5′ UTR. A series of small binary vectors, the pEAQ series, has been developed to facilitate direct cloning of heterologous sequences into the *HT* cassette using either restriction enzyme or GATEWAY-based cloning [4]. The pEAQ vectors also contain an expression cassette for the P19 suppressor of gene silencing [5]. All the initial cloning work is carried out in *Escherichia coli*. The pEAQ-based constructs are then transferred to *Agrobacterium tumefaciens* and suspensions of the resulting bacteria are used to infiltrate leaves of *Nicotiana benthamiana* to initiate plant-based expression. Expression of the inserted sequence is generally achieved 3–10 days post-infiltration (dpi).

The pEAQ vector system has proved highly effective for rapid and transient expression of a variety of proteins including vaccine candidates, antibodies, and active enzymes [4, 6–11]. Some of these examples have required the co-expression of multiple polypeptide chains. For a recent review on the variety of recombinant proteins produced in plants using the pEAQ system, *see* [12].

Expression of multiple proteins using the pEAQ vector system can be achieved in two ways. In the first approach, two or more independent cultures of *A. tumefaciens* each containing a pEAQ construct expressing a single protein can be mixed and the mixture can be used to infiltrate *N. benthamiana* leaves. The advantage of this approach is that it is very flexible, allowing various gene combinations to be rapidly tested. The disadvantage is that it is not possible to guarantee that all the cells have received all the constructs unless high-density bacterial suspensions are used for inoculation (Fig. 1). This can create problems in terms of the efficiency

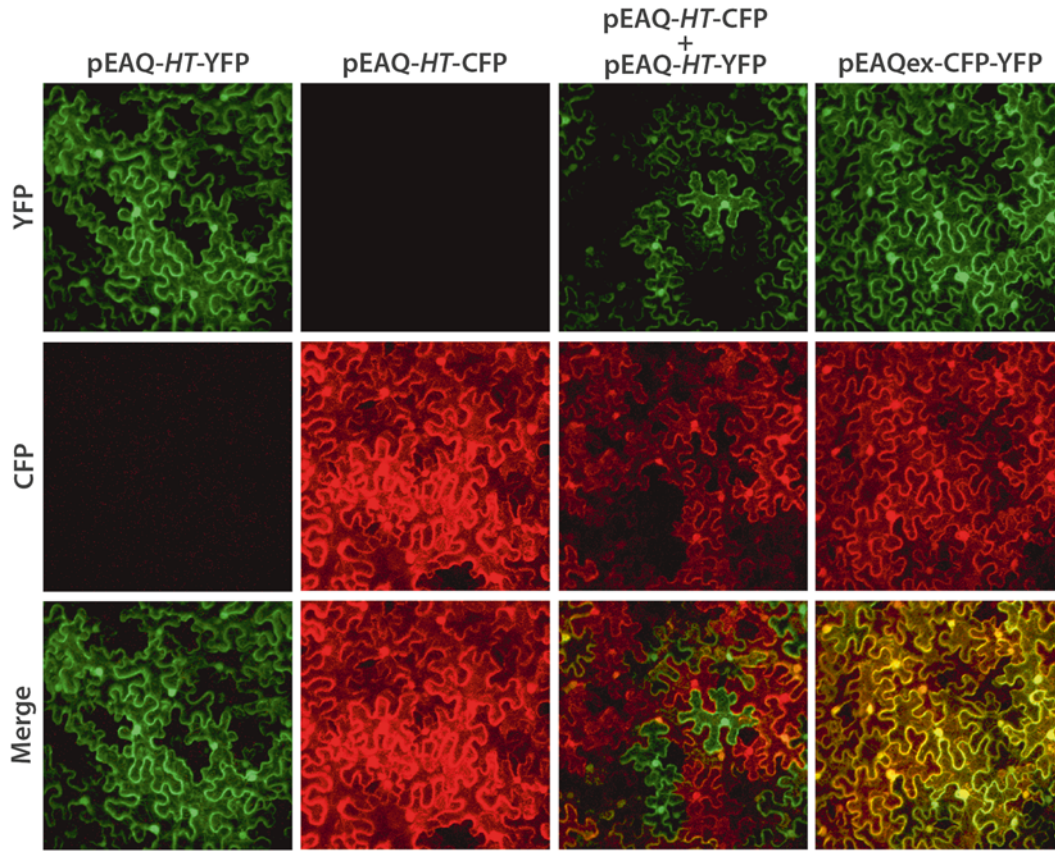



**Fig. 1** Plant tissue visualized using confocal microscopy at 6 dpi. Expression of two reporter genes, EYFP (yellow fluorescent protein) and ECFP (cyan fluorescent protein) was performed in the following manner: Column 1: individual expression of EYFP ($OD_{600} = 0.2$), Column 2: individual expression of ECFP ($OD_{600} = 0.2$), Column 3: expression of EYFP and ECFP from separate plasmids by mixing cultures in 1:1 ratio (final $OD_{600} = 0.4$), and Column 4: co-expression of EYFP and ECFP from the same T-DNA ($OD_{600} = 0.2$). *Green* signal shows expression of EYFP, *red* signal shows expression of ECFP, and *yellow* signal depicts co-expression of EYFP and ECFP

of infiltration and can have cost implications if large amounts of *A. tumefaciens* have to be grown. The alternative approach is to express multiple genes from a single construct. With this system, multicistronic vectors are not compromised by the constraints imposed by replication, as made possible by the pEAQ vector system by way of its modular design, which allows the incorporation of multiple *HT* cassettes on the same T-DNA [4, 13]. This approach is effective at ensuring all proteins are co-expressed in the same cell (Fig. 1) and allows infiltration with lower density bacterial suspensions. The main disadvantage of this approach is that vector design is more complicated. In practice, it is probably best to carry out preliminary screening experiments using the mixing approach and, once an appropriate combination of genes has been identified, construct the more complex vectors to optimize the

efficiency of expression. Introduction of multiple sequences on the same T-DNA can be achieved through the use of the basic transient expression vector "pEAQexpress" and is explained in Subheading 3.2.

## 2 Materials

### 2.1 Media, Buffers, and Solutions

1. Luria-Bertani (LB) medium: 10 g/L Bacto-tryptone, 10 g/L NaCl, and 5 g/L Yeast extract, pH 7.0 with NaOH.
2. LB agar: as LB with 10 g/L agar added.
3. SOC: 20 g/L Bacto-tryptone, 5 g/L yeast extract, 0.58 g/L NaCl, 0.19 g/L KCl, 2.03 g/L $MgCl_2$, 2.46 g/L magnesium sulfate 7-hydrate, 3.6 g glucose.
4. MMA: 10 mM MES (2-[*N*-morpholino]ethanesulfonic acid), pH 5.6 with NaOH, 10 mM $MgCl_2$, 100 μM acetosyringone (3′5′-dimethoxy-4′-hydroxyacetophenone).
5. Kanamycin, gentamycin (for GATEWAY cloning only), and rifampicin.

### 2.2 Enzymes and Kits

1. Restriction enzymes: *Age*I, *Asc*I, *AsiS*I, *Fse*I, *Mlu*I, *Not*I, *Nru*I, *Pac*I, *Sma*I, *Stu*I, *Xho*I, *Xma*I, with appropriate reaction buffers (New England Biolabs).
2. Alkaline Phosphatase, Calf Intestinal (New England Biolabs).
3. T4 DNA ligase and reaction buffer (New England Biolabs).
4. BP clonase II (Invitrogen) (for GATEWAY cloning).
5. LR clonase II (Invitrogen) (for GATEWAY cloning).
6. Proofreading DNA polymerase.
7. DNA gel extraction kit (Qiagen).
8. Plasmid extraction kit (Qiagen).
9. Quick Blunting Kit (New England Biolabs) (for cloning of more than two genes).
10. Site-directed mutagenesis kit (Agilent Technologies) (for modulation of relative expression levels).

### 2.3 Bacterial Strains

1. One Shot® TOP10 chemically competent *E. coli* (Invitrogen) is used for propagation of recombinant plasmids.
2. One Shot® ccdB survival 2T1R chemically competent *E. coli* (Invitrogen) is used for propagation of pEAQ-DEST plasmids before recombination with entry clones.
3. Electrocompetent *A. tumefaciens* strain LBA4404 [14] is used for plant transformation.

### 2.4 Other

1. 3–4-week-old *N. benthamiana* plants.
2. Gene-specific primers for PCR with appropriate restriction sites (for restriction enzyme-based cloning) or with *attB* sites (for GATEWAY cloning).
3. GATEWAY donor vector (Invitrogen) (for GATEWAY cloning).
4. Reagents for PCR including buffer, dNTPs, $MgCl_2$.
5. 1 % agarose gel and materials for gel electrophoresis.
6. Spectrophotometer.
7. Heat block.
8. Shaker incubator.
9. Centrifuge.
10. Electroporator and cuvettes.
11. Vacuum desiccator or 1 mL plastic syringe.
12. Greenhouse.

## 3 Methods

### 3.1 Creation of Expression Plasmids

#### 3.1.1 Choice of Expression Vector

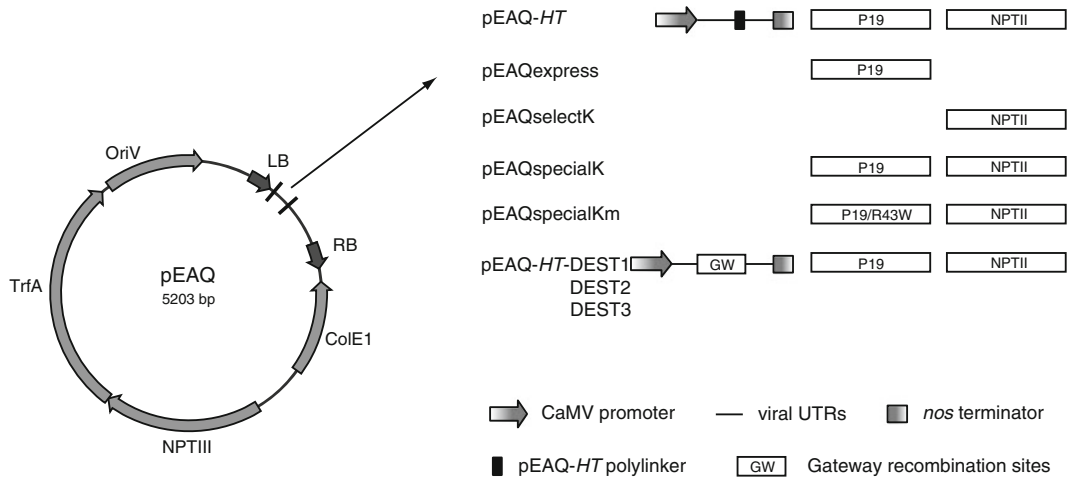
1. Using the features described in Table 1, choose the most appropriate pEAQ vector for the expression of the gene of interest. Further details regarding the construction of the pEAQ vectors can be obtained from Sainsbury et al. [4].
2. Clone the insert by either restriction enzyme-based cloning (Subheading 3.1.2) or GATEWAY recombination (Subheading 3.1.3).

#### 3.1.2 Restriction Enzyme-Based Cloning

1. Generate the insert, i.e. DNA encoding the desired gene from start to end, with appropriate restriction sites at both ends using PCR (*see* **Notes 1** and **2**).
2. In separate reactions, digest the insert and plasmid pEAQ-*HT* with appropriate restriction enzymes (*see* Fig. 2 and Table 2) in buffer according to the enzyme manufacturer's recommendation (*see* **Note 3**). For expression using pEAQ vectors other than pEAQ-*HT*, the insert should first be cloned in the polylinker of pEAQ-*HT* (*see* Fig. 2). Then, the entire expression cassette can be moved to other pEAQ vectors using the appropriate restriction enzymes for the multiple cloning site (*see* Fig. 2).
3. Heat-inactivate the restriction enzymes, if possible, according to the manufacturer's instructions.
4. Dephosphorylate the linearized vector with alkaline phosphatase following the manufacturer's instructions (*see* **Note 4**). Resolve digests on a 1 % agarose gel (*see* **Note 5**) and purify the digested vector and inserts using a DNA gel extraction kit.

**Table 1**
**ThepEAQ vector series and their features**

| Plasmid name | GenBank accession | Features |
|---|---|---|
| pEAQ-*HT* | GQ497234 | Designed for easy and quick cloning of a "gene of interest" into the CPMV-*HT* system. Its T-DNA comprises:<br>• The CPMV-*HT* expression cassette with a polylinker (Figure 2) to insert the "gene of interest."<br>• The suppressor of gene silencing P19.<br>• Neomycin phosphotransferase II (*nptII*) to confer resistance to kanamycin. |
| pEAQexpress | GQ497230 | Designed for cloning multiple CPMV-*HT* expression cassettes in the same vector for transient expression only. Its T-DNA comprises:<br>• A multiple cloning site for insertion of multiple expression cassettes digested using enzymes *Pac*I and *Asc*I.<br>• The suppressor of gene silencing P19. |
| pEAQselectK | GQ497231 | Designed for expression from a CPMV-*HT* expression cassette in the *absence* of a suppressor of silencing. Its T-DNA comprises:<br>• A multiple cloning site for insertion of the expression cassettes.<br>• *nptII* to confer resistance to kanamycin. |
| pEAQspecialK | GQ497232 | Designed for expression from a CPMV-*HT* expression cassette in the *presence* of a suppressor of silencing. Its T-DNA comprises:<br>• A multiple cloning site for insertion of the expression cassette.<br>• The suppressor of gene silencing P19.<br>• *nptII* to confer resistance to kanamycin. |
| pEAQspecialKm | GQ497233 | Best suited for stable expression of proteins in whole plants. Its T-DNA comprises:<br>• A multiple cloning site for insertion of the expression cassette.<br>• The *modified* suppressor of gene silencing P19/R43W [16].<br>• *nptII* to confer resistance to kanamycin. |
| pEAQ-*HT*-DEST1 | GQ497235 | Designed for easy and quick cloning using the Gateway® system to express *wild-type* protein in plants. Its T-DNA comprises:<br>• The CPMV-*HT* expression cassette with attR sites to introduce the "gene of interest" from the entry clone via recombination.<br>• The suppressor of gene silencing P19.<br>• *nptII* to confer resistance to kanamycin. |
| pEAQ-*HT*-DEST2 | GQ497236 | Designed for easy and quick cloning using the Gateway® system to express *N-terminally His-tagged* protein in plants. Its T-DNA comprises:<br>• The CPMV-*HT* expression cassette with attR sites to introduce the "gene of interest" from the entry clone via recombination.<br>• The suppressor of gene silencing P19.<br>• *nptII* to confer resistance to kanamycin. |
| pEAQ-*HT*-DEST3 | GQ497237 | Designed for easy and quick cloning using the Gateway® system to express *C-terminally His-tagged* protein in plants. Its T-DNA comprises:<br>• The CPMV-*HT* expression cassette with attR sites to introduce the "gene of interest" from the entry clone via recombination.<br>• The suppressor of gene silencing P19.<br>• *nptII* to confer resistance to kanamycin. |




pEAQ Multiple cloning site:***Pac***I-*Ase*I-*Stu*I-*Xho*I-*Sbf*I-*Mfe*I-*Avr*II-*Bsp*EI-*Age*I-*Sna*BI-*Sma*I-*Xma*I-***Asc***I-***Asi***SI-***Mlu***I

pEAQ-*HT* polylinker: ***Age*I/*Nru*I** - 6xHis - ***Xma*I/*Sma*I** - 6xHis - ***Xho*I/*Stu*I**

**Fig. 2** Schematic representation of the pEAQ vector series

**Table 2**
**Restriction enzyme pairs to use for restriction enzyme-based cloning into pEAQ-*HT* depending on whether a *His-tag* is required**

| | | |
|---|---|---|
| For expression of *wild-type* protein | *Nru*I or *Age*I at the 5′ end | *XhoI* or *Stu*I at the 3′ end |
| For expression of protein with a *His-tag* at the N-terminus | *XmaI* or *Sma*I at the 5′ end | *XhoI* or *Stu*I at the 3′ end |
| For expression of protein with a *His-tag* at the C-terminus | *Nru*I or *Age*I at the 5′ end | *XmaI* or *Sma*I at the 3′ end |

Each position contains the option of using a restriction enzyme that leaves an overhang or a blunt end

5. Determine the concentration of extracted DNA using a spectrophotometer, or by agarose gel electrophoresis with a DNA mass ladder.
6. Combine the digested vector and insert in molar ratio 1:3 in ligase buffer, add T4 DNA ligase and incubate according to the manufacturer's recommendations.
7. Transform chemically competent *E. coli* using standard heat-shock transformation methods (*see* **Note 6**; mix cells with DNA on ice, heat shock at 42 °C for 30 s, return to ice, add 250 μL liquid SOC or LB, shake at 37 °C and ~250 rpm for 1 h) and plate onto LB agar plates with kanamycin (50 μg/mL) selection. Incubate the plates overnight at 37 °C to allow transformed colonies to appear.

**Table 3**
**Choices of pEAQ-*HT*-DEST vectors depending whether the protein is required to be wild-type or His-tagged**

| For expression of *wild-type* protein | Do the LR reaction of your entry clone with pEAQ-*HT-DEST1* |
|---|---|
| For expression of protein with a *His-tag* at the N-terminus | Do the LR reaction of your entry clone with pEAQ-*HT-DEST2* |
| For expression of protein with a *His-tag* at the C-terminus | Do the LR reaction of your entry clone with pEAQ-*HT-DEST3* |

8. Screen colonies by PCR or restriction analysis. Grow a positive clone overnight in liquid LB medium with 50 μg/mL kanamycin.
9. Extract the plasmid using a plasmid extraction kit or other standard method for sequencing (to confirm insertion) and transformation of *A. tumefaciens*.
10. Transform this expression plasmid into *Agrobacterium* (Subheading 3.3); or for expression of multiple genes, follow the methods in Subheading 3.2.

*3.1.3 Cloning by Gateway® Recombination*

1. Generate the insert, i.e. DNA encoding the desired gene from start to end, with bacteriophage lambda attachment B (*attB*) sites at both ends using PCR (*see* **Notes 1** and **2**).
2. Using BP clonase II according to the manufacturer's directions, transfer the PCR fragment to a Gateway® donor vector via site-directed recombination. The resultant plasmid is the entry clone.
3. Transform chemically competent *E. coli* using heat-shock transformation methods (*see* **Note 6**) and plate onto LB agar plates with gentamycin (7 μg/mL) selection. Incubate the plates overnight at 37 °C to allow transformed colonies to appear. Resulting colonies are highly likely to be positive for the insert, but may be screened by PCR or restriction analysis.
4. Grow a positive clone overnight in liquid LB medium with 7 μg/mL gentamycin.
5. Extract the entry clone using a plasmid extraction kit or other standard method.
6. Using LR clonase II according to the manufacturer's directions, transfer the gene of interest from the entry clone to the appropriate pEAQ-*HT*-DEST vector (*see* Table 3).
7. Transform chemically competent *E. coli* using heat-shock transformation methods (*see* **Note 6**) and plate onto LB agar plates

with kanamycin (50 μg/mL) selection. Incubate the plates overnight at 37 °C to allow transformed colonies to appear.

8. Screen colonies by PCR or restriction analysis. Grow a positive clone overnight in liquid LB medium with 50 μg/mL kanamycin.
9. Extract the plasmid using a plasmid extraction kit or other standard method for sequencing (to confirm insertion) and transformation of *A. tumefaciens*.
10. Transform this expression plasmid into *Agrobacterium* (Subheading 3.3); or for expression of multiple genes, follow the methods in Subheading 3.2.

### 3.2 Cloning of Two or More Genes onto One T-DNA Construct

The pEAQ vector system allows for the efficient co-expression of multiple proteins from a single plasmid, thereby ensuring co-expression in every cell (Fig. 1). It is recommended that genes should first be cloned into pEAQ-*HT* and tested individually and in combination by co-infiltration before cloning onto the same plasmid.

#### 3.2.1 Cloning of Two Genes into pEAQexpress

Start with sufficient plasmid preparations of pEAQexpress (Fig. 3) and pEAQ-*HT* containing gene A (pEAQ-*HT*-A) and gene B (pEAQ-*HT*-B).

1. Digest pEAQexpress vector with *Mlu*I and *Asi*SI, and digest pEAQ-*HT*-A with *Asc*I and *Pac*I to create compatible sticky ends.
2. Purify the vector and insert by agarose gel purification, using a DNA gel extraction kit.
3. Ligate the *Asc*I-*Pac*I insert and *Mlu*I-*Asi*SI vector at a molar ratio of 3:1.
4. Using heat-shock transformation methods (*see* **Note 6**), transform chemically competent *E. coli* and plate on LB agar containing 50 μg/mL kanamycin. Incubate overnight at 37 °C to allow colonies to appear.
5. Colonies may be screened using PCR or restriction digest. Use a positive clone to prepare the intermediate vector pEAQex-A.
6. Digest pEAQex-A and pEAQ-*HT*-B with *Asc*I and *Pac*I.
7. Purify the vector and insert by agarose gel purification.
8. Ligate the insert and vector at a molar ratio of 3:1.
9. Using heat-shock transformation methods (*see* **Note 6**), transform chemically competent *E. coli* and plate on LB agar containing 50 μg/mL kanamycin. Incubate overnight at 37 °C to allow colonies to appear.
10. Colonies may be screened using PCR or restriction digest. Use a positive clone to prepare the expression vector pEAQex-A-B.

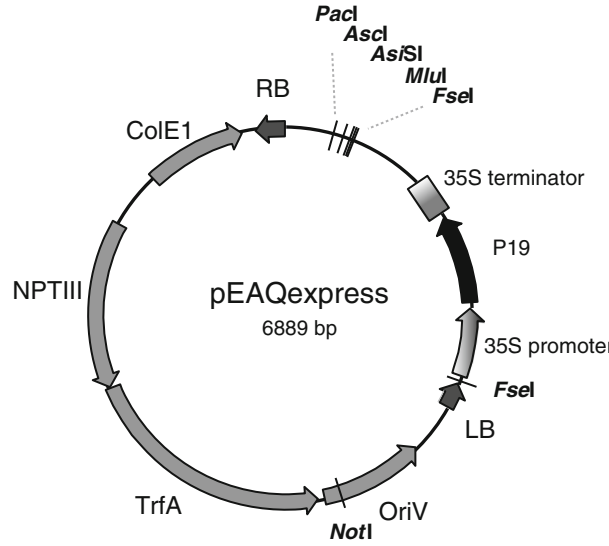



**Fig. 3** Schematicrepresentation of the pEAQexpress vector

Note that the two genes can be cloned sequentially in two ways to create pEAQex-A-B or pEAQex-B-A. Both of these constructs should allow for co-expression of the two genes A and B.

### *3.2.2 Cloning of Four Genes into pEAQexpress*

Cloning of more than two genes onto one pEAQ vector can be complex and exact strategies will be dependent on the availability of unique restriction sites in each individual case. The following is an example of a strategy used for cloning four genes (A, B, C, and D) onto a single construct.

1. Prepare two pEAQexpress (Fig. 3) clones for co-expression of two gene pairs: pEAQex-A-B and pEAQex-C-D as described in Subheading 3.2.1.
2. Digest pEAQex-A-B with *Pac*I and digest pEAQex-C-D with *Fse*I.
3. Use the Quick Blunting Kit to create blunt ends, then purify the linearized plasmids using a plasmid extraction kit.
4. Digest both linearized plasmids with *Not*I which cuts within the oriV sequence of the pEAQ backbone, creating a 5′-overhang.
5. Purify the fragments containing the genes of interest (A-B and C-D) by gel extraction. Then, ligate the two fragments at an equimolar ratio at 16 °C overnight.
6. Transform chemically competent *E. coli* and plate on LB agar containing kanamycin (50 μg/mL). Incubate overnight at 37 °C to allow colonies to appear.

7. Screen for positive transformants by PCR using one primer specific for gene A or B, and a reverse primer specific for gene C or D. Alternatively, confirm positive transformants by digestion of extracted plasmid with restriction enzymes.

#### 3.2.3 Modulation of Relative Expression Levels of Two Proteins of Interest

Whilst most applications strive for the highest yields possible, it is sometimes advantageous to co-express two proteins of interest at different levels. For instance, in the case of expression of metabolites in an enzymatic pathway, low levels of a substrate may be needed or in expression of a multimeric protein, varying amounts of different subunits may be required. Using the pEAQ vectors, expression can be modulated by selectively removing the *HT* mutation [3] within the 5′UTR of one of the expression cassettes, thereby reducing the expression level of that gene. This can be achieved by site-directed mutagenesis (*see* **Note 7**). Expression cassettes of genes with and without the *HT* mutation can then be combined in pEAQexpress to allow targeted manipulation of relative expression levels of two or more proteins within the same cell. This strategy has been successfully used to produce multimeric virus-like particles of bluetongue virus in plants [10]. Variation of the 3′UTR of pEAQ-*HT* vectors has also been recently explored for modulation of protein expression levels and this has been discussed in [15].

### 3.3 Transformation of Agrobacterium

1. Once the expression construct is generated, it can be introduced into electrocompetent *A. tumefaciens* strain LBA4404 using standard electroporation procedures at 2.5 kV.
2. Recover agrobacterial cells at 28 °C for 1 h in SOC medium.
3. Plate the cells on LB agar containing rifampicin 50 μg/mL (to select for LBA4404) and kanamycin 50 μg/mL (to select for carried plasmid).
4. Incubate the plates at 28 °C. Transformed colonies are visible on plates in 2–3 days.
5. Continue to Subheadings 3.4 and 3.5 for transient expression in *N. benthamiana*, or to Subheading 3.7 for stable expression.

### 3.4 Preparation of Agrobacterium Suspensions for Infiltration

1. Prepare 10–100 mL LB with appropriate antibiotics to select for the *Agrobacterium* strain (rifampicin 50 μg/mL for LBA4404) and carried plasmid (kanamycin 50 μg/mL for pEAQ vectors). The volume of the culture depends on the scale of your experiment. Generally, a 10-mL culture is enough to infiltrate 4–5 *N. benthamiana* leaves (approx. 5 g fresh-weight tissue).
2. Inoculate the liquid culture by picking a single colony from a plate. Grow the culture at 28 °C in a shaking incubator until the optical density (OD) at 600 nm is ≥2. Typically, inoculate the culture in the afternoon and grow overnight.

3. Spin cells at 4000 × *g* for 10 min at room temperature to pellet them and discard the supernatant (*see* **Note 8**).
4. Resuspend cells gently in the required volume of MMA (*see* **Note 9**) to make a solution of final $OD_{600} = 0.4$. For co-expression of two constructs, prepare solutions of individual $OD_{600} = 0.8$ which when mixed 1:1 will result in a final $OD_{600} = 0.4$ for each construct.
5. Leave the solutions at room temperature for 0.5–3 h to allow MMA buffer to induce the virulence of agrobacteria.

### 3.5 Infiltration of Leaves with Agrobacterium Suspensions

There are two different methods for introducing agrobacterial suspensions into leaves. The method of choice largely depends on the scale of the experiment.

#### 3.5.1 Syringe-Infiltration (Small-Scale Expression; 1–10 Plants)

For best results, work with 3–4-week-old (from pricking out stage) *N. benthamiana* plants and choose the youngest fully expanded leaves for infiltration.

1. To infiltrate *N. benthamiana* leaves, prick the leaf surface with a sterile needle or a sterile pipette tip (*see* **Note 10**). For maximum yield, infiltrate all sections of the leaf by pricking the leaf at multiple spots.
2. Aspirate the *Agrobacterium* suspension into a sterile 1-mL plastic syringe (take care to avoid bubbles) and, place the syringe over the leaf wound while keeping a finger behind the leaf for support.
3. Gently press the solution into the intercellular space. Infiltrated regions of the leaf will appear darker than the rest of the leaf for a few minutes post infiltration.

#### 3.5.2 Vacuum-Infiltration (Large-Scale Expression; >10 Plants)

Using this approach, more than one plant or even a tray of several plants can be infiltrated at the same time depending on the size of the desiccator unit.

1. Cover the base of a 3–4-week-old *N. benthamiana* plant such that the soil is retained in the pot during the infiltration procedure.
2. Invert the plant into a beaker containing the *Agrobacterium* suspension such as all or most leaves are completely submerged in solution.
3. Place the beaker containing the *Agrobacterium* suspension and the inverted plant in the centre of a vacuum desiccator unit.
4. Close and seal the desiccator and apply negative pressure of 25 in. of mercury (170 mbar) for 60 s. Bubbles will appear as the air is sucked out from intercellular spaces in the leaf.

5. Break the vacuum gently. At this point, the *Agrobacterium* suspension will enter evacuated intracellular spaces, making the leaves look darker. Wait for a few seconds before returning the infiltrated plant to the growth room.

### 3.6 Monitoring Expression Levels in N. benthamiana Leaves

Harvesting of *N. benthamiana* leaves is typically done at 3–10 dpi. A time-course should be done to assess optimum expression as expression levels might vary depending on the nature of your protein (*see* **Note 11**). Tissue can be processed fresh or snap-frozen and stored at −80 °C. Methods for monitoring protein expression depend on the nature of the experiment. For reporter proteins such as the green fluorescent protein (GFP), expression is visible in vivo under UV illumination. For active enzymes, expression of a product that is formed upon action of the enzyme may be monitored. For other proteins, expression may be monitored by detection of the protein in a crude extract of the infiltrated tissue using protein-specific antibodies. Protocols may then be developed for purification of the desired protein from infiltrated tissue using techniques such as centrifugation, affinity chromatography, and density gradients.

It is recommended to include inoculation with a GFP-expressing construct (such as pEAQ-*HT*-GFP) as a control in each experiment. Using the *HT* system, expression of GFP in *N. benthamiana* is visible at 2 dpi upon UV illumination of leaves, reaches its peak at 5 dpi, and remains at this level until day 7, after which the leaves start showing symptoms of necrosis.

### 3.7 Stable Expression of Proteins in N. benthamiana

pEAQ vectors can also be used for stable integration of a desired gene into the plant genome such that it is maintained through subsequent generations. Transgenic seed thus produced can be stored for years and used for bulk production of the encoded recombinant protein with minimal effort. The binary vector pEAQspecialKm (Table 1) is tailored toward stable expression since it encodes a modified version of the P19 suppressor of silencing known to enhance transgene expression without affecting the growth and development of the plant from transgenic seed [16],

For transgenic expression, clone the desired gene into pEAQ-specialKm, following methods described in Subheading 3.1 and transform *A. tumefaciens* following methods described in Subheading 3.3.

1. Transform leaf tissue with *A. tumefaciens* using the "leaf disc method." Transfer regenerated and rooted plantlets to soil. Young leaves may be harvested from regenerated plants for analysis of expressed proteins from time to time.
2. To obtain subsequent generations of transgenic plants by self-fertilization, cover flowers with a small paper bag before they start developing and collect seed from the seed pods that develop from the taped flowers.

3. Plate transgenic seed on MS-agar and place plates in the growth chamber for 4–6 weeks to allow development of seedlings.
4. Subsequently, transfer seedlings to soil for generation of plants. Since seedlings are grown in the absence of any antibiotic selection, screen leaf tissue for presence of the transgene using methods such as visualization under UV light for detection of GFP or immuno-detection of proteins.

*N. benthamiana* plants are grown in greenhouses maintained at 25 °C with supplemental lighting to provide 16 h of daylight throughout the year. Plants are watered daily.

## 4 Notes

1. PCR for amplification of the gene of interest should be carried out using a high fidelity polymerase. The size of the amplified product should be verified by agarose gel electrophoresis and the DNA should subsequently be purified by extraction from agarose.
2. DNA must be kept on ice and handled with gloves to avoid degradation. DNA may be stored at –20 °C. Care must be taken to avoid contamination at all times.
3. Buffers for digestion with restriction enzymes should be chosen according to the manufacturer's recommendations to optimize enzyme activity and avoid star activity. In case of digestion with two enzymes with non-compatible buffers, sequential digests should be performed and a spin column should be used to isolate DNA in-between digests.
4. Calf intestinal alkaline phosphatase from New England Biolabs may be used with any of the restriction enzyme buffers from the same supplier. Therefore, following heat inactivation of the restriction enzymes, phosphatase may be added directly to the reaction mixture of the digest and incubated at 37 °C for a further 60 min.
5. DNA fragments may be routinely separated on 1 % agarose gels. Fragments of >10 kb resolve better on 0.8 % agarose while for fragments of <500 bp, 1.2 % agarose provides better separation.
6. Special care must be taken during handling of chemically competent *E. coli* to not pipette cells up and down too much. Cells must be thawed on ice and all tubes must be chilled before use. Media must be pre-warmed before use.
7. Site-directed mutagenesis may be performed using a Site-directed mutagenesis kit and by following the manufacturer's instructions closely.

8. Remove all traces of the antibiotic-containing media by decanting the supernatant and blotting tubes on tissue.
9. Resuspension to $OD_{600}$ of 0.4 usually requires a volume of MMA 4–5 times larger than the volume of the starting culture. Although each culture tends to grow at a different rate, allowing cultures to grow to stationary phase generally ensures that all cultures have similar densities. Resuspension can be achieved by vortexing and pipetting up and down.
10. While pricking leaves with a needle/pipette tip for syringe infiltration, care should be taken to not shear leaf tissue. Pricks should be 1–4 cm apart, depending on the degree to which the resuspended agrobacterium spread through the interstitial spaces.
11. Time of harvest must be optimized to achieve the best accumulation levels of the protein, which is based on the stability of the protein and health of the infiltrated tissue. Leaves may show slight discoloration due to expression of proteins from agrobacteria. If leaves become necrotic, the experiment should be repeated with diluted agrobacterial cultures and leaves should be harvested before the onset of necrosis.

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# Chapter 4

# Transient Protein Expression by Agroinfiltration in Lettuce

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## Abstract

Current systems of recombinant protein production include bacterial, insect, and mammalian cell culture. However, these platforms are expensive to build and operate at commercial scales and/or have limited abilities to produce complex proteins. In recent years, plant-based expression systems have become top candidates for the production of recombinant proteins as they are highly scalable, robust, safe, and can produce complex proteins due to having a eukaryotic endomembrane system. Newly developed "deconstructed" viral vectors delivered via *Agrobacterium tumefaciens* (agroinfiltration) have enabled robust plant-based production of proteins with a wide range of applications. The leafy *Lactuca sativa* (lettuce) plant with its strong foundation in agriculture is an excellent host for pharmaceutical protein production. Here, we describe a method for agroinfiltration of lettuce that can rapidly produce high levels of recombinant proteins in a matter of days and has the potential to be scaled up to an agricultural level.

**Key words** Agroinfiltration, Plant-made pharmaceutics, Lettuce, Antibodies, Large-scale production, Transient expression, Vaccines, Recombinant protein, Plant biology, Biotechnology, *Agrobacterium tumefaciens*

## 1 Introduction

Currently, recombinant proteins for human health applications are produced by various cell culture technologies including mammalian, insect, and bacterial cell-based systems [1, 2]. However, these systems require capital-intensive cell culture facilities and have restricted production capacity. As production cost is one of the most important determinants of a drug's accessibility, its market acceptability, and profitability, there is an urgent need to develop low-cost alternative production platforms [3]. Plant-based expression systems offer several advantages over cell culture-based systems including lower startup costs due to the lack of need for cell culture facilities and bioreactors, faster production times, and greater scalability, with commercial agriculture as a potential source for biomass production [4–6]. Additionally, plant-based expression

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systems have a reduced risk of transmitting human pathogens because plant cells rarely host human pathogens and no animal-derived products are required for plant culture [1, 7]. Lastly, plant cells have eukaryotic endomembrane systems that are capable of performing mammalian-like post-translational modifications and assembly of multi-subunit proteins [8].

Recombinant proteins can be expressed in plants from the nuclear genome or the plastid genome. Nuclear transformation is preferred for producing plant-made pharmaceutical proteins (PMPs) that require post-translational modification for their functionality [9]; while plastid transformation reduces the risk of transgene spread through pollination because plastids are not present in the pollen of most species [10]. This is particularly important for food crops such as lettuce, as transgene escape through pollen poses a serious risk of food contamination with recombinant DNAs and their products [1, 2]. With stable expression, from either the nuclear or plastic genome, foreign genes can be heritable through succeeding generations in the form of seed, allowing transgenic plants to be grown on an agricultural scale [9]. The drawbacks are the vast amount of time required to generate and select stable transgenic plant lines [1, 3], and biosafety issues associated with growing plants in the field [1].

Recombinant proteins can also be expressed transiently, where the gene of interest (GOI) directs the production of the target protein during its transient presence in the host cell [11]. The most efficient way of transgene delivery for plant transient expression is via agroinfiltration [12, 13], during which transgene-carrying *Agrobacterium tumefaciens* bacteria are infiltrated into the interstitial space of the mesophyll to deliver the transgene into plant cells. This approach allows greater protein production levels and shorter production lead times. For example, lettuce has been reported to yield transient expression levels 20 times higher than that of stably expressed plants at a much shorter timescale [14]. Importantly, with transient expression environmental risks are minimized as infiltrated plants are grown in enclosed facilities, as opposed to fields [13]. While transient expression does not yield transgenic seed, meaning that infiltration has to be repeated for each batch of recombinant protein production [12], the vastly improved yield, biosafety, and development timescale make transient expression the preferred choice for large-scale production of recombinant proteins in plants.

Several vector systems can be used with agroinfiltration including viral vectors or the more recently developed "deconstructed" viral vectors [15, 16]. The deconstructed viral vector strategy can drive high levels of target protein production owing to efficient replication and transcription, yet eliminates the unnecessary functions of the viral genome and greatly reduces the biosafety and environmental risks associated with live plant viruses [17, 18].

Two noteworthy deconstructed viral vectors are the MagnICON vectors, which are RNA replicons based on the genomes of tobacco mosaic virus (TMV) and the non-competing potato virus X (PVX) with plant promoters [19]; and thegeminiviral vectors, a DNA replicon system based on the bean yellow dwarf virus (BeYDV) [20–22]. The geminiviral system consists of three components: the pBY-GOI vector containing the replicon of the target gene, the pREP110 vector coding for the Rep/RepA protein necessary for replication, and the p19 vector coding for a post-transcriptional silencing suppressor. P19 has been shown to significantly enhance the expression and accumulation of recombinant protein in *Nicotiana benthamiana* plants [20, 22]. However, this enhancement was not detected when lettuce was used as a production host [23, 24]. The three geminiviral vectors can be combined into a single super vector that contains one or more replicons to produce a single protein, several different proteins or a protein with several distinct subunits [21]. The geminiviral vector system may have a broader range of plant host species than the MagnICON system, and has been successfully used to produce PMPs in lettuce [23, 25].

After *A. tumefaciens* is transformed with the appropriate vector, it can be infiltrated into plant cells by either syringe or vacuum infiltration [12, 13]. Syringe infiltration is popular for laboratory production of protein owing to its ease and the ability to infiltrate various parts of a single plant with many different combinations of *Agrobacterium* strains [24]. Vacuum infiltration was initially developed for plant species such as lettuce that are not amenable for syringe infiltration [12, 13], but it also allows for multiple whole plants to be infiltrated rapidly and simultaneously, which is beneficial for large-scale operation. During vacuum infiltration, plants are first placed in a vacuum chamber upside down with their aerial parts submerged in an *Agrobacterium* culture [12]. The pressure is subsequently lowered to allow the gas to escape the interstitial space between plant cells. GOI-carrying *Agrobacterium* cells can then infiltrate this space when the pressure inside the chamber is equalized again with the atmosphere [12]. It is through this method that large-scale transgene delivery and protein production can be achieved, as a specially designed facility can vacuum infiltrate metric tons of plants at once.

Historically, tobacco and the related *N. benthamiana* plant have been the plants of choice for agroinfiltration. Tobacco-related plants offer several advantages as hosts for transient recombinant protein expression due to their well-characterized biology, their high biomass yield, and the number of expression vectors available for these species [1]. Tobacco plants, however, can produce high levels of phenolic and toxic alkaloids. These compounds foul purification resins and are very difficult to remove from the target protein, and ultimately complicate downstream processing [25].

Lettuce (*Lactuca sativa*) tissue contains negligible levels of phenolic and alkaloid compounds, but has been shown to produce high levels of recombinant protein [23, 26]. It is for this reason that lettuce has received abundant attention over the past decades as it has been explored as a host for protein production in the form of transgenic lines and transient expression with conventional non-viral vectors [27–33]. In recent years, lettuce-based transient expression platforms have been greatly improved with the use of deconstructed plant virus vectors. For example, high levels of monoclonal antibodies (mAbs), virus-like particles (VLPs), and subunit vaccines have been produced in lettuce using geminiviral vectors [23, 25, 34, 35]. More recently, the utility of commercially produced lettuce for producing high levels of functional PMPs has been demonstrated [23]. Here, a scalable protocol that demonstrates the transient expression of recombinant proteins in lettuce by agroinfiltration is presented.

## 2 Materials

### 2.1 Plant Material

1. Humidity dome.
2. Fertilizer: 15-16-17.
3. Peat pellets.
4. Seeds from a *Lactuca sativa* leafy cultivar, such as Black Seeded Simpson cultivar, Grand Rapids cultivar, or Oak Leaf cultivar (*see* **Note 1**).
5. T.O. Plastics SFT-1020-OPEN-NH standard flat carry tray.
6. T.O. Plastics STI-804 standard insert.
7. Hand-held seeder (e.g. Vibro Hand Seeder VHS-1).
8. Linear Fluorescent bulbs F40T12 CW Alto.

### 2.2 Bacterial Culture

1. *Agrobacterium tumefaciens* strain GV3101 carrying the GOI.
2. 100 mg/ml (1000×) carbenicillin disodium (*see* **Note 2**).
3. 100 mg/ml (1000×) kanamycin sulfate.
4. LB medium: 1 % tryptone, 0.5 % yeast extract, 1 % NaCl. Use 1.5 % agar for plates.
5. Difco™ Nutrient Broth (Becton, Dickinson and Company (BD), Franklin Lakes, NJ, USA).
6. Petri dishes.
7. 16.65 mg/ml (333×) rifampicin.
8. YenB medium: 1 % nutrient broth, 0.5 % yeast extract. Use 1.5 % agar for plates.
9. Shaker incubator.
10. Spectrophotometer.

### 2.3 Infiltration

1. 3 L tub.
2. Vacuum desiccator.
3. Desiccator plate/shelf.
4. MES buffer (1×): 10 mM MES Hydrate (FW 195.24), 10 mM $MgSO_4$ Hydrate (FW 246.47). Mix in filtered water and adjust pH to 5.5 with NaOH. Sterilize by passing through a 0.2 μM filter.
5. Vacuum Pump.

## 3 Methods

Plants are grown inside a climate controlled room at 25 °C, 60–80 % humidity, and with a 16/8 h day/night cycle under artificial fluorescent lighting.

### 3.1 Plant Growth (See Note 3)

1. Place 32 peat pellets inside the flat carry tray. Add 1–2 l water and allow the pellets to expand to their full size (Fig. 1a).
2. Using a hand-held seeder, add 1–2 *L. sativa* seeds to the center of each pellet and add enough water to cover the bottom quarter of the peat pellet (*see* **Note 4**).
3. Cover the tray with a humidity dome (Fig. 1b) and allow the seeds to germinate for 1 week in the growth chamber. After 1 week, move the dome so that a small opening is created (*see* **Note 5**).
4. After 2 weeks, remove the dome and add 2 l of 1.48 g/l fertilizer to the tray (Fig. 2). Continue to grow plants under the same environmental conditions, adding fertilizer every 2 days or as necessary (*see* **Note 6**).
5. Continue to fertilize until plants are ready for infiltration at 6 weeks of age (Fig. 3).

### 3.2 A. tumefaciens Culturing and Preparation for Vacuum Infiltration

1. Streak *A. tumefaciens* GV3101 strains containing geminiviral vector modules carrying the gene of interest on LB-Agar plates with 100 μg/ml kanamycin. Grow for 48 h at 30 °C (*see* **Note 7**).
2. Inoculate a 7 ml liquid YenB starter culture from a single colony for each strain. Grow at 30 °C in a shaker (230 rpm) for 16–20 h, until the OD600 reaches at least 1.0 (measure OD600 with a spectrophotometer).
3. Use the culture to inoculate a new 250 ml YenB subculture with 100 μg/ml kanamycin for each strain, so that the OD600 of the new culture is 0.025. The following formula can be used to calculate the volume of starter culture needed to transfer to the subculture.

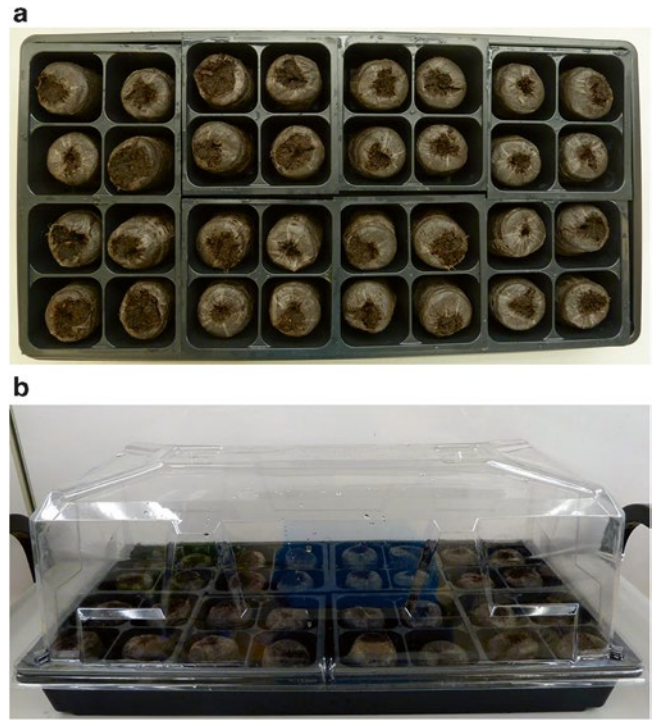


**Fig. 1** Peat pellets and growth trays without (**a**) or with humidity dome (**b**). Thirty-two peat pellets are placed inside the growth tray and two liters of water are added to allow peat pellets expanding to their full size. After sowing with lettuce seeds, the tray is covered with a dome to facilitate germination

**Fig. 2** Lettuce plants (*L. sativa*, var. Rouge d'Hiver) 2 weeks after seeding

$$V_{\text{starterculture}} = \frac{(\text{OD}_{600}\,0.025)(250\text{ml})}{\text{OD}_{600}}$$

4. Grow the new culture at 30 °C and 230 rpm for 16–20 h or until the OD600 reaches at least 1.0. Centrifuge the cultures at room temperature and 6500 × *g* for 30 min. Remove the supernatant.

**Fig. 3** Lettuce plants (*L. sativa*, var. Rouge d'Hiver) 6 weeks after seeding

5. Resuspend the pellet in 25 ml MES buffer and measure the OD600 (*see* **Note 8**).
6. If multiple vector modules are used, mix equal amounts of each vector-carrying *Agrobacterium* strain together in 3 l of MES buffer. Use the following formula to calculate the volume of each of the three strains to transfer. The final OD600 of each strain should be 0.07, for a total OD600 of 0.21 (*see* **Note 9**):

$$V_{\text{subculture}} = \frac{(\text{OD}_{600}\,0.07)(300\text{ml})}{\text{OD}_{600}}$$

### 3.3 Vacuum Infiltration

1. Place a 3 L tub containing the *Agrobacterium* strains into the bottom half of the vacuum desiccator.
2. Place a plant upside down on the desiccator plate and dip it into the 3 l tub with the plate resting on top. Seal the desiccator and connect it to the vacuum pump (Fig. 4).
3. Turn on the pump and allow it to maintain the desiccator at an interior pressure of 10 kPa for 5 min. Then, slowly open the release valve to allow the pressure to equalize and the *Agrobacterium* to infiltrate the submerged plant tissue. Repeat this step until the entire leaf area becomes translucent.
4. When complete, remove the plant from the desiccator (Fig. 5) and place it upright in a pot (Fig. 6). Move it back to the growth chamber and monitor expression (*see* **Note 10**).

### 3.4 Isolation and Characterization of Recombinant Proteins

1. For marker proteins such as Green Fluorescent Protein (GFP) and Red Fluorescent Protein (DsRed), expression can be monitored simply by placing the plants in a dark room and illuminating them with a UV lamp. The intensity of the fluorescence can be observed visually and recorded by photography (Fig. 7).

**Fig. 4** Vacuum infiltration of lettuce leaves with *Agrobacterium tumefaciens*. Strain GV3101 of *A. tumefaciens* containing target protein-expressing vectors are resuspended in infiltration buffer in a tub. The tub is placed in a vacuum desiccator and a 6-week-old lettuce plant is placed upside down so that the aerial parts of the plant are submerged into the infiltration mixture. Agroinfiltration is achieved by applying a vacuum at 10 kPa twice for 5 min

**Fig. 5** A lettuce plant after infiltration. The agroinfiltrated plant is taken out of the vacuum desiccator and extra liquid on the leaves is drained. Leaves appear translucent when they are well infiltrated

**Fig. 6** Agroinfiltrated lettuce plant. The agroinfiltrated plant is put back upright in a peat pellet holder and moved back to the growth chamber for target protein expression

**Fig. 7** Expression of GFP in vacuum agroinfiltrated lettuce leaves. *Lettuce* (*L. sativa*, var. Rouge d'Hiver) leaves were infiltrated with GV3101 harboring the GFP expression cassette in Geminiviral vectors. Leaves were photographed under UV light at 4 days post infiltration

2. For other target proteins, they need to be extracted from plant tissue typically by homogenization of the tissue in a buffer supplemented with protease inhibitors [4, 23, 36]. Target protein-specific purification schemes can be developed by using a combination of non-chromatographic method such as precipitation and chromatographic methods including ion-exchange, hydrophobic interaction, size-exclusion, and affinity chromatography [37].

For example, purification of mAbs from plant tissue can be achieved by a combination of ammonium sulfate precipitation and protein A column chromatography [23, 36, 38–40]. An ion exchange chromatography can then be used as a polish step to remove residual DNA, endotoxin, or protein A [36]. Purity and molecular weight can be determined further by Coomassie staining following SDS-PAGE. Additionally, provided there are antibodies available, western blotting can be used to confirm protein identity [23, 36]. ELISA and Bradford assays can be used in conjunction to measure total protein yield (e.g. mg target protein/g leaf fresh weight (LFW)) as well as the percent total soluble protein (% soluble protein) [4, 23, 25]. The functionality of the purified target protein can be measured in target protein-specific functional assays [39, 41].

## 4 Notes

1. Leafy cultivars are the best for infiltration. Cultivars like "Iceberg" head are either too tough, too high in water content, or too cumbersome to infiltrate efficiently.
2. All antibiotics must be filter-sterilized.
3. By far the most important factor influencing yield and quality of recombinant protein is the quality of the host plants. Therefore, as plants are growing pay extra attention to their color (avoid using plants that are yellowing or beginning to become necrotic at the stem or leaf) and their quality (do not use plants that have begun to wilt or feel thin to touch, as these may be dehydrated). Small changes in plant growth conditions will result in significant changes in protein quantity and quality.
4. Multiple seeds are used to maximize germination.
5. The dome is used to maintain high humidity conditions for the germinating seeds with little air flow. After the first week the dome is moved slightly to allow the humidity levels to drop and air to flow through.
6. Over-watering here will lead to large amounts of root growth that will eventually penetrate the peat pellets and cover the trays. The key to watering here is to wait until the tray dries but the peat pellets are still moist, then add more fertilizer.

7. The Geminiviral vector system requires a module coding for Rep/RepA and the module coding your gene of interest. However, the use of a post-translational silencing suppressor like p19 has not been shown to increase yield in lettuce.
8. Resuspending in approximately 1/10 of the culture volume of MES concentrates the cells and allows you to work with smaller volumes.
9. The final OD value for each module as 0.07 is based on the use of GFP as the gene of interest. This value is variable depending on the type of protein you are expressing. If there are low expression levels at this particular OD, it can be raised to attempt to increase the final concentration of protein obtained from the plants. Similarly, in the case of expression of multiple subunit proteins one can correct not only high and low expression but can alter the ratio between the subunits themselves in order to optimize yield.
10. For proteins expressed using a geminiviral vector system, expression typically peaks at 4 days post infiltration (dpi).

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# Chapter 5

# Production of Recombinant Proteins in the Chloroplast of the Green Alga *Chlamydomonas reinhardtii*

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## Abstract

Chloroplast transformation in the green algae *Chlamydomonas reinhardtii* can be used for the production of valuable recombinant proteins. Here, we describe chloroplast transformation of *C. reinhardtii* followed by protein detection. Genes of interest integrate stably by homologous recombination into the chloroplast genome following introduction by particle bombardment. Genes are inherited and expressed in lines recovered after selection in the presence of an antibiotic. Recombinant proteins can be detected by conventional techniques like immunoblotting and purified from liquid cultures.

**Key words** Chloroplast transformation, *Chlamydomonas reinhardtii*, Recombinant protein, Green algae, Chloroplast biotechnology, Particle bombardment

## 1 Introduction

Recombinant proteins can be produced in platforms ranging from bacterial cells to whole animals such as pigs and goats [1]. Amongst the platforms used for the production of recombinant proteins is the unicellular green algae *Chlamydomonas reinhardtii* whose nucleus [2] and chloroplast [3] can be readily transformed. The particle bombardment device or glass beads are most commonly used to transform both the chloroplast and the nucleus, whilst carbide whiskers and electroporation have proven to be effective for nuclear transformation only [4–7]. Valuable proteins including bacterial and viral antigens, antibodies, and immunotoxins have been successfully produced in *C. reinhardtii* [8, 9], some with demonstrated biological activity [11–14] and some of them at pilot scale [10].

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Some of the advantages of producing proteins in the chloroplasts include: potential for targeted insertion of the genes of interest, a lack of silencing mechanism, potential for high levels of recombinant protein (2–20 % total soluble protein in *C. reinhardtii*) and, because of the prokaryote-like nature of the chloroplast, multiples genes can be expressed in the form of operons [15, 16]. Because algae grow relatively fast and under contained conditions, chloroplast transformation in *C. reinhardtii* offers an additional advantage over chloroplast transformation in land plants. Perhaps one of the main drawbacks of expressing genes in the chloroplast of *C. reinhardtii* is that protein-coding sequences require codon optimization to comply with codon usage in the organelle [17].

Transformation can be achieved using selectable markers that include: aminoglycoside 3′ adenyl transferase (*aadA*), a gene of bacterial origin that confers resistance to spectinomycin and streptomycin [18]; or 3′-aminoglycoside phosphotransferase type VI (*aphA-6*), another bacterial gene that confers resistance to kanamycin and amikacin [19, 20]. In its origins, chloroplast transformation was routinely achieved using plasmid p228 (Chlamydomonas Center, University of Minnesota), which by introducing a point mutation in the 16S rRNA gene, confers resistance to spectinomycin [21]. However, the occurrence of spontaneous point mutations in the 16S rRNA and the need to bombard with two vectors in parallel have led to infrequent use of p228 in favor of alternative vectors carrying a selectable marker gene.

After transformation, several rounds of selection are carried out with the aim of eliminating all copies of the wild-type genome and rescuing strains containing the genes of interest. Some studies have also shown that it is possible to select transformed lines by re-establishing photosynthesis. For this, mutants with defects in photosystem I, which are sensitive to light-induced oxidative damage, can be recovered by introducing the wild-type version of the defective gene that gave rise to the mutation, e.g. *psbA* or *psbB* [22, 23].

Here, we describe the procedure for *C. reinhardtii* chloroplast transformation followed by protein detection. For illustrative purposes, we use as an example the expression of recombinant green fluorescent protein (GFP) from *Aequorea victoria*. The *gfp* gene is contained in a transformation vector carrying the selectable marker *aphA-6*, which confers resistance to kanamycin. The genes are introduced into the chloroplast by bombarding DNA-coated gold or tungsten particles followed by selection of transformed cells in media containing kanamycin [24]. Genes are detected using PCR followed by protein detection by immunoblotting using a specific antibody for the protein of interest or antibodies against tags such as 6xHis or 3XFLAG.

## 2 Materials

### 2.1 Chloroplast Transformation Vectors

Insertion of transgenes in the chloroplast genome occurs by recombination of homologous sequences [25]. Vector p322, derived from a chloroplast genome library (Chlamydomonas Center, Duke University, Durham, NC, USA), carries a 5.5 kbp *Eco*RI-*Xho*I fragment from the *C. reinhardtii* chloroplast genome comprising the region *psbA exon 5-5S rRNA* inserted in pBlueScript KS+. Even though vector p322 has been extensively used, recently, different vectors, targeting other regions of the chloroplast genome, are being used [26]. All of them require the presence of sequences that are homologous to endogenous sequences in the chloroplast genome. The methodology we describe below refers to the use of vector p320-aphA6-GFP (p320 is a p322 derivative, lacking a *BamHI* site in the original multiple cloning site of pBlueScript KS+). In this vector, the expression for *aphA*-6 is under control of the *psbA* promoter and *rbcL* terminator, while the expression of *gfp* is under the control of the *atpA* promoter and the *psbA* terminator (Fig. 1) (*see* **Note 1**).

### 2.2 Biological Material and Growth Media

1. *Chlamydomonas reinhardtii* strain CC-125 mating type *mt+*. This is the wild-type strain used routinely for chloroplast transformation; mating type *mt+* is responsible for the inheritance of chloroplast DNA so this strain must be used if the introduced genes are to be conserved. This strain contains mutations in the genes *nit1* and *nit2*. These mutations render the strain unable to grow on nitrate as the sole nitrogen source so ammonium must be used. While *nit1* encodes a nitrate reductase, *nit2* is a regulatory gene that is required for expression of *nit1* (*see* **Note 2**).

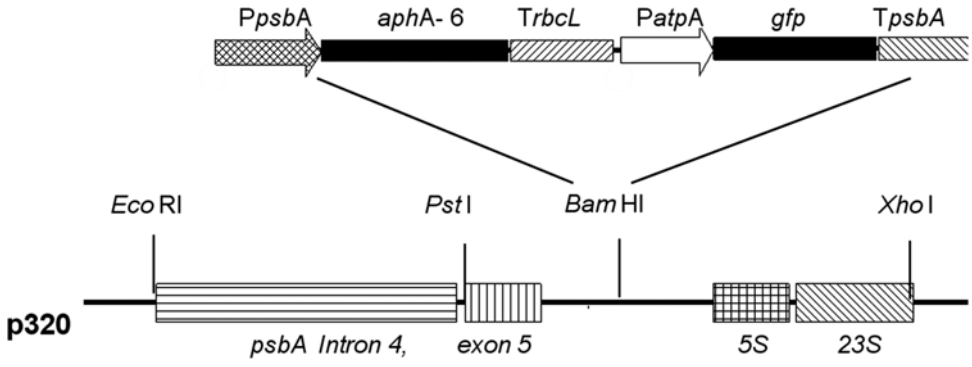


**Fig. 1** p320-aphA6-GFP vector used for chloroplast transformation in *Chlamydomonas reinhardtii.* p320-aphA6-GFP targets the insertion of the expressions cassettes for *aphA-6* and *gfp* to the region between intron 4/exon 5 from *psbA* and the ribosomal genes 5S rRNA/5′ 23S RNA. P*psbA* promoter from *psbA*, T*rbcL* terminator from *rbcL*, P*atpA* promoter from *atpA*, T*psbA* terminator from *psbA*

2. Tris-Acetate-Phosphate medium (TAP) medium: 2.42 g/L Tris-Base, 25 mL/L Solution #1 (salts), 0.375 mL/L of Solution #2 (phosphates), 1.0 mL/L Hutner's trace elements (*see* **Note 3**), 1.0 mL/L glacial acetic acid. Mix all components well in deionized water, adjust pH to 7.0 with acetic acid and bring to a final volume of 1 L. The solution is sterilized for 20 min at 121 °C and 15 psi. For solid medium, add 8 % (w/v) noble agar before sterilization. For supplementation with antibiotics, let the medium cool after sterilization (to around 42 °C) before adding the desired antibiotic. For kanamycin, use 100 and 20 μg/mL for solid and liquid media, respectively. For spectinomycin use 150 and 50 μg/mL for solid and liquid media, respectively. After sterilization media can be stored for a month at room temperature.
3. Solution # 1 (salts): 15.0 g/L $NH_4Cl$, 4.0 g/L $MgSO_4 \cdot 7H_2O$, 2.0 g/L $CaCl_2 \cdot 2H_2O$. This solution does not require sterilization and can be stored at room temperature for several months.
4. Solution # 2: for 100 mL weigh 28.8 g of $K_2HPO_4$ and 14.4 g of $KH_2PO_4$. Dissolve in 80 mL of deionized water and adjust the final volume to 100 mL. Sterilization is not required and the solution has a long shelf life.

### 2.3 Biomass Growth and General Items

1. 250 and 500 mL flasks.
2. 50 mL plastic tubes.
3. 90 × 15 mm Petri dishes.
4. Sterile water.
5. Sterile toothpicks.
6. Centrifuge.
7. Microcentrifuge.
8. Vortex.
9. Shaker.
10. Laminar flow chamber.
11. Diurnal incubator.

### 2.4 Particle Delivery

1. Biolistic PDS-1000/He Particle Delivery System (BIO-RAD).
2. Macrocarriers (BIO-RAD).
3. Rupture disks (1100 psi) (BIO-RAD).
4. Stopping screens.
5. Tungsten particles M-10, 0.7 μm (BIO-RAD).
6. Spermidine 0.1 mM in sterile deionized water (*see*·**Note 4**).
7. $CaCl_2$ 2.5 M in sterile deionized water (*see* **Note 5**).

8. Chloroplast transformation vector 1.0 μg/μL (*see* **Note 6** and Subheading 3.1).
9. HPLC-grade 100 % ethanol and HPLC-grade 70 % ethanol (prepared with sterile deionized water).
10. Helium, grade 4.5.
11. 50 % sterile glycerol.

### 2.5 Screening for the Presence of Transgenes in Transformed Lines

1. Chelex-100 resin 5 % (w/v) in sterile water. Chelex-100 resin is insoluble in water so mix well before use. The solution can be stored at –20 °C (*see* **Note 7**).
2. Taq 5X Master Mix (New England BioLabs) (*see* **Note 8**).
3. Specific primers for the gene of interest (10 μM) (*see* **Note 9**).
4. Agarose gel 1 % (w/v) prepared in TBE buffer.
5. TBE buffer: for 1 L weigh 108 g Tris-Base, 55 g Boric acid, 40 mL EDTA 0.5 M pH 8. Mix all components in 800 mL of deionized water and bring to 1 L.
6. DNA weight markers.
7. Heat block.
8. PCR machine/thermocycler.
9. Equipment for agarose gel electrophoresis.

### 2.6 Detection of Recombinant Proteins

1. Extraction buffer: 25 mM HEPES, 100 mM NaCl, 5 mM $MgSO_4$, 10 % glycerol, 1/10 of 10 % Triton X-100. For 50 mL of extraction buffer weigh 0.29 g of HEPES, 0.29 g of NaCl, 0.03 g of $MgSO_4$, 5 mL of glycerol, 5 mL of 10 % Triton X-100. Dissolve all the components in 40 mL of deionized water and adjust pH to 7.5 with 2.5 M KOH. Bring the final volume to 50 mL.
2. Equipment for polyacrylamide gel casting and electrophoresis.
3. Semidry blotter equipment.
4. Mortar and pestle, left at –80 °C for 24 h.
5. Stock acrylamide solution. We normally use Acrylamide/Bis-acrylamide 19:1 from Sigma-Aldrich (*see* **Note 10**).
6. Gel Buffer: 3 M Tris-HCl, 1 M HCl, 0.3 % SDS, pH 8.45. For 100 mL of Gel Buffer solution weigh 36.3 g of Tris, 8.33 mL of 12 M HCl, 0.3 g of SDS and adjust pH to 8.45 with 3 M Tris-HCl and 12 M HCl if required.
7. Glycerol.
8. 10 % ammonium persulfate (APS). For 1 mL of ammonium persulfate solution weigh 0.1 g of ammonium persulfate and adjust the volume to 1 mL with sterile deionized water in a 1.5 mL tube. Make fresh every time.

9. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED).
10. Anode buffer 10× (1 M Tris-base, 0.225 M HCl, pH 8.9). For 1 L of anode buffer weigh 121.14 g of Tris-base, 18.75 mL of 12 M HCl and dissolve in 800 mL of sterile deionized water. Adjust pH to 8.9 with 1 M Tris-base and 12 M HCl if required.
11. Cathode buffer (1 M Tris-base, 1 M Tricine, 1 % SDS, pH 8.25). For 1 L of cathode buffer weigh 121.14 g of Tris-base, 179.17 g of Tricine, 1 g of SDS and adjust pH to 8.25 with 1 M Tris-Base if required.
12. Fixing solution (50 % methanol, 10 % acetic acid, 100 mM ammonium acetate). For 1 L of fixing solution weigh 7.7 g of ammonium acetate, 100 mL of acetic acid and 500 mL of methanol. Mix all components in 900 mL of deionized water and then bring to a final volume of 1 L.
13. Sample Buffer (150 mM Tris–HCl pH 7.0, 12 % SDS (w/v), 30 % glycerol (w/v), 6 % 2-mercaptoethanol (v/v), 0.05 % Coomassie dye (w/v)). For 10 mL of sample buffer add 1.5 mL of 1 M Tris–HCl pH 7.0, 3 g of glycerol, 0.6 mL of 2-mercaptoethanol and 0.005 g of Coomassie dye (*see* **Note 11**).
14. Protein stain solution (0.025 % Coomassie dye in 10 % acetic acid). For 1 L of protein stain solution weigh 0.025 g of Coomassie dye and mix with 100 mL of acetic acid in 900 mL of deionized water. This solution can be reused; we normally use it up to five times.
15. Destain solution (10 % acetic acid). For 1 L of destain solution add 100 mL of acetic acid in 900 mL of deionized water. This solution can be reused until saturation is reached.
16. Protein transfer buffer. For 1 L of protein transfer buffer weigh 5.82 g of Tris base, 2.93 g of glycine and 0.735 g of SDS in 700 mL of deionized water, add 200 mL of methanol and adjust the volume to 1 L (*see* **Note 12**).
17. Nitrocellulose membrane 0.2 μm pore size.
18. Filter paper for blotting.
19. Phosphate-buffered saline (PBS). To prepare a 10× PBS solution, weigh 2 g of $KH_2PO_4$, 11.5 g of $Na_2HPO_4$, 2 g of KCl and 80 g of NaCl. Dissolve in 800 mL of deionized water and then bring to a final volume of 1 L. Sterilize by autoclaving at 121 °C 15 psi for 15 min.
20. Phosphate buffer saline-Tween (PBST). For 1 L of PBST solution add 100 mL of 10× PBS and 5 mL of 10 % Tween 20 to 895 mL of deionized water.
21. Blocking solution (5 % (w/v) non-fat dry milk in PBST) (*see* **Note 13**).

22. Primary antibody solution. The primary antibody should be diluted in PBST at the appropriate experimentally determined concentration. In this case, for GFP we used a mouse anti-GFP monoclonal antibody (Abcam, Cat. ab184611) at a 1/10,000 dilution. This solution can be used several times (2–4 times gives us good results). Store at 4 °C for short time (1–2 weeks) and at −20 °C for longer periods.
23. Secondary antibody solution. The secondary antibody should be diluted in PBST at the appropriate experimentally determined concentration in PBST. In this case we used a Goat Anti-Mouse IgG1 antibody coupled to horseradish peroxidase (HRP) (Abcam) at a 1/15,000 dilution. This solution should be prepared fresh each time.
24. Enhanced Chemiluminescent Detection (ECL) kit (BioRad-USA).

## 3 Methods

### 3.1 Preparing Biological Material for Bombardment

1. Inoculate a 250-mL flask containing 50 mL of TAP medium with 1 $cm^2$ square of the solid medium where a fresh (2–3 weeks) culture of algae is growing. Incubate at 25 °C under constant illumination (17,000 lx) at 200 rpm for 2–3 days (*see* **Note 14**).
2. Inoculate a 500-mL flask containing 200 mL of TAP medium with the 2–3-day culture until a cell density of $2 \times 10^4$ cell/mL is obtained. Grow the cells under photoperiod (16 h light/8 h dark) at 25 °C until a cell density of $2 \times 10^6$ cell/mL is reached, which is usually in 3–4 days (*see* **Note 14**).
3. Divide the culture into four 50 mL samples, centrifuge at $3500 \times g$ for 5 min in 50-mL plastic tubes, and discard the supernatant by decantation. Resuspend each pellet in 0.5–1 mL of new sterile TAP media, and combine the cells from the four cultures into a new single 50 mL tube. Centrifuge again, discard the supernatant, and resuspend the pellet in enough TAP medium to obtain a cell density of $1 \times 10^8$ cell/mL. A volume in the range of 5–10 mL is usually needed.
4. Place 250 μL of the $1 \times 10^8$ cell/mL culture in the centre of each of eight to nine 90 × 15 mm Petri dishes containing solid TAP medium supplemented with an appropriate antibiotic for selection.
5. Let the liquid culture dry on the plates for 2 h at room temperature in the dark (*see* **Note 15**).
6. Place another 250 μL of the $1 \times 10^8$ cell/mL culture in the centre of a 90 × 15 mm Petri dish containing solid TAP medium without antibiotics. This will serve as a viability control.

### 3.2 Microparticles Preparation

1. Weigh 30 mg of tungsten microparticles in a 1.5 mL tube (*see* **Note 16**).
2. Add 1 mL of 70 % ethanol and vortex for 3–5 min.
3. Leave the particles in 70 % ethanol for 15 min.
4. Centrifuge for 5 s and discard the supernatant using a micropipette, taking care not to disturb the pellet.
5. Wash the pellet with 1 mL of sterile water, vortex for 1 min, leave the particles to sediment for 1 min and centrifuge for 20 s. Discard the supernatant and repeat this **step 3** more times.
6. Add 500 μL of 50 % sterile glycerol and mix by inverting the tube several times. Particles in 50 % glycerol can be stored at room temperature for up to a week. We have stored them for up to a month at –20 °C.

### 3.3 DNA Coating of Microparticles

1. For the next step, particles must be at room temperature. If the particles were stored in the freezer, let them stand for 30–40 min at room temperature, then vortex them for 5 min to disaggregate particle lumps that may have formed.
2. Pipette 50 μL of microparticles into a 1.5 mL tube.
3. Leave the tubes open and vortex them vigorously, taking care not to splash the solution out of the tube. We use a clamp to hold the open tube to the vibrating part of a vortex. Increase the speed gradually until the particles, while being agitated, remain suspended in the solution. While continuing to vortex, add in this order: 5.0 μL of DNA (1.0 μg/μL), 50 μL of 2.5 M $CaCl_2$, and 20 μL of 0.1 mM spermidine.
4. Continue vortexing for 2–3 min, then let the particles precipitate for 1 min and centrifuge at maximum speed for 20 s.
5. Carefully discard the supernatant and wash the pellet with 140 μL of 70 % ethanol, taking care not to disturb the particles. Discard the ethanol after a few seconds. If the pellet was disturbed, briefly centrifuge the tube and discard the supernatant.
6. Add 100 μL of absolute ethanol and mix by carefully pipetting up and down 3–4 times.
7. Pipette 10 μL of DNA-coated microparticles onto each of 8–9 macrocarriers, spreading them over a circle of 0.5-cm diameter and try to disaggregate any lumps that may form (*see* **Note 17**).

### 3.4 Particle Bombardment and Rescue of Transformed Lines

All components of the particle deliver system must be cleaned with 70 % ethanol prior to use, including the safety chamber. The particle delivery system must be installed following the manufacturer's instructions.

1. Turn on the particle delivery system and open the helium valve completely. The helium pressure must be over 1100 psi.

2. Turn on the vacuum pump.
3. Load a 1100-psi rupture disk onto a rupture disk holder and screw it into place.
4. Place a sterile stopping screen in the centre of macrocarrier launch assembly.
5. Place a macrocarrier with the DNA-coated microparticles in the macrocarrier holder with the macrocarrier insertion tool.
6. Place the macrocarrier holder in the launch assembly. Lock the macrocarrier holder with the macrocarrier cover lid.
7. Place the macrocarrier launch assembly in the top level slot, closest to the rupture disk holder.
8. Put the plate holder in the lowest level slot so that there is a distance of 9 cm from the launch assembly to the plate holder.
9. Place a Petri dish containing the algae in solid TAP media (prepared as described in Subheading 3.1) onto the plate holder. The Petri dish must be open.
10. Close the chamber door and turn on the vacuum switch until a vacuum of 30 in. Hg is reached.
11. Turn the vacuum switch to hold.
12. Press and hold the fire button until the rupture disk bursts.
13. Turn the vacuum switch to air safe and when the chamber pressure reaches 0 in. Hg, open the door and cover the Petri dish with its lid. Discard the spent macrocarrier, rupture disk and stopping screen. Repeat the process of bombardment until all the macrocarriers are used. Make sure to leave one culture unbombarded to be used as a negative control.
14. When finished, clean the particle delivery device and purge the He pipeline.
15. Incubate the bombarded and control cultures overnight in the darkness at 25 °C.
16. Transfer the cultures to 25 °C under a 16 h light/8 h dark photoperiod for 2–4 weeks.
17. Transformed cells form bright green colonies 2–4 weeks after bombardment among pale yellowish/whitish dead cells (Fig. 2). Check regularly for signs of contamination. If cultures become contaminated, discard them.
18. In a laminar flow chamber, pick individual transformed colonies with a sterile toothpick and streak them separately onto fresh plates containing solid TAP medium supplemented with the appropriate antibiotic.
19. Let the cultures grow for 1–2 weeks and repeat **steps 17** and **18** three more times in order to obtain homoplastomic strains.

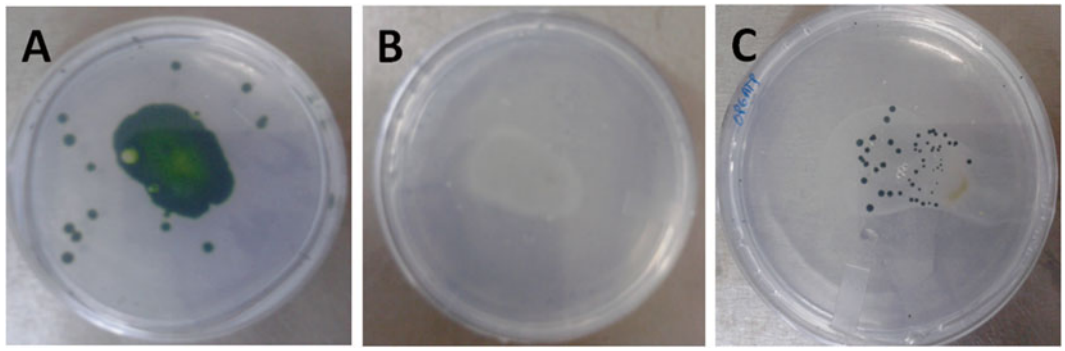


**Fig. 2** *Chlamydomonas reinhardtii* cultures 3 weeks after bombardment with chloroplast transformation vector p320-aphA6-GFP. (**a**) Wild-type cells growing in medium without antibiotic; (**b**) Wild-type cells in the presence of kanamycin; (**c**) Transformed cellsforming colonies in the presence of kanamycin

### *3.5 Screening Transformed Cells Using the Polymerase Chain Reaction*

1. In a laminar flow hood, with the tip of a sterile toothpick touch the surface of a culture of transformed algae growing on solid medium and resuspend the cells in 50 µL of Chelex-100 resin solution in a 200-µL PCR tube.
2. Vortex for 30 s.
3. Incubate at 98 °C for 8–10 min and then cool down on ice for 1 min.
4. Vortex for 10 s and then centrifuge for 5 min at maximum speed.
5. Take 0.5 µL of the supernatant as template for a 25 µL PCR. PCRs for the amplification of *gfp* and *aphA-6* amplification, using the primers in **Note 9**, should be performed under the following conditions: 30 s at 95 °C; 30 cycles of 20 s at 95 °C, 20 s at 60 °C, 30 s at 68 °C; one final extension of 5 min at 68 °C (*see* **Note 18**).
6. Analyze 3 µL of the PCR product in a 1 % agarose gel (Fig. 3) (*see* **Note 19**).

### *3.6 Detection of GFP in Transformed Algae*

This method is used to separate proteins with molecular mass in the range of 1–100 kDa.

1. Grow transformed strains under constant illumination at 25 °C and 100 rpm in 200 mL liquid TAP medium with appropriate antibiotic until a cell density of $2 \times 10^6$ cell/mL is reached.
2. Harvest the cells by centrifuging at $1700 \times g$ for 5 min and discard the supernatant.
3. Resuspend the pellet in 1–2 mL extraction buffer and pour into a mortar (previously frozen at −80 °C for 24 h). Grind with the pestle until a fine powder is obtained. Add 9 mL of extraction buffer and continue grinding until the sample becomes liquid.

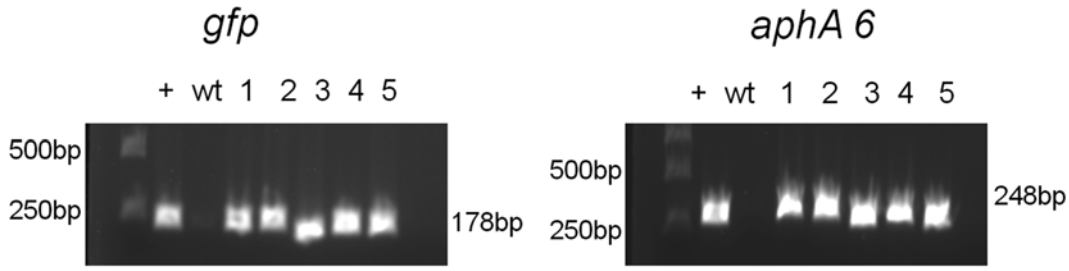


**Fig. 3** Electrophoresis gel of PCR products from transformed colonies, using primers designed to amplify fragments of *gfp* and aphA-6 (*see* **Note 9**). Control (+) corresponds to transformation vector; lanes 1–5 correspond to PCR products of transformed colonies. wt corresponds to a wild-type untransformed strain

4. Transfer the ground cells to a 50-mL tube and centrifuge at 6800 × *g* for 20 min at 4 °C. Transfer the supernatant to a 15-mL tube and keep on ice.
5. Quantify protein from the supernatant with a Bradford assay [27].
6. Assemble the glasses of the gel casting apparatus to prepare a polyacrylamide gel following the manufacturer's instructions. We recommend preparing two gels, one for staining with Coomassie brilliant blue and one for protein transfer and immunodetection.
7. For a 16 % acrylamide-gel, mix the following components in a 50-mL tube: 3 g of glycerol, 10 mL of Stock acrylamide solution, 10 mL of gel buffer, 100 µL of APS (10 %), 10 µL of TEMED. Add water to a final volume of 30 mL and gently agitate the tube to ensure complete mixing. With a pipette tip add enough solution to fill 3/4 of the assembled glasses to cast the resolving gel. Slowly add 1 mL of isopropanol on top of the solution. When the acrylamide has formed a gel remove the isopropanol by blotting with a piece of filter paper and immediately add 1 mL of 4 % acrylamide-gel to cast the stacking gel (to prepare 1 mL of 4 % acrylamide solution, mix 3 mL of gel buffer, 90 µL of 10 % APS, and 9 µL TEMED). Add water to a final volume of 12 mL and allow the acrylamide to polymerize.
8. Assemble the electrophoresis chamber and fill the inner well completely with cathode buffer and the outer well with the anode buffer just to the level indicated in the chamber.
9. Prepare samples of 20 µg of protein in 15–20 µL. To each sample add an equal volume of sample buffer and mix well by pipetting up and down without forming bubbles. Heat the samples at 98–100 °C for 5 min and then cool on ice for 10 min. Spin down to recover any evaporated droplets and load the sample into the wells of the cast gel. Run for 2 h at 95 V.

10. Disassemble the electrophoresis equipment, recover the gel, and discard the stacking gel. Place one of the gels in a plastic container, add enough fixing solution to cover the gel, and incubate for 30 min at room temperature with gentle agitation on an orbital shaker. Discard the fixing solution, rinse briefly with water, add enough stain solution to cover the gel, and incubate with agitation at room temperature for 5 h or overnight. To destain, remove the staining solution and add enough destain solution to cover the gel. Incubate with agitation for 3–5 h at room temperature. Check the gel every hour and look for the appearance of stained discrete bands; the rest of the gel becomes clear. Changing the destain solution several times, every 10–15 min, can help to reduce the time required for destaining.
11. For the blotting of proteins place the second gel in a plastic container, then add previously cooled (to 4 °C) protein transfer buffer and incubate for 30 min at room temperature with gentle agitation on an orbital shaker. In a separate container place the nitrocellulose membrane, cut to the exact size of the gel, and the filter paper sheets, and soak with enough transfer buffer to cover them for 30 min.
12. After incubation, assemble the "sandwich" for transfer in this order: filter paper, nitrocellulose membrane, gel, and filter paper. Remove all air bubbles between the membrane and gel and between the paper sheets and gel.
13. Place the "sandwich" in a transfer cell oriented as follow: anode, filter paper, nitrocellulose membrane, gel, filter paper, and cathode.
14. Run the blotting apparatus at a constant voltage of 25 V for 1 h.
15. When the transfer has ended, disassemble the "sandwich" and put the gel in stain solution to make sure that all proteins were transferred.
16. Wash the nitrocellulose membrane in blocking solution for 2 h at 37 °C with gently agitation.
17. Discard the blocking solution, and wash the membrane five times with PBST for 5 min with gently agitation at room temperature.
18. Incubate the membrane in the primary antibody solution for 1 h at room temperature.
19. Wash the membrane five times for 5 min in PBST with gentle agitation at room temperature.
20. Incubate the membrane for 1 h in the secondary antibody solution (antibody diluted in PBST) at room temperature.
21. Wash the membrane five times for 5 min in PBST with gentle agitation at room temperature.

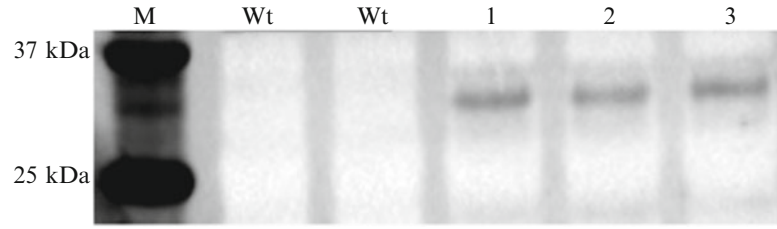


**Fig. 4** Immunodetection of GFP in *Chlamydomonas reinhardtii* transformed lines. *M* molecular weight marker Precision Plus Protein WesternC Standards (Bio-Rad). Wt is total soluble protein of a wild-type strain, lanes 1–3 contain total soluble protein from three different transformed strains

22. Wash the membrane with sterile deionized water for 5 min.
23. Develop the membrane using an Enhanced Chemiluminescent Detection (ECL) kit according to the manufacturer's instructions (Fig. 4).

## 4 Notes

1. The expression cassette *Ppsba-aphA-6-TrcbL* was obtained from pSK.KmR [19]. The *aphA-6* sequence can be consulted at the NCBI GeneBank with the accession number X07753.1R. The *gfp* gene is a synthetic gene, synthesized and codon-optimized by DNA 2.0 under our group's request.
2. An ample collection of *C. reinhardtii* strains, plasmids, media composition, and components can be accessed at the Chlamydomonas Resource Center (University of Minnesota, http://chlamy.org).
3. For convenience, we normally purchase the Hutner's trace elements solution from the Chlamydomonas resource center (http://chlamycollection.org/media/). However, the solution can also be prepared following the instructions in the web page from the same center (http://www.chlamy.org/trace.html). Dissolve the following salts in the indicated volume of deionized water: 50 g of EDTA disodium salt in 250 mL of boiling water, 22 g of $ZnSO_4{\cdot}7H_2O$ in 100 mL, 11.4 g of $H_3BO_3$ in 200 mL, 5.06 g of $MnCl_2{\cdot}4H_2O$ in 50 mL, 1.61 g of $CoCl_2{\cdot}6H_2O$ in 50 mL, 1.57 g of $CuSO_4{\cdot}5H_2O$ in 50 mL, 1.10 g of $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ in 50 mL, 4.99 g of $FeSO_4{\cdot}7H_2O$ in 50 mL (prepare $FeSO_4$ last to avoid oxidation). Mix all solutions, except EDTA, and bring to boil, and then add the EDTA solution. The mixture should turn green. When all components are dissolved, cool down to 70 °C and keeping the temperature at 70 °C, add 85 mL of hot 20 %

KOH solution. Bring the final volume to 1 L with warm deionized water. The solution should be clear green initially. Stopper the flask with a cotton plug and let it stand for 1–2 weeks, shaking it once a day. The solution should eventually turn purple and leave a rust-brown precipitate, which can be removed by filtering with filter paper (two layers of Whatman #1). Repeat the filtration process if necessary until the solution is free of the precipitate. Store in the refrigerator or freeze aliquots of a convenient volume.

4. Spermidine stock solution is prepared at 1 M and stored at −20 °C. When needed, the stock solution is diluted to assay concentration. Repetitive events of thawing and freezing must be avoided.
5. Calcium chloride is ideally prepared fresh the same day it will be used; there is no need for sterilization.
6. Ideally, the transformation vector concentration should be 1 μg/μL or higher. However, we have used concentrations of 0.5 μg/μL, adjusting the volume needed for particle coating to 10 μL (*see* Subheading 3.3**step 3**). We use the HiSpeed Plasmid Maxi Kit (Qiagen, USA) to purify the transformation vector from a 100–150 mL bacterial culture.
7. Chelex-100 is the commercial brand of BIO-RAD for styrene divinylbenzene copolymer. Resins from other manufacturers can be used, although we have only used Chelex-100 [28].
8. We usually use Taq 5X Master Mix (New England Biolabs), but Extract-N-Amp Plant PCR Kit (Sigma-Aldrich) yields similar results. However, we prefer to use Taq 5X Master mix (New England Biolabs), because it is robust enough, gives us consistently good results and works well in combination with Chelex-100 resin.
9. Use primers that amplify the whole length of the gene when genes are <1000 bp. When working with genes longer than 1 kb, is desirable to design primers to cover a region of 150–500 bp. In this particular case, primers for *gfp* amplify a fragment of 178 bp (gfp-Fw: GAAGGAGAAGGTGACGCAAC; gfp-Rv: CCTTCTGGCATAGCTGATTTG), primers for *aphA-6* amplify a fragment of 248 bp (aphA6-Fw: CGGAAACAGCG TTTTAGAGC;aphA6-Rv:GGTTTTGCATTGATCGCTTT).
10. For 100 mL of stock solution of acrylamide, weigh 48 g of acrylamide and 1.5 g of bisacrylamide and dissolve in 100 mL of water. Filter the solution using a 0.2 mm filter and store at 7–10 °C because crystallization occurs at 4 °C. Use safety protection when manipulating acrylamide due its neurotoxic effects.
11. Sample buffer can be prepared as shown in Subheading 2; however, we use Laemmli Sample Buffer (Biorad-USA).

12. For protein blotting we use Trans-blot SD Semi-Dry Transfer Cell (Bio Rad-USA).
13. Blocking solution can be acquired in different commercial presentations; however, we prepare our own solution using non-fat skimmed milk.
14. When growing in liquid media, cultures must be dark green. For this step cultures should reach a cellular density of $1.5 \times 10^7$ cells/mL. Optimal cellular density can easily be tracked by taking daily samples of the culture and measuring the optical density at 750 nm. When an optical density of 1.0 is obtained, the culture is ready for the next step. Our culture rooms are illuminated with LEDs with a light intensity of 17,000 lx. LEDs are relative low cost and do not increase room temperature.
15. It is important to dry *Chlamydomonas* liquid cultures in solid media, in order to guarantee adhesion and avoid splashing during the process of bombardment. The plates can be let to dry open in a laminar flow cabinet.
16. Gold particles can also be used, however, we have consistently obtained good results with tungsten particles.
17. As absolute ethanol dries quickly, the particles must be placed onto the macrocarriers rapidly. In the past we used to place the macrocarriers in a desiccator to dry them more quickly. We have since found that by placing the macrocarriers in a Petri dish with lid and using them immediately after the particles have dried, the transformation efficiency is not affected.
18. A conventional PCR reaction can be performed using Taq polymerase from various manufacturers. A typical 25 μL PCR using Taq 5X Master Mix requires as follows: 10 μM primer forward 0.5 μL, 10 μM primer reverse 0.5 μL, template DNA 0.5–2 μL, Taq 5X Master Mix 5 μL, nuclease-free water up to 25 μL. Thermocycling conditions for a routing PCR are: initial denaturalization 95 °C for 30 s; 30–32 cycles at 95 °C for 15 s, lowest primer annealing temperature for 30 s, extension 68 °C for 1 min; final extension 68 °C for 5 min.
19. The expected PCR product sizes for *gfp* and *aphA-6* are 178 bp and 248 bp, respectively, as shown in Fig. 3.

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# Part II

## Product Improvements

# Chapter 6

# Efficient, Antibiotic Marker-Free Transformation of a Dicot and a Monocot Crop with Glutamate 1-Semialdehyde Aminotransferase Selectable Marker Genes

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## Abstract

Antibiotic-free, efficient in vitro selection in plant genetic engineering can improve risk perception and speed up pre-market scrutiny of genetically modified crops. We provide a protocol for genetic transformation of two important crops, durum wheat and alfalfa, using a bacterial and a plant-derived selectable marker gene encoding mutated, gabaculine-insensitive glutamate 1-semialdehyde aminotransferase (GSA) enzymes. These methods can potentially be applied, with minor adaptations, to many other monocot and dicot crop plants.

**Key words** Alfalfa, Durum wheat, Gabaculine, GMO, *Hem*L, MsGSAgr, Non-antibiotic selection, Plant genetic engineering

## 1 Introduction

Due to concerns about perceived risks of selective marker genes for human and environmental health, new alternative selection systems have been proposed for the efficient transformation of crop plants, based on genes conferring resistance to chemical agents other than clinically useful antibiotics or common herbicides [1]. In particular, gabaculine (3-amino-2,3-dihydrobenzoic acid hydrochloride, $C_7H_9NO_2 \cdot HCl$) is a potent inhibitor of glutamate 1-semialdehyde aminotransferase (GSA), a key enzyme of the tetrapyrrole pathway, and is toxic to a wide range of plants [2]. Gabaculine-resistant forms of GSA have therefore been used as selective markers in plants. For example, the *hem*L gene from a gabaculine-resistant mutant of the cyanobacterium *Synechococcus* PCC6301, strain GR6, encoding a gabaculine-insensitive form of GSA has been successfully employed as a selectable marker in tobacco [3] and alfalfa (*Medicago sativa*, [4]). Recently, the GSA gene has been isolated

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from *M. sativa*, point-mutated to induce gabaculine resistance, and demonstrated to be an efficient selectable marker in alfalfa, tobacco [5] and durum wheat (*Triticum turgidum* var. *durum*) [6]. Since the *GSA* gene is present in all plants, its use may be perceived as less harmful compared with other selectable markers.

In this chapter, we provide detailed protocols to carry out plant genetic transformation using gabaculine selection with both the cyanobacterial *hem*L and the point-mutated *M. sativa GSA* (*MsGSAgr*) genes. The methods include biolistic transformation of a monocotyledonous crop plant, durum wheat, as well as *Agrobacterium*-mediated transformation of a dicotyledonous crop plant, ,alfalfa (*M. sativa*). These protocols could be applicable to a wide range of crop species. Furthermore, since *MsGSAgr* has been efficiently transformed into three distantly related crops (tobacco, alfalfa, and wheat), we suggest that GSA can be isolated from any species of interest, point-mutated to induce gabaculine resistance, and used as an "endogenous" selection system for plant transformation.

## 2 Materials

The pH of culture media should be adjusted to 5.85 with NaOH or HCl prior to autoclaving. Stock solutions for tissue culture and *Agrobacterium* growth are prepared using double-distilled (dd) water. Working solutions of macronutrients, micronutrients, and iron (*see* **Note 1**) must be sterilized by autoclave and stored at 4 °C. Thermolabile compound solutions (vitamins, plant growth regulators, gabaculine, and antibiotics) must be sterilized with syringe filters (*see* **Note 2**) under a laminar air flow cabinet, stored at −20 °C in aliquots, and added to sterile culture media once the media have cooled to 50–55 °C under a laminar air flow cabinet.

Gabaculine is soluble in water. It is sold in very little quantities, so it is best handled by adding water to the vial containing gabaculine powder with a syringe (*see* **Note 3**).

### 2.1 Biolistic Transformation of Wheat

#### 2.1.1 Plant Material and Vectors

1. Durum wheat immature embryos. We used the cultivar Varano for its high regeneration frequency in tissue culture among Italian durum wheat varieties [7].
2. Plasmid pAPCK-*hemL* (7718 bp) or pAPCK-*MsGSAgr* (5700 bp), each available from the authors upon request. These plasmids carry the coding sequence of the mutant *hem*L gene of *Synechococcus* PC6301 strain GR6, or the point-mutated *GSA* gene of *M. sativa*, respectively, under the control of a dual *CaMV 35S* promoter and *nos* terminator. They also contain genes for ampicillin resistance for selection in *E. coli*.

#### 2.1.2 Culture Media

1. Callus Induction Medium (MS): 4.3 g/L Murashige and Skoog (MS) basal salts, 40 g/L maltose, 0.50 mg/L thiamine-HCl, 150 mg/L aspargine, 3.5 g/L Phytagel, 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D).
2. Hypertonic Medium: 4.3 g/L MS basal salts, 40 g/L maltose, 0.50 mg/L thiamine-HCl, 150 mg/L aspargine, 171.15 g/L sucrose, 3.5 g/L Phytagel, 2 mg/L 2,4-D.
3. Recovery Medium: 4.3 g/L MS basal salts, 2.5 g/L Phytagel, 2 mg/L 2,4-D.
4. Selection-Regeneration Medium: 4.3 g/L MS basal salts,2.5 g/L Phytagel, 15 μM gabaculine, 0.2 mg/L 2,4-D.
5. Rooting Medium: 2.15 g/L MS basal salts, 15 μM gabaculine.

#### 2.1.3 Additional Items for Transformation

1. 70 % ethanol.
2. 20 % sodium hypochlorite.
3. Sterile water.
4. Stereo dissecting microscope.
5. Diurnal growth chamber set at 25 °C.
6. Gold particles.
7. 100 % ethanol.
8. Centrifuge.
9. 0.1 M spermidine.
10. 2.5 M $CaCl_2$.
11. PDS 1000\He particle gun and accessories (BioRad, Richmond, CA, USA).
12. Rooting culture tubes.
13. Pots.
14. Sterile mixture of 2:1 peat:vermiculite.
15. Clear plastic bags.
16. Greenhouse.
17. Petri dishes (100 × 15 mm).
18. Forceps.

### 2.2 Agrobacterium-Mediated Transformation of Alfalfa

#### 2.2.1 Plant Material and Vectors

1. Young, healthy, fully expanded leaves from alfalfa plants of the genotype Regen-SY [8, 9], or another genotype with high in vitro regeneration ability.
2. Binary vector pPZP201BK-*hemL* or pPZP201BK-*MsGSAgr* ([4, 5]; available upon request). These vectors carry the coding sequence of the mutant *hem*L gene of *Synechococcus* PC6301 strain GR6, or the point-mutated *GSA* gene of *M. sativa*, respectively, under the control of a dual *CaMV 35S* promoter and *nos* terminator. They also contain genes for ampicillin and kanamycin resistance for selection in *E. coli* and *Agrobacterium*.

#### 2.2.2 Culture Media

1. *Agrobacterium* growth medium:
   Standard LB liquid and solid media: 10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract. Adjust the pH to 7.0. For solid medium add 15 g/L agar. Autoclave. Add 50 mg/L kanamycin, 50 mg/L rifampicin, 100 mg/L carbenicillin.
2. Somatic embryo regeneration medium:
   The B5H Medium [10], with or without 100 or 400 mg/L cefotaxime and/or 30 µM gabaculine: 150 mg/L $NaH_2PO4{\cdot}H_2O$, 134 mg/L $(NH_4)_2SO_4$, 3000 mg/L $KNO_3$, 500 mg/L $MgSO_4{\cdot}7H_2O$, 895 mg/L $CaCl_2{\cdot}H_2O$, 0.75 mg/L KI, 3 mg/L $H_3BO_3$, 10 mg/L $MnSO4{\cdot}H_2O$, 2 mg/L $ZnSO_4{\cdot}7H_2O$, 0.25 mg/L $Na_2MoO_4{\cdot}2H_2O$, 0.025 mg/L $CuSO_4{\cdot}5H_2O$, 0.025 mg/L $CoCl_2{\cdot}6H_2O$, 10.8 mL/L Na Fe EDTA stock solution (*see* **Note 1**), 800 mg/L L-glutamine, 100 mg/L serine, 10 mg/L glutathione, 1 mg/L nicotinic acid, 1 mg/L pyridoxine-HCl, 10 mg/L thiamine-HCl, 100 mg/L myo-inositol, 1 mg/L 2,4D, 0.2 mg /L kinetin, 1 mg/L adenine, 30 g/L sucrose. Adjust the pH to 5.5 with KOH. For solid medium, add 3 g/L Gelrite (Duchefa).
3. Embryo conversion medium (MMS Medium, [11]): MS medium (4.3 g/L MS basal salts) containing NN vitamins [12] (5 mg/L nicotinic acid, 0.5 mg/L pyridoxine-HCl, 0.5 mg/L thiamine-HCl, 0.5 mg/L folic acid, 0.05 mg/L biotin), 2 mg/L glycine, 100 mg/L myo-inositol, 30 g/L sucrose, 2 g/L Gelrite (Duchefa), 400 mg/L cefotaxime, with or without 30 µM gabaculine. Adjust the pH to 5.8 with NaOH.

#### 2.2.3 Additional Items for Transformation

1. Incubator and shaker set at 28 °C.
2. Spectrophotometer.
3. Centrifuge.
4. Laminar air flow cabinet.
5. 70 % ethanol.
6. Sterile water.
7. 0.5 % sodium hypochlorite.
8. Sterile filter paper.
9. Razor blade.
10. Aluminum foil.
11. Magenta GA-7 vessels (77 × 77 × 97 mm).
12. Pots.
13. Sterile mixture of 1:1:1 soil:sand:peat.
14. Clear plastic bags.
15. Diurnal growth chamber at 25 °C.

16. Greenhouse.
17. Petri dishes (100 × 15 mm).
18. Acetosyringone.

### 2.3 Molecular Analysis

1. Plant DNA extraction kit.
2. Primers for the *hem*L gene (hemL-F 5′-GCAGTTTGAGG CGGGCTTTA-3′ and NosR 5′-ATCGCAAGACCGGCAAC AG-3′, [4]) or for the *MsGSAgr* gene (GSA-F 5′-CCAGCTTT GGTGCACCTTGTC-3′ and NosR 5′-ATCGCAAGACCGG CAACAG-3′, [5]).
3. Reagents for PCR (*Taq* DNA polymerase, PCR buffer, dNTPs, $MgCl_2$).
4. Thermocycler.
5. 1.5 % agarose gel with DNA stain.
6. Buffers, markers, and equipment for agarose gel electrophoresis.

## 3 Methods

### 3.1 Biolistic Transformation of Wheat

#### 3.1.1 Obtaining Wheat Embryogenic Calli

1. Collect immature caryopses from durum wheat spikes harvested 15–18 days post-anthesis.
2. Surface-sterilize seeds with 70 % ethanol for 5 min and 20 % sodium hypochlorite for 15 min, then rinse twice in sterile water.
3. Aseptically excise immature embryos varying in size between 0.8 and 1.5 mm diameter under a stereo dissecting microscope using sterile forceps, and place them with the scutellar portion exposed on the basal Callus Induction Medium in Petri dishes (100 × 15 mm), in number of 50 per dish.
4. Incubate at 25 °C in the dark for 7–10 days to obtain embryogenic calli (*see* **Note 4**).

#### 3.1.2 Bombardment

1. Apply an osmotic treatment to the wheat calli by incubation at 25 °C in the dark on Hypertonic Medium for 4 h prior to bombardment (*see* **Note 5**).
2. Prepare a stock solution of microcarriers by mixing 0.030 g of fine gold powder (1 μm diameter) with 500 μL of cold absolute ethanol.
3. Dispense 35 μL of stock solution into 1.5 mL tubes, then rinse in sterile water, centrifuge for 2 min at 10,000 × *g*, and resuspend in 35 μL 70 % ethanol.
4. To coat the gold microcarriers with DNA, combine in a centrifuge tube 35 μL gold particles, 40 μL 0.1 M spermidine, 250 μL 2.5 M $CaCl_2$, and a quantity of plasmid vector (*p*V) calculated as follows:

$pV\ (\mu L) = 2.27 \cdot pV\ (kbp) / pV(\mu g/\mu L)$
Add sterilized water to a final volume of 544.8 µL.

5. Vortex the tube for 10 min at 4 °C, then centrifuge at 16,000 × *g* for 5 min and discard the supernatant. Add 600 µL of cold absolute ethanol, centrifuge for 5 min at 16,000 × *g* and remove the supernatant. Finally, add 40 µL of cold absolute ethanol.
6. Spread 10 µL of the plasmid DNA-gold suspension in the center of each macrocarrier immediately prior to bombardment, then evaporate for 10 min.
7. Perform gene delivery on 40–60 wheat calli placed on Petri dishes (60 × 15 mm) using the helium-driven PDS-1000\He particle gun biolistic delivery system and disposable components supplied by Bio-Rad, according to the manufacturer's instructions. Bombard at 1100 psi pressure under vacuum of 26 in. (66 cm) Hg, with the following technical parameters: 8 mm between rupture disk and macrocarrier, 10 mm between macrocarrier and stopping screen, 7 cm between stopping screen and the Petri dish with the target calli.

### *3.1.3 Selection and Regeneration*

1. Keep the bombarded explants overnight at 25 °C in the dark on the Hypertonic Medium.
2. Transfer bombarded calli onto fresh substrate in 100 × 15 mm Petri dishes and keep them for 3 weeks in the dark at 25 °C on the Recovery Medium without selection, with weekly transfers to fresh medium (*see* **Note 6**).
3. Transfer bombarded calli to Selection-Regeneration medium with gabaculine.
4. Regenerate calli by culturing in a growth chamber with a 16 h-light and 8 h-dark photoperiod at 25 °C. In 5–7 weeks transformed calli will form green spots and then develop into green shoots.
5. Perform sub-cultures on fresh Selection-Regeneration medium every 7 days.
6. Perform two control experiments by culturing some non-transformed calli on the same selection condition of the bombarded explants (negative controls) and on a non-selective medium (positive controls). Transfer calli that developed green shoots to complete selection in culture tubes containing Rooting Medium supplemented with gabaculine. Conditions in the growth chamber during rooting are the same as for the selection-regeneration phase. Roots regenerate in 2–4 weeks.
7. Transfer plantlets showing healthy growth under selection from rooting tubes to pots containing a sterile mixture of 2:1 peat-vermiculite.

8. Keep the pots in the growth chamber for 2 weeks with decreasing humidity (by protecting the plant with plastic bags and gradually opening them), then transfer to a greenhouse suitable for transgenic plants (*see* **Note 12**).

### 3.2 Alfalfa Agrobacterium-Mediated Transformation

#### 3.2.1 Culture of Agrobacteriun Strain AGL1

1. Streak a fresh LB plate containing 50 mg/L rifampicin, 100 mg/L carbenicillin, and 50 mg/L kanamycin, with *A. tumefaciens* AGL1 transformed with the *pPZP201BK-hemL* or *pPZP201BK-MsGSAgr* binary vector from glycerol stock. Incubate overnight at 28 °C in the dark.
2. Inoculate a single colony in 6 mL of fresh liquid LB medium containing antibiotics. Incubate overnight at 28 °C and 200–250 rpm in the dark.
3. Inoculate 50 μL of the overnight *Agrobacterium* culture in 30 mL liquid LB medium containing antibiotics. Incubate overnight at 28 °C and 200–250 rpm in the dark.
4. Prepare *Agrobacterium* cultures by inoculating different amounts (e.g. 20, 30, and 50 μL) of this culture into 30 mL of liquid LB medium containing antibiotics (*see* **Note 7**). Incubate overnight at 28 °C and 200–250 rpm in the dark. Repeatedly measure the OD600 of the *Agrobacterium* suspensions until it reaches 1.0. Centrifuge the *Agrobacterium* culture at 5150 × *g* for 10 min and keep the pellet at 4 °C.
5. Resuspend the pellet with liquid B5H medium, using twice the volume of the centrifuged suspension (i.e. 60 mL) in order to obtain an OD600 of 0.5. Add acetosyringone to a final concentration of 100 μM.

#### 3.2.2 Inoculation of Agrobacterium and Co-culture

1. Keep the leaves in double-distilled (dd) water on ice and transfer them as soon as possible to the laminar air flow cabinet. Wash the leaves in 70 % ethanol for 10 s, rinse three times with sterile dd water.
2. Sterilize with a solution 0.5 % sodium hypochlorite for 20 min. Stir or invert the containers every 2–3 min. Rinse with abundant sterile dd water three times.
3. Cut the trifoliate leaves into pieces of about 0.5 $cm^2$ on sterile filter papers wetted with sterile dd water (*see* **Note 8**).
4. Transfer the cut explants to 90 × 20 mm Petri dishes containing liquid B5H medium until ready for infection.
5. With a pipettor remove the B5H liquid medium from the plates containing alfalfa explants and add 25–30 mL of the diluted *Agrobacterium* culture.
6. Co-cultivate for 30 min in the dark (cover the plates with aluminum foil) in the air flow cabinet.

7. Remove the *Agrobacterium* liquid culture with a pipettor and rinse with fresh B5H liquid medium.
8. Blot the explants dry on sterile filter papers and transfer them to 90 × 20 mm Petri dishes (15–20 explants per plate) containing solid B5H medium without selective agents. Place the leaf explants with the adaxial side down (*see* **Note 9**).
9. Co-cultivate for 3 days in a tissue culture chamber in the dark or low light (*see* **Note 10**).

#### 3.2.3 Selection and Regeneration

1. Rinse the explants with liquid B5H medium containing cefotaxime (100 mg/L). The same solution volume can be reused sequentially for up to three plates.
2. Blot the explants onto sterile filter paper and transfer them on fresh solid B5H medium containing 30 μM gabaculine (except for the positive control) and 400 mg/L cefotaxime.
3. After 2 weeks of culture, transfer the explants (which will have formed callus clumps) onto fresh solid B5H medium with 30 μM gabaculine and 400 mg/L cefotaxime.
4. After 2 weeks, transfer the material on solid MMS medium containing gabaculine and cefotaxime.
5. Pick single, well conformed mature somatic embryos and transfer them to fresh solid MMS medium containing cefotaxime for conversion to plantlets (*see* **Note 11**). Somatic embryos will emit shoots and roots in about 1–2 weeks.
6. Transfer the plantlets to Magenta vessels containing 50 mL solid MMS medium with cefotaxime. Transfer plantlets showing healthy growth under selection to pots containing a sterile mixture of 1:1:1 soil:sand:peat.
7. Keep in the growth chamber for 2 weeks at 24 °C with decreasing humidity (by protecting the plant with plastic bags and gradually opening them), and finally transfer to a greenhouse suitable for transgenic plants (*see* **Note 12**).

### 3.3 Molecular Analyses

#### 3.3.1 Molecular Screening of Transgenic Plants

1. Isolate total genomic DNA from young leaf samples of $T_0$ plants regenerated from gabaculine selection and from non-transgenic controls, using any commercial DNA extraction kit for plants or other standard methods.
2. Set up each PCR reaction containing 100 ng template DNA, 250 nM of each primer pair, 200 μM of each dNTP, 1.5 mM MgCl2, 1× PCR Buffer (e.g. 10 mM Tris–HCl, pH 8.3; 10 mM KCl), and 1 unit of Taq DNA polymerase.
3. Cycling is as follows: denaturation at 94 °C for 3 min, followed by 35 cycles of: 94 °C for 1 min, annealing at 62 °C (for *MsGSAgr*) or 67 °C (for *hem*L) for 1 min, extension at 72 °C for 2 min, and final extension at 72 °C for 10 min.

4. Analyze the amplification products by electrophoresis in 1.5 % (w/v) agarose gels. The GSA-F and NosR primers give a product of 1130 bp and the hemL-F and NosR primers give a product of 208 bp (*see* **Note 13**).

## 4 Notes

1. Prepare Na Fe EDTA stock solution as follows: dissolve 7.46 g $Na_2EDTA$ and 5.56 g $FeSO_4 \cdot 7H_2O$ by heating at about 50 °C in 1 L of dd water. Store in the dark.
2. Use cellulose acetate or cellulose nitrate filters with a membrane pore size of 0.22 μm.
3. To prepare a 50 mM stock solution, inject 5.694 mL of dd water into a vial containing 50 mg gabaculine. Once solubilized, store at −20 °C in 0.5 mL aliquots. Even if gabaculine was considered neurotoxic [13], the MSDS provided by the manufacturer describes this molecule as non-hazardous. In any case, it should be solubilized and aliquoted under a fume hood. The concentrations in plant growth media then become very low.
4. Use only good quality calli, showing a round shape and a bright yellow color.
5. This treatment promotes plasmolysis and reduces cellular turgor in order to minimize mechanical damage caused by gold microcarriers.
6. This serves to increase the survival of transformants.
7. Using different inoculum volumes helps to obtain the desired OD by the following morning in at least one culture.
8. Remove the leaf margins, where dead cells are located, to facilitate bacterial infection of fresh wounded tissue. Generally, 5–6 explants can be obtained from a medium size trifoliate leaf.
9. Press the explants down so that the entire adaxial surface of the explants is in contact with the medium.
10. It is not necessary to incubate plates in the dark; it is enough to cover the plates with aluminum foil.
11. Avoid picking embryos that may have originated from the same transgenic cell (duplicated events) by taking embryos from clearly separate tissue portions.
12. If necessary, supply supplementary light by sodium lamps.
13. The reverse primer designed on the Nos terminator prevents amplification of the endogenous GSA gene.

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# Chapter 7

# Transient Expression of Mammalian Genes in *N. benthamiana* to Modulate *N*-Glycosylation

**Alexandra Castilho and Herta Steinkellner**

## Abstract

*Nicotiana benthamiana* has shown great success as a platform for the production of recombinant proteins. Here, we describe methods to transiently express high levels of recombinant proteins and simultaneously modulate their glycosylation pattern toward human-like structures. The method aims to generate recombinant proteins with a targeted largely homogeneous glycosylation profile for structure–function studies.

**Key words** Transient expression, *Nicotiana benthamiana*, Glycoengineering, Recombinant proteins

## 1 Introduction

Plants have been proposed as bioreactors for recombinant protein production because they can generate large amounts of protein at low cost and carry out mammalian-like posttranslational modifications [1, 2]. In particular, *Nicotiana benthamiana* plants can transiently express a target protein within 1 week of delivery of the appropriate DNA; and optimization of transient expression systems based on plant viral vectors promote high-level accumulation of a wide range of proteins for multiple applications [3]. Essential advances have made this process robust and fully scalable. The strategy relies on *Agrobacterium tumefaciens* as a vector to deliver DNA copies of one or more viral RNA/DNA replicons into plant leaves by vacuum infiltration (magnifection). The viral machinery induces the production of substantial amounts of RNA and finally protein. This technology has been proven successful and several antibodies and vaccine antigens produced by magnifection are currently in clinical development [3].

The great majority of therapeutic products in the market are glycoproteins. Glycosylation is considered to be an important quality attribute, particularly in the case of therapeutic proteins, due to its strong effect on quality, immunogenicity, pharmacokinetics,

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and potency. Moreover, many human glycoproteins are terminally sialylated, a particularly complex glycoform. These negatively charged sugars affect the protein biological activity and serum half-life [4]. Sialylated glycoproteins are usually expressed in mammalian cells, however control over these structures is challenging.

In eukaryotes, the Golgi apparatus plays a central role in protein glycosylation since this is the organelle where many glycosylation enzymes are located. Despite the functional and morphological differences between animal and plant cells, there is a strong conservation among them in terms of Golgi organization and the proteome. Thus, plants share a remarkably high degree of homology to mammalian protein *N*-glycosylation and concerns with plant-specific *N*-glycosylation have been addressed extensively. Over the past years, we have witnessed outstanding achievements on modulating plant *N*-glycosylation toward the production of recombinant proteins with human-like structures at great uniformity [1, 2]. It is well described that plants, and specifically *N. benthamiana*, have a high degree of tolerance for modulation of their glycosylation pathways [1, 2].

Due to a limited repertoire of glycosylation enzymes, plants are unable to perform some glyco-modifications typical of mammalian-derived protein, such as bisected, multi-antennary, β1,4-galactosylated and sialylated *N*-glycans. Notwithstanding, it has been shown that by overexpressing mammalian glycosylation enzymes a number of human-like glycoforms can be generated in plants. However, correct subcellular targeting of each protein is fundamental. This enables activity to be coordinated with the plant glycosylation pathway, without interfering with endogenous enzymes.

All Golgi-resident *N*-glycan processing enzymes characterized thus far are type II transmembrane proteins consisting of an amino terminal cytoplasmic tail, a transmembrane domain, and a stem region (the cytoplasmic, transmembrane, and stem region; CTS), and a large luminal catalytic domain [5]. While enzyme activity is a function of the catalytic domain, the CTS region is responsible for sub-Golgi targeting [5–8] and thus has profound implications on the final glycosylation pattern. CTS fusions to fluorescent proteins demonstrate that there is a clear sequential distribution of glycosyltransferases across the Golgi stacks [8] (Table 1). The ability to swap CTS domains and thus target the glycosylation enzyme to different sub-compartments provides precise control over glycosylation pathways. This has been demonstrated in the course of generating bisected, branched, and galactosylated structures [9–15].

In this chapter, we describe an efficient strategy to produce plant-derived glycoproteins with *N*-glycan profiles that resemble their human counterparts.

The procedure combines two expression systems: (1) viral-based vectors [16–18] to transiently express high amounts of the

**Table 1**
**List of cytoplasmic, transmembrane, and stem regions (CTS) of type II proteins that can be used for different subcellular targeting of mammalian glycosyltransferases expressed in plants**

| Subcellular targeting | Type II transmembrane protein | Accession | CTS region (aa) |
|---|---|---|---|
| ER | α-Glucosidase I (GCSI) | AAL17718.1 | 1–90 |
| ER/*cis*-Golgi | Arabidopsis Golgi α1,2-mannosidase A (GMI-A) | NP_175570.1 | 1–88 |
| *cis*/ medial-Golgi | Arabidopsis Golgi α1,2-mannosidase C (GMI-C) | NP_564345.1 | 1–109 |
| | Arabidopsis xyloglucan α1,6-xylosyltransferase (XT1) | AEE80384.1 | 1–10 |
| | Arabidopsis *N*-acetylglucosaminyltransferase I (GnTI) | CAC80700.1 | 1–77 |
| medial-Golgi | Golgi mannosidase II (GMII) | AED92095.1 | 1–92 |
| | Xyloglucan β1,2-galactosyltransferase (Kam1) | AEC06999 | 1–120 |
| | α1,3-Fucosyltransferase 11 (FUT11) | AEE76217.1 | 1–66 |
| | α1,3-Fucosyltransferase 12 (FUT12) | AEE32462.1 | 1–66 |
| | β1,2-Xylosyltransferase (XylT) | CAB90610.1 | 1–90 |
| | Arabidopsis α1,3-galactosyltransferase 16 (GALT2) | NM_111519.2 | 1–118 |
| | Arabidopsis β1,2-*N*-acetylglucosaminyltransferase II (GnTII) | CAC08806.1 | 1–76 |
| | Human β1,4-*N*-acetylglucosaminyltransferase III (GnTIII) | NP_001091740 | 1–34 |
| | Human β1,4-galactosylatransferase (B4GALT1) | NM_001497 | 1–43 |
| *trans*-Golgi | Arabidopsis xyloglucan α1,2-fucosyltransferase (FUT1) | AF154111.1 | 1-80 |
| | Arabidopsis α1,3-galactosyltransferase 15 (GATT1) | AEE30744. | 1–60 |
| | Arabidopsis α1,4-fucosyltransferase (FUT13) | AEE35261.1 | 1–52 |
| | Rat α2,6-Sialyltransferase (ST) | M18769.1 | 1–52 |

Protein accession numbers and corresponding CTS amino acids (aa) are also listed

target protein (protein of interest POI); and (2) binary vectors to express glycosylation genes for glycan modeling. Once the necessary genes are cloned, cDNAs are co-delivered into the plant cell by agro-infiltration.

Our strategy combines advantages of three biological systems: (1) the transfection efficiency of *A. tumefaciens*; (2) high expression of target proteins by viral vectors; and (3) plant glycosylation repertoire. Notably, the method does not require stable genetic modification of plants.

Here, the *N. benthamiana*glycosylation mutant ΔXTFT serves as expression host, as it lacks plant-specific *N*-glycan residues, i.e. core α1,3-fucose and β1,2-xylose [19]. In the simplest method two vectors are delivered, a magnICON®-based vector for the high expression of a gene/protein of interest (POI), and a binary vector

**Table 2**
**List of proteins used for modulation of plant glycosylation**

| name | Gene | Acc. |
|---|---|---|
| GNE | Mouse UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine-kinase | NM053765 |
| NANS | Human *N*-acetylneuraminic acid phosphate-synthase | AF257466 |
| CMAS | Human CMP-*N*-acetylneuraminic acid synthase | AL832975 |
| GalT | Human β1,4-galactosyltransferase | NM_001497 |
| CTS | Mouse CMP-sialic acid transporter | BC012252 |
| ST | Rat α2,6-sialyltransferase | M18769.1 |
| GnTIV | Human α1,3-mannosyl- β1,4-N-acetylglucosaminyltransferase IVa | NP_036346 |
| GnTV | Human α1,6-mannosyl-β1,6-N-acetylglucosaminyltransferase V | NM_002410 |
| GnTII | Arabidopsis α1,6-mannosyl-β1,2-N-acetylglucosaminyltransferase II | AJ249274 |

Accession numbers are listed

carrying the glycan-modifying enzyme. An example is *in planta* produced human erythropoietin (EPO) with bisected glycans, obtained by the co-expression of a magnICON® vector carrying EPO cDNA and a binary vector that caries cDNA of human β1,4-N-acetylglucosaminyltransferase III (GnTIII) [9]. For more complex structures, several glycosyltransferases need to be added to the infiltration mix. For example, the synthesis of multi-antennary sialylated glycans in plants involves the co-expression of mammalian glyco-genes necessary for (1) GlcNAc branching, (2) β1,4-galactosylation as well as for the (3) synthesis, transport and transfer of sialic acid. The formation of this highly complex oligosaccharide structure requires the coordinated expression of nine human proteins acting in different subcellular compartments at different stages of the glycosylation pathway (Table 2) [20].

An obvious approach to simplify the procedure (transient or stable) is to reduce the number of binary vectors that need to be co-delivered into plants. Several strategies to deliver multi-gene have been reported [21–23].

## 2 Materials

All solutions are prepared using Milli-Q ultrapure water and analytical grade reagents. All reagents are at room temperature (unless indicated otherwise).

### 2.1 Plant Cultivation

1. *N. benthamiana*seeds (glycosylation mutant ΔXTFT).
2. Plastic pots and trays.
3. Growth chamber with controlled temperature, photoperiod, and humidity.
4. Sterilized soil with perlite (2:1 mixture).
5. Fertilizer.

### 2.2 DNA Sequences, Cloning Vectors, and Others

1. Gene of interest: cDNA from reporter genes can either be purchased or made synthetic from, e.g. GeneArt® Gene Synthesis (http://www.lifetechnologies.com/), preferentially in a species codon-optimized version.
2. Glycan-modifying genes: Genes necessary to modulate glycosylation of plant-produced proteins are listed in Table 2. Table 1 summarizes CTS sequences used for domain swapping. Sequences can be directly amplified from genomic DNA or purchased from, e.g. ImaGenes (http://www.imagenes-bio.de).
3. Plant Viral vectors: can be purchased for IconGenetics (www.icongenetics.org). Alternative vectors based on regulatory viral sequences have been described as well (e.g. pEAQ vectors) [18].
4. Binary vectors: several expression vectors are commercially available with multiple cloning sites, e.g. pGreen, pPZP, pBI121, and pCAMBIA just to name a few.
5. Conventional and type II restriction enzymes.
6. DNA Polymerase.
7. T4 DNA Ligase.

### 2.3 Bacteria Stocks and Cultivation

1. Bacteria strains: *Escherichia coli* DH5α; *Agrobacterium tumefaciens* GV3101::pMP90RK and UIA143.
2. 29 and 37 °C incubators with horizontal shakers.
3. Inoculation tubes.
4. Antibiotics: Depends on the resistance cassettes present in expression vectors.
5. LB media (1 L): 5 g NaCl, 5 g yeast extract, 10 g Tryptone/peptone. Autoclaved to sterilize.
6. Agar plates (1 L): 5 g NaCl, 5 g yeast extract, 10 g Tryptone/peptone, 15 g Agar. Autoclave to sterilize and leave to cool down to 60 °C. Add appropriated antibiotic and pour into Petri dishes. Leave at room temperature to polymerize. 1 L of LB medium plus agar gives around 20 plates.
7. Chemical or electro-competent bacteria cells for plasmid transformation.
8. Sterile glycerol.

### 2.4 Agro-infiltration

1. 1 M MES pH 5.6 (100 mL): 19.5 g MES dissolved in 90 mL of water. Adjust pH to 5.6 with NaOH and add water up to 100 mL.
2. 1 M $MgSO_4$ (100 mL): 12 g $MgSO_4$ dissolved in 100 mL of water.
3. Infiltration buffer (1 L): 10 mM MES pH 5.6, 10 mM $MgSO_4$. 10 mL of 1 M MES pH 5.6, 10 mL of 1 M $MgSO_4$, add water up to a final volume of 1 L. Store at 4 °C.
4. Spectrophotometer.
5. Centrifuge.
6. Disposable syringes.

### 2.5 Intercellular Fluid (IF)

1. 1 M Tris–HCl pH 7.5 (1 L): 121.14 g Tris dissolved in 900 mL of water. Adjust the pH with HCl. Make up to 1 L with water. Store at 4 °C.
2. 1 M $MgCl_2$ (100 mL): 22 g $MgCl_2$ dissolved in 100 mL of water.
3. 0.5 M EDTA (0.5 L): 18.6 g of disodium ethylenediamine tetraacetate·$2H_2O$ (EDTA) dissolved in a volume of 300 mL. Adjust pH to 8.0 with NaOH, add water up to 500 mL.
4. IF buffer solution (1 L): 100 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM EDTA. 100 mL of 1 M Tris–HCl pH 7.5, 10 mL of 1 M $MgCl_2$ and 4 mL of 0.5 M EDTA. Adjust volume up to 1 L with water. Store at 4 °C.
5. Vacuum pump.
6. Nalgene Vacuum Desiccator or similar.
7. Centrifuge.
8. 50 mL falcon tubes.
9. Fine mesh.
10. SDS-PAGE equipment and related material.

## 3 Methods

The transient expression of high levels of recombinant proteins and the simultaneous modulation of their glycosylation pattern comprises a number of separate procedures. These include (1) generation of expression vectors; (2) preparation and propagation of *N. benthamiana* plants; (3) delivery of recombinant DNA into plant cells by agro-infiltration; and finally (iv) isolation and glycan analysis of the POI.

All procedures are carried out at room temperature unless otherwise specified.

### 3.1 Preparation of Expression Vectors (Viral and Binary Vectors)

#### 3.1.1 Cloning of Target Genes

Optimized cloning protocols are based on the use of type II restriction enzymes that cut outside the recognition site. This has many advantages allowing restriction and ligation to be performed together as well as directional cloning preventing re-ligation of the empty vector [24, 25]. Examples of viral vectors for the expression of reporter proteins are displayed in Fig. 1a.

1. Amplify the gene of interest by PCR with specific primers each carrying a *Bsa*I restriction site with a different cleavage site complementary to the two *Bsa*I restriction sites of the recipient expression vector. Alternatively to PCR, a synthetic gene sequence can be made with flanking *Bsa*I restriction sites (*see* **Note 1**).
2. Digest PCR product with *Bsa*I and ligate into a viral vector digested the same way (*see* **Note 2**).
3. Transform competent bacterial cells by conventional methods.

#### 3.1.2 Cloning Mammalian Genes for Glycan Modulation

Genes used to modulate the glycosylation pathway in plants are listed in Table 2. These genes can be cloned either in their full-length cDNA version or, when necessary, the catalytic domain can be fused to an appropriate CTS region (Table 1) for altering sub-Golgi targeting. Binary vectors designed to express different genes necessary to modulate plant glycosylation are represented in Fig. 1b.

1. Cloning of DNA fragments in a binary vector of choice, transformation of bacterial cells, and analysis of positive clones are performed by conventional cloning methods and will not be described here. The major features of binary vectors used here are depicted in Fig. 1b (*see* **Note 3**).

After cloning, plasmids are transformed into competent *A. tumefaciens* cells. Prepare stocks by mixing 1 mL of overnight culture with an equivalent volume of 100 % (v/v) sterile glycerol. Stocks are stored at –80 °C.

### 3.2 Plant Cultivation

#### 3.2.1 Soil Preparation

1. Mix soil with perlite in a 2:1 ratio. Close plastic bags with the soil mixture tightly with tape.
2. Optional: Sterilize the soil, by freeze–thawing the bags (–20 °C/RT) three times prior to use.
3. Fill plastic pots with a humid soil–perlite mixture, using tap water.

#### 3.2.2 Plant Propagation (Fig. 2)

Grow all plants in a growth chamber with a constant temperature of 24 °C, 60 % relative humidity, and a 16 h light/8 h dark photoperiod.

1. Sow approximately 30 non-sterile *Nicotiana benthamiana* seeds in 9×9×9 cm pots, cover with a plastic lid, and allow seedlings to grow for 2 weeks.

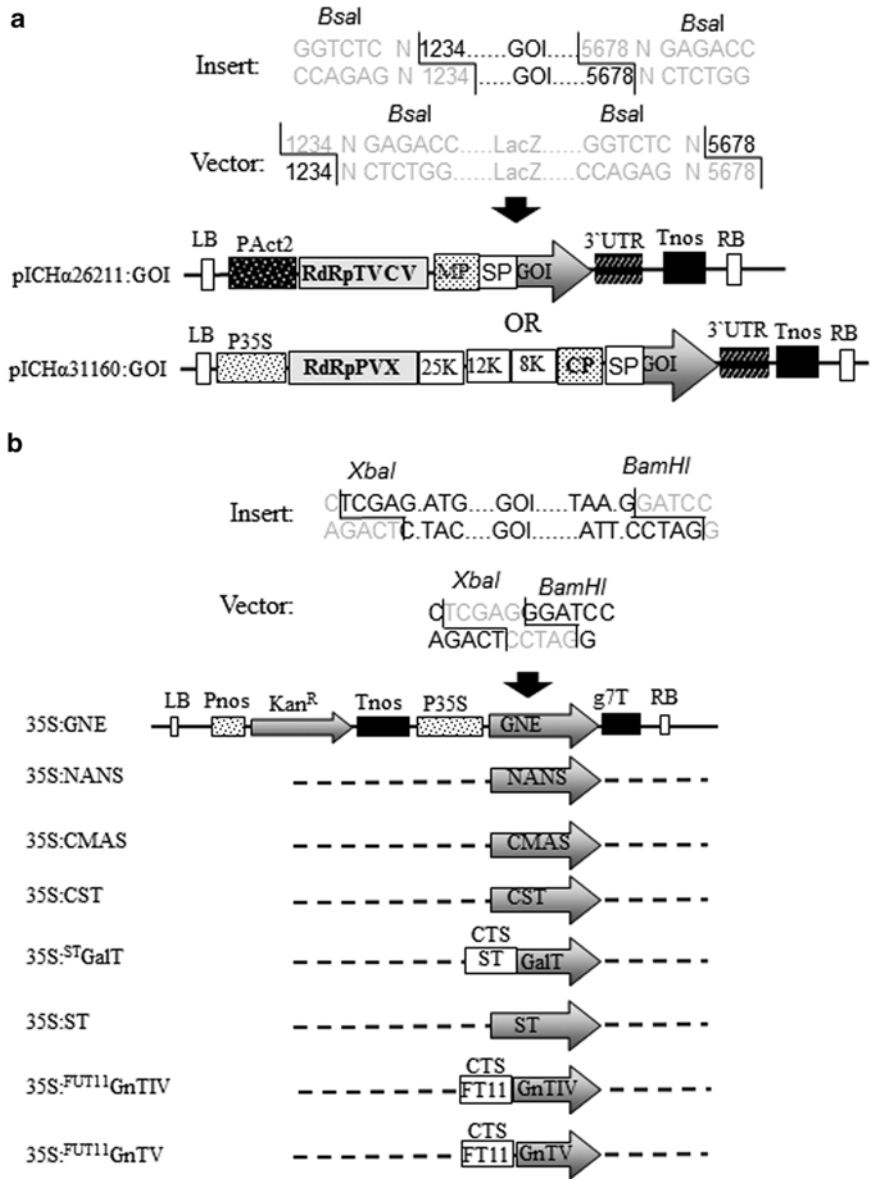


**Fig. 1** Schematic representations of the T-DNA constructs used to transiently express and modulate glycosylation of reporter proteins expressed in *Nicotiana benthamiana*. (**a**) MagnICON vectors used to express gene of interest (GOI) in *Nicotiana benthamiana.* As examples the modified TMV-based and PVX-based magICON vectors (pICH26211, pICH31160) with a targeting sequence for the secretory pathway (SP) are shown. Gene sequence lacking endogenous signal peptide (insert) is assembled into expression cassettes using type II restriction enzymes (e.g. *Bsa*I). (**b**) Schematic representation of the main features of binary vectors used to modulate *Nicotiana benthamiana N*-glycosylation. For gene names *see* Tables 1 and 2. Full gene sequence or quimeras (insert) are assembled into expression cassettes using common restriction enzymes (e.g. *Xba*I-*Bam*HI). *PAct2* Arabidopsis actin 2 promoter, *TVCV* turnip vein clearing virus RNA-dependent RNA polymerase, *MP* movement protein, *PVX Pol* potato virus X replicase, *25K, 12K and 8K* 25 kDa, 12 kDa and 8 kDa movement protein, *CP* coat protein, *SP* barley α amylase signal peptide for apoplast targeting, *Pnos* nopaline synthase gene promoter, *Tnos* nopaline synthase gene terminator, *3′ UTR* TVCV or PVX 3′-untranslated region, *P35S* cauliflower mosaic virus 35S gene promoter, *g7T* agrobacterium gene 7 terminator, *KanR* neomycin phosphotransferase 2 gene, *LB* left border, *RB* right border


**Fig. 2** Plant cultivation steps. Seeds are sown in pots with sterilized soil (**a**). After approximately 2 weeks, seedlings are separated (one plant per pot) and trays are covered with thin plastic film (**b**). Four to five weeks old plants are ready to be infiltrated (**c**)

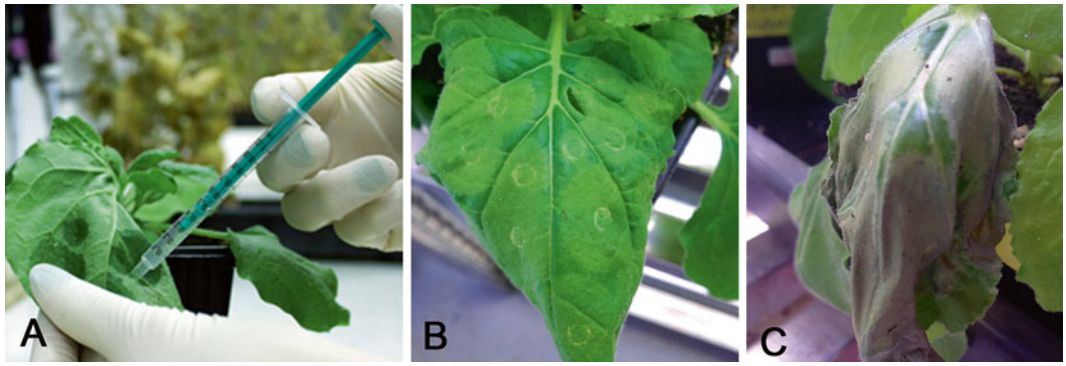


**Fig. 3** Agro-infiltration. Agrobacteria mixture is delivered into plant cells manually using a needleless plastic syringe (**a**). Infiltrated leave (**b**); in some cases leaves exhibit a necrotic phenotype (**c**). Do not use necrotic leaves for further analyses, in such cases harvest earlier

2. Transfer each individual seedling to a single pot. Arrange single plant pots in trays and cover with a thin plastic film to form a humid chamber.
3. After 2–3 weeks remove the plastic film and grow the plants for an additional 4–5 weeks. Plants should be watered and fertilized according to growth chamber conditions.

### *3.3 Agro-infiltration*

Transient protein expression is accomplished by introducing the heterologous gene to as many plant cells as possible without heritably modifying the host plant. The agro-infiltration system is widely used in a range of plant species. The method facilitates the delivery of several transgenes into the same cell enabling simultaneous expression of several proteins. *See* Fig. 3.

1. Inoculate 5 mL of LB medium supplemented with the appropriate antibiotics, with 20 μL glycerol stock of the desired agrobacteria strains (each containing the gene(s) of interest and the glyco-genes). Cultures for all the *Agrobacterium* strains should be initiated at the same time. Incubate cells overnight at 29 °C, continuously shaking at 180 rpm.

2. Harvest cells by centrifugation at low speed (1600 × *g* for 4 min) and resuspend pellets carefully in infiltration buffer.
3. Measure optical density at 600 nm ($OD_{600}$) and calculate the appropriate volume of agrobacteria suspensions that should be added to the infiltration mixture to reach the desired final concentration (*see* example in Table 3). Normally, the target gene is infiltrated at a final $OD_{600}$ of 0.1 and glyco-modifying genes at 0.05 (*see* **Note 4**).
4. Inject bacterial suspension through the stomata on the lower epidermal surface of fully expanded leaves using a 1 mL needleless plastic syringe applying gentle pressure (*see* **Notes 5** and **6**).
5. Water plants and let infiltrated plants grow for 4–7 days (*see* **Note 7**).

### *3.4 Isolation of Secreted Recombinant Proteins from the Intercellular Fluid (IF)*

If a secreted protein needs to be analyzed, we recommend isolation of intercellular fluid (apoplastic fluid). The following steps are carried out at 4 °C. *See* Figs. 4 and 5.

1. Harvest leaves 3–7 days post infiltration (dpi), weigh, and place in an appropriate sized beaker (*see* **Notes 7** and **8**).
2. Submerge leaf material in IF buffer and place the beaker in a Nalgene Vacuum Desiccator (*see* **Note 9**).

**Table 3**
**Example of scheme of infiltration mixtures to express a reporter protein with di-sialylated (mix 1), tri-sialylated (mix 2 and 3), and tetra-sialylated (mix 4) glycans**

| Construct | $OD_{600}$ | Mix 1 | Mix 2 | Mix 3 | Mix 4 |
|---|---|---|---|---|---|
| Reporter protein | 1.2 | 333 µL | 333 µL | 333 µL | 333 µL |
| GNE | 1.1 | 182 µL | 182 µL | 182 µL | 182 µL |
| SAS | 1.2 | 166 µL | 166 µL | 166 µL | 166 µL |
| CMP | 0.8 | 250 µL | 250 µL | 250 µL | 250 µL |
| CST | 0.8 | 250 µL | 250 µL | 250 µL | 250 µL |
| $^{ST}$GalT | 0.7 | 286 µL | 286 µL | 286 µL | 286 µL |
| ST | 0.8 | 250 µL | 250 µL | 250 µL | 250 µL |
| $^{FUT11}$GnTIV | 0.7 | | 286 µL | | 286 µL |
| $^{FUT11}$GnTV | 0.7 | | | 286 µL | 286 µL |
| Buffer | – | 2.28 mL | 2.57 mL | 2.57 mL | 2.85 mL |
| Final vol. | – | 4 mL | 4 mL | 4 mL | 4 mL |

In the final mixture the bacteria for expressing the reporter glycoprotein are at an $OD_{600}$ of 0.1 (range 0.02–0.3) while for expressing of glyco-genes, the bacteria are at an $OD_{600}$ of 0.05 (range 0.004–0.3)

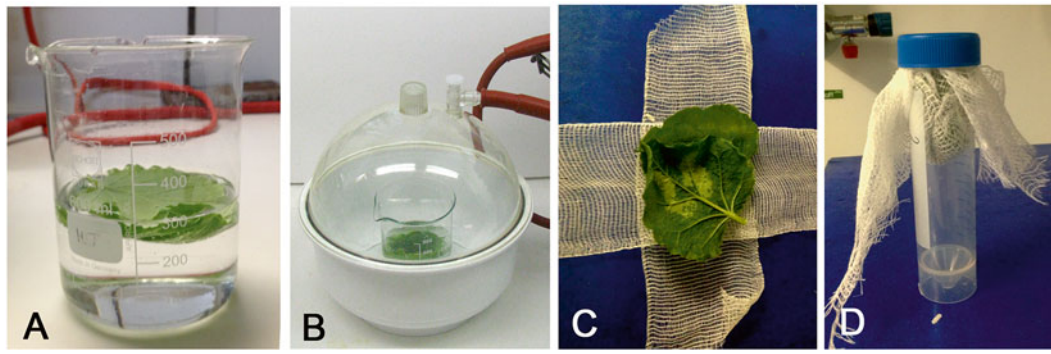


**Fig. 4** Isolation of secreted recombinant protein from the intercellular fluid (IF). Leaves are submerged in IF buffer (**a**) and vacuum is applied (**b**). A mesh holds leaf material (**c** and **g**) and IF is collected by centrifugation

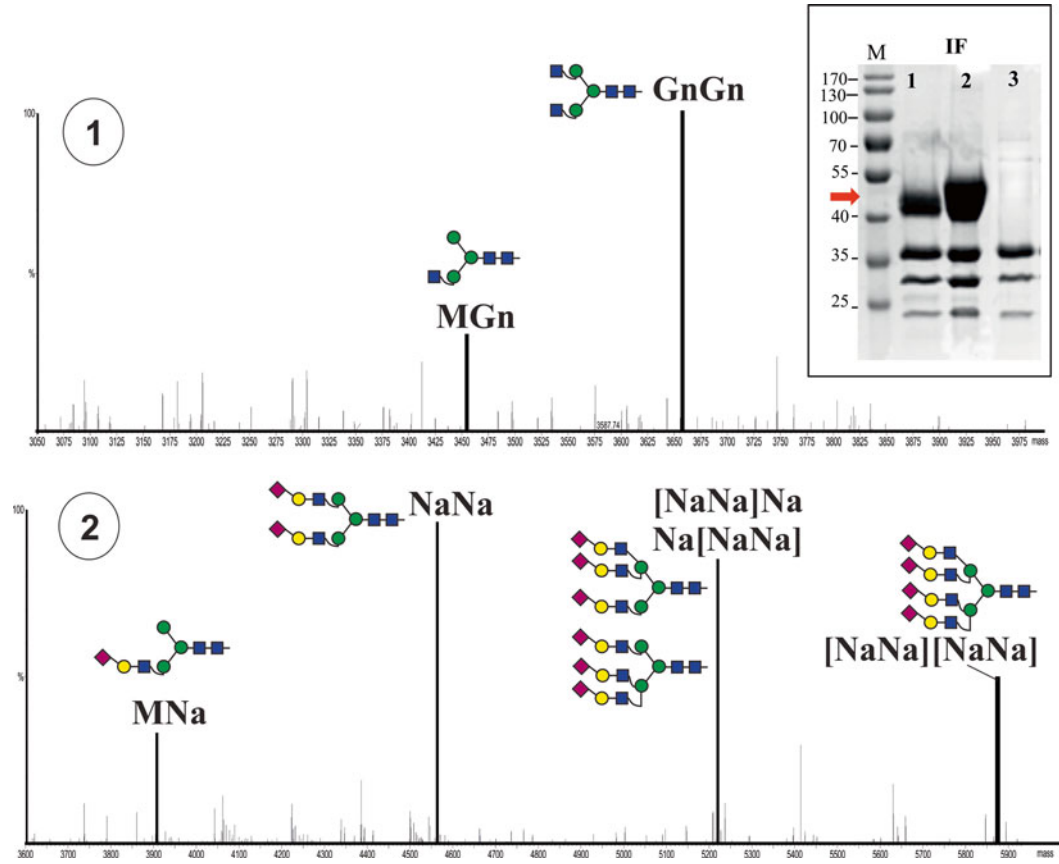


**Fig. 5** Recombinant protein analysis. Secreted recombinant proteins collected from the intercellular fluid (IF) are separated by SDS-PAGE and stained with Coomassie brilliant blue (*left* panel). In this example a recombinant protein (~40 kDa) was expressed without (lane 1) and with (lane 2) modulation of glycosylation toward highly sialylated structures. Mobility shift reflects differences in glycosylation, which isconfirmed by mass spectrometry (*right* panel). Lane 3 shows the secreted proteins after infiltration with an empty vector (control sample)

3. Apply vacuum, hold for approximate 2 min and then slowly release. Repeat twice (*see* **Note 10**).
4. Blot plant tissue in absorbent paper to remove excess buffer solution.
5. Wrap leaves in mesh and place in 50 mL tubes. Use the tube lid to hold the mesh containing the leaves at the top to allow separation and recovery of IF from the leaf material. Collect IF by low speed centrifugation (900 × *g* for 10 min at 4 °C) (*see* **Notes 11** and **12**).
6. Collect and store IF in a clean tube (*see* **Notes 13** and **14**).

## 4 Notes

1. Other type II restriction enzymes such as *Bpi*I and *Esp*3I can be used as an alternative to *Bsa*I. This can be useful when the gene of interest has an internal *Bsa*I site.
2. Several signal peptides can be used to target the protein of interest to the secretory pathway with the final destination of secretion. Some vectors supplied by IconGenetics are already carrying signal peptide sequences such as the alpha amylase from barley. Other signal peptides are suitable as well. When no other signals are present, proteins are usually directed to the apoplast. When other destinations are envisaged (e.g. ER, vacuoles), additional targeting sequences are mandatory.
3. The modulation of plant glycosylation toward complex structures requires the expression of multiple genes. This method reports the co-infiltration of several binary vectors each carrying one of the necessary genes. To simplify the procedure, the number of binary vectors that need to be co-delivered into plants can be reduced by assembling the different expression cassettes in a single construct. A number of strategies are now available for multi-gene vector construction, such as the pRCS/pSAT multisite vector system using rare-cutters, homing endonucleases, or artificial restriction enzymes to increasing the cloning capacity [21, 26]; combinatorial DNA assembly using Golden Gate cloning [22, 25]; or GoldenBraid, a standardized assembly system based on type II restriction enzymes [27].
4. Concentration of bacterial strains can be optimized for a particular experiment. Also, sometimes a "fine tuning" is needed for the generation of tailor-made mammalian glycoforms. Such fine tuning is not predictable a priori, it largely depends on the *N*-glycan structure required and the protein itself. This can be accomplished by altering the mixture of glycosylation enzymes

in the agro-infiltration procedures. The expression levels of certain "foreign" glycosylation enzymes may interfere with the endogeneous glycosylation machinery. This has, for example, been observed with the expression of human β1,4-galactosyltransferase, where low expression levels are advantageous. Also, incompletely processed structures are synthesized. It has been reported that some glycosyltransferases impair the function of GnTII [28]. In this case it is advisable to add GnTII to the infiltration mixture.

5. Normally, 1 mL of agrobacteria suspension is enough to manually infiltrate two fully expanded *N. benthamiana* leaves. Usually 4- to 5-week-old plants are selected for infiltration. To avoid cross-contamination, gloves should be changed or sterilized with ethanol between infiltrations. Also, for health and safety reasons eye protection should be worn during this process.
6. Vacuum infiltration can be performed as an alternative to syringe (manual) infiltration. This is particularly useful when large amounts of protein are required and several plants need to be transfected. The procedure is similar to that described for the collection of secreted proteins (Subheading 3.4). Whole plants are placed upside down in a beaker containing the agrobacteria suspension and vacuum is applied [17].
7. Plant phenotype should be scored around 3 days post infiltration (dpi). Some bacteria can induce leaf necrosis (Fig. 3b versus d). To avoid this, leaves should be harvested prior to necrosis or the concentration of one or more agrobacteria should be reduced. It is advised to perform time course experiments to determine optimal bacterial concentrations and harvesting dates.
8. This method is useful with many plant tissues such as leaves, roots, shoots, stems, flowers, fruits, embryos, and seedlings.
9. Normally EDTA or Tris buffer solution is suitable, though any buffer may be more or less appropriate for a given plant or protein of interest. However, it is generally recommended to maintain conditions which avoid oxidation, precipitation, proteolysis or denaturation of the protein of interest. Thus, pH, temperature and other variables should be monitored and altered as needed.
10. Vacuum pressure and time may need to be adjusted to the experiment. However, exposure to a vacuum environment for durations of around a few seconds to 10 min has proven especially effective. In some cases, it is necessary to expose the leaves to a vacuum environment repeatedly. Normally, one to three separate exposures are effective.

11. Depending on the amount of leaf material, larger centrifugation bottles can be used. The procedure can be scaled-up.
12. With this procedure one *N. benthamiana* leaf yields about 500–600 μL of buffer containing proteins secreted to the apoplast. Centrifugation at low speed is preferential and although normally it does not completely remove the infiltrated buffer, it avoids contamination of the IF with chloroplasts ("greenish fluid").
13. Depending on the expression levels and subsequent analyses, the IF may be concentrated. In some cases, purification of POIs is necessary. Quality of IF can immediately be analyzed by SDS-PAGE stained with Coomassie blue (Fig. 5, left panel). Glycan analysis using appropriate protocols will elucidate the glycosylation structure (Fig. 5 right panel).
14. The *N*-glycan composition of the POI can be determined by liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) after treating samples with PNGase A to release glycans. Alternatively, when several glycosylation sites need to be analyzed LC-ESI-MS can be used for glycol-profiling of individual glycopeptides obtained by in-gel digestion of the POI (e.g. using trypsin) [29]. *N*-Glycans of endogenous proteins can be analyzed using total soluble protein extracts from *N. benthamiana* leaf material by matrix-assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF-MS) as described elsewhere [30].

## Acknowledgments

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# Chapter 8

# Companion Protease Inhibitors for the In Situ Protection of Recombinant Proteins in Plants

**Stéphanie Robert, Philippe V. Jutras, Moustafa Khalf, Marc-André D'Aoust, Marie-Claire Goulet, Frank Sainsbury, and Dominique Michaud**

## Abstract

We previously described a procedure for the use of plant protease inhibitors as "companion" accessory proteins to prevent unwanted proteolysis of clinically useful recombinant proteins in leaf crude protein extracts (Benchabane et al. Methods Mol Biol 483:265–273, 2009). Here we describe the use of these inhibitors for the protection of recombinant proteins *in planta*, before their extraction from leaf tissues. A procedure is first described involving inhibitors co-expressed along—and co-migrating—with the protein of interest in host plant cells. An alternative, single transgene scheme is then described involving translational fusions of the recombinant protein and companion inhibitor. These approaches may allow for a significant improvement of protein steady-state levels in leaves, comparable to yield improvements observed with protease-deficient strains of less complex protein expression hosts such as *E. coli* or yeasts.

**Key words** Clinically useful recombinant proteins, Heterologous protein expression, Recombinant protein degradation, Companion protease inhibitors, Protein stabilization

## 1 Introduction

A key challenge in plant molecular pharming is to ensure the integrity and overall quality of the recombinant protein product [1]. Unlike many less complex pharmaceuticals, proteins have a natural tendency toward structural heterogeneity, often resulting in a complex mixture of protein variants differing in their primary or tertiary structures [2]. Such heterogeneity, commonly a result of unequal glycosylation or instability associated with partial hydrolysis [3, 4], is generally viewed as an indicator of poor quality by regulatory agencies, and can thus impede approval of the protein product. While proteolytic enzymes are involved in various physiological processes in plants [5, 6], their abundance in most tissues often represents a burden to the effective production of proteins.

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Studies have reported, for instance, the detrimental impact of host resident proteases against recombinant proteins travelling the plant cell secretory pathway [3]. A well-known case is the proteolytic processing of secreted mammalian antibodies in Golgi vesicles, which results in complex patterns of antibody chain fragments together with the expected full-size antibodies [7, 8]. Other examples are the clinically useful trypsin/chymotrypsin inhibitors bovine aprotinin and human $\alpha_1$-antichymotrypsin (hACT), which undergo partial trimming at the C- and/or N-terminal ends when retained in the ER or allowed to migrate downstream toward the apoplast [9, 10].

Different strategies have been proposed to stabilize recombinant proteins in plant systems, such as their targeting to alternative subcellular compartments using appropriate peptide sorting signals [2, 4] or the addition of a stabilizing fusion partner to drive their sequestration in protein bodies [11]. The antisense-mediated repression of endogenous protease biosynthesis [12] and the co-expression of "companion" pseudosubstrate inhibitors acting as molecular baits for endogenous proteases [4, 13] have also been proposed to create cellular environments with reduced protease activity favorable to recombinant protein accumulation. Whereas the antisense-mediated repression approach targets specific transcript sequences and might be of limited use in whole plant systems that express complex protease complements encoded by different nucleic acid sequences, companion protease inhibitors may present a relatively broad activity spectrum and be useful to inhibit complete populations of functionally related proteases in host tissues [4, 13]. Companion inhibitors can be used either to protect the expressed proteins *ex planta* upon extraction or secretion [14, 15] or to increase their steady-state levels *in planta* before biomass harvesting [16–19].

Some years ago we described a procedure for the use of companion protease inhibitors to prevent recombinant protein loss in leaf crude protein extracts [20]. Here we describe procedures for the use of these inhibitors as in situ stabilizing partners for co-localized recombinant proteins, before their recovery from plant tissues. We use as an example the widely adopted expression host *Nicotiana benthamiana* [21] transiently co-expressing secreted mammalian proteins and cysteine protease inhibitors of the plant cystatin protein family [22].

## 2 Materials

### 2.1 Plants

1. Forty-two-day-old, greenhouse-grown *N. benthamiana* plants are used as an example in this article (*see* **Note 1**). Sowing and growth parameters for *N. benthamiana* plants used as protein expression platforms are detailed in refs. 23, 24.

### 2.2 Gene Constructs and Vectors

1. Vectors for *Agrobacterium*-mediated expression of the protein of interest and companion protease inhibitor, or a fusion of the two, are required. As examples, this chapter describes the human blood-typing IgG antibody C5-1 [25], the antifibrinolytic drug bovine aprotinin [26] and the human serpin hACT [27] as recombinant protein models, expressed alone or along with tomato cystatin SlCYS8 [28] as a companion protease inhibitor. All four proteins are expressed fused to a native or heterologous N-terminal signal peptide allowing for cellular secretion (*see* **Note 2**). Transgene constructs and binary vectors for *inplanta*expression are described in refs. 17, 29, 30.

### 2.3 Agrobacteria

1. *Agrobacterium tumefaciens* cells, strain AGL1 (*see* **Note 3**) harboring the above identified vectors are used as source bacteria for leaf agroinfiltration (*see* Subheadings 3.1.1 and 3.2.2, below).
2. Bacteria transformed with the pEAQexpress vector engineered to express the viral protein p19 [31] are used in parallel to suppress transgene silencing [32].
3. Bacteria carrying "empty" (no transgene insert) versions of the same vectors that are used as recipients for the protein-encoding transgenes are infiltrated as blank controls.

### 2.4 Special Laboratory Tools and Materials

1. Vacuum pump and dessicator for leaf agroinfiltration (*see* ref. 24 for a visual illustration).
2. Mini-Beadbeater-24 homogenizer (BioSpec Products, Bartlesville, OK, USA).
3. 2-mL screw cap microtubes (Sarstedt, Montréal, QC, Canada).
4. 3-mm tungsten carbide beads (Qiagen, Mississauga, ON, Canada).
5. Bio-Rad Mini-Protean III Electrophoresis Unit™ for protein gel electrophoresis (Bio-Rad, Mississauga, ON, Canada).
6. Bio-Rad Minitransfer Unit™ for protein electrotransfer to nitrocellulose sheets (Bio-Rad).
7. Hybond C nitrocelluluse sheets (GE Healthcare, Baie d'Urfé, QC, Canada).
8. Phoretix 2-D Expression software, v. 2005 for gel image analysis (NonLinear USA, Durham, NC, USA).
9. Standard reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (*see* refs. 33, 34).
10. Gel image scanner for protein band densitometry.
11. Temperature-controlled shaking incubator.
12. Refrigerated centrifuge.

13. Basic spectrophotometer.
14. Plant diurnal growth chamber.
15. Circular hole punch (~1-$cm^2$).
16. Aluminum foil.
17. Polyvinyl-polypyrrolidone.
18. Bradford protein assay kit (Bio-Rad).

### 2.5 Buffers and Other Solutions

Buffers and inocula are made up as aqueous solutions. Working buffers and step-by-step protocols for the widely used techniques of SDS-PAGE and immunoblotting are described in refs. 33, 34, respectively. For immunodetection, simple procedures involving alkaline phosphatase- or peroxidase-labeled secondary antibodies and appropriate reagents for protein band detection are usually provided by the suppliers.

1. Bacterium inocula: Glycerol stocks of *Agrobacterium* cells grown in liquid LB medium containing 50 μg/mL kanamycin and 100 μg/mL carbenicillin.
2. Leaf infiltration buffer: 10 mM MES-KOH (2-[*N*-morpholino] ethanesulphonic acid), pH 5.6, containing 10 mM $MgCl_2$ and 100 μM acetosyringone. Prepare fresh.
3. Leaf extraction buffer: 50 mM Tris–HCl, pH 7.5, containing 150 mM NaCl and the cOmplete protease inhibitor cocktail (one tablet per 10 mL of extraction buffer) (Roche, Laval, QC, Canada). Prepare fresh.

## 3 Methods

### 3.1 Co-expressed Companion Inhibitors

A procedure is here described to evaluate the effectiveness of a candidate companion inhibitor in protecting protease-susceptible recombinant proteins transiently expressed in leaves of *N. benthamiana* (*see* **Note 4**). In brief, the procedure consists of: (1) infiltrating *N.benthamiana*leaves with agrobacterial suspensions harboring the appropriate transgene-encoding vectors; (2) recovering leaf proteins after a 6-day incubation phase in growth chamber for heterologous protein expression; and (3) assessing the impact of companion inhibitor expression on accumulation and integrity of the recombinant protein. Light and heavy chains of the C5-1 antibody expressed alone or in combination with tomato cystatin SlCYS8 are taken as an example for demonstration purposes (Fig. 1).

#### 3.1.1 Bacterial Growth and Leaf Agroinfiltration

1. Grow bacterium inocula to stable phase under agitation at 28 °C in LB medium containing 50 μg/mL kanamycin and 100 μg/mL carbenicillin.

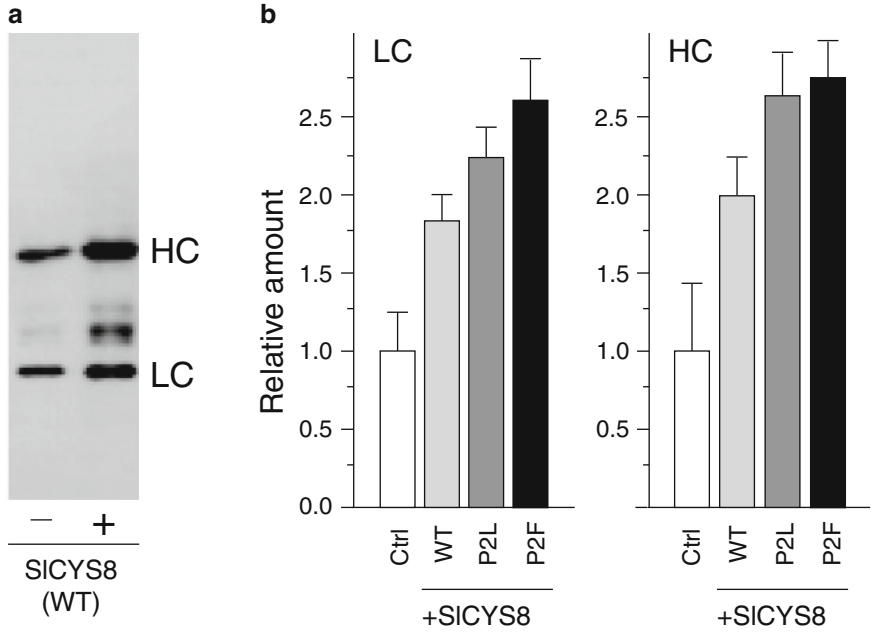


**Fig. 1** Transient expression of the human blood-typing IgG antibody C5-1 in leaves of *Nicotiana benthamiana*. The antibody was expressed alone or along with different forms of tomato cystatin SlCYS8 [28]. (**a**) The C5-1 light (LC) and heavy (HC) chains were immunodetected using appropriate primary and secondary antibodies (*see* ref. 19), following 12 % (w/v) SDS-PAGE in reducing conditions and electrotransfer onto a nitrocellulose membrane. (**b**) The antibody chain signals were quantified by densitometry and compared to signal intensities measured for the antibody chains expressed alone (*Ctrl*; arbitrary control value of 1.0). Each bar is the mean of three independent (biological replicate) values ± SE

2. Centrifuge growth cultures at room temperature for 5 min at 2000 × *g*, and resuspend bacterial pellets in leaf infiltration buffer to an $OD_{600}$ of ~0.4.
3. Blend equal volumes of bacterial suspensions harboring the expression vectors for C5-1, silencing suppressor p19 and companion inhibitor SlCYS8, and let stand for 2 h at room temperature before infiltration. A control inoculum is prepared in parallel by mixing bacteria for C5-1 and protein p19 with bacteria carrying the recipient (empty) vector used for SlCYS8 construct engineering.
4. Immerse an *N. benthamiana* plant upside down in a vacuum dessicator reservoir filled with appropriate bacterial mixture (*see* **Note 5**), after covering the plant culture pot with aluminum foil to prevent medium contamination with soil particles. Take care to prevent mechanical damage to leaf tissues.
5. Put the dessicator lid on the reservoir, turn on the vacuum pump, and draw the vacuum until all bubbles are released from the surface of the leaves.

6. Slowly open the dessicator valve to release pressure and allow entrance of agrobacteria into the apoplast interstitial space of the submerged leaves.
7. Remove the plant from the desiccator, return it to an upright position, and repeat **steps 4**–7 for the other plants to infiltrate.

### 3.1.2 Protein Transient Expression and Recovery

1. Incubate infiltrated plants for 6 days at 20 °C in a growth chamber, under a 16 h:8 h light/dark photoperiod, to allow for cell transfection and transient protein expression (*see* **Note 6**).
2. Collect three ~1-cm² foliar disks on each test plant using a circular hole punch, and put them in a 2-mL microtube containing one tungsten carbide bead for tissue disruption and 10 mg polyvinyl-polypyrrolidone to neutralize phenolics. Tissue material must be harvested from leaves of comparable developmental age to avoid confounding effects due to age-dependent transcription and protein turnover rates *in planta* [19]. Leaf disks can be quick-frozen in liquid nitrogen and stored for several weeks at –80 °C if not used immediately.
3. Add 400 µL of cold leaf extraction buffer to each tube and shake leaf disks for 30 s using a Mini-Beadbeater apparatus.
4. Let mixtures stand on ice for 1 min, and repeat **step 3** once to complete tissue disruption.
5. Centrifuge mixtures at 4 °C for 10 min at 20,000 × *g* and recover the supernatant for further analysis.
6. Assay total soluble proteins according to Bradford [35], using the Bio-Rad protein assay kit or other standard reagents.
7. Standardize protein content among the extracts by adding leaf extraction buffer.

### 3.1.3 Estimation of Protein Yield Improvement

1. Resolve leaf proteins by SDS-PAGE in reduced conditions using the Bio-Rad Mini-Protean III Electrophoresis Unit™ (*see* ref. 33 for a generic procedure). Load 10 µg of leaf protein [in up to 25 µL] in wells of a 1.0-mm thick gel, and perform electrophoretic migration at 150 V until the bromophenol blue tracking dye reaches the bottom of the gel.
2. Transfer resolved proteins onto a nitrocellulose Hybond C membrane using the Bio-Rad Minitransfer Unit™ (*see* ref. 34 for a generic procedure).
3. Reveal the light and heavy chains of C5-1 by immunodetection with appropriate antibodies and reagents for color development [19] (Fig. 1a).
4. Estimate the relative intensity of C5-1 light and heavy chains in test and control extracts by densitometry of the immunoblot signals, using a Microtek ScanMaker II digitalizer and the image analysis software Phoretix 2-D (Fig. 1b) (*see* **Note 7**).

### 3.2 Translational Fusion Inhibitors

A recently described variation of the companion protease inhibitor approach consists of expressing the recombinant inhibitor fused to the protein of interest [29]. This approach is well suited in those cases where expression level of the recombinant protein is negatively affected by companion inhibitor co-expression (presumably due to competition for cellular resources [29]), and/or when structural robustness of the inhibitor confers increased stability to the recombinant protein *in planta*. The general scheme for this approach consists of: (1) designing a stable protein–inhibitor translational fusion; and then (2) expressing the transgene hybrid sequence and monitoring recombinant protein expression as described above for the co-expressed inhibitor approach (Subheading 3.1). Bovine aprotinin and hACT expressed alone, together with tomato cystatin SlCYS8, and/or fused to SlCYS8 are shown as an example for demonstration purposes (Fig. 2).

#### 3.2.1 Translational Fusion Design

Recombinant fusion proteins are engineered by simply ligating DNA coding sequences of the proteins, including in most cases a peptide linker sequence between them to avoid undesired steric interference between the two fusion partners [36]. Fusion proteins should be designed based on current knowledge about the

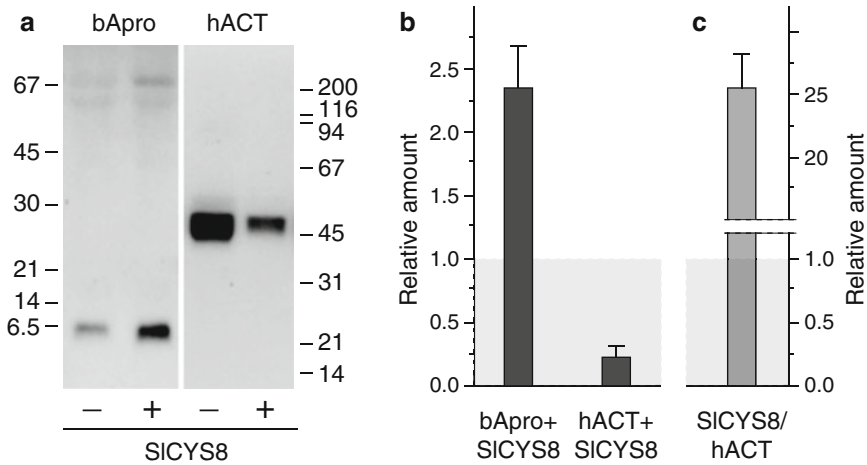


**Fig. 2** Transient expression of bovine aprotinin (bApro) and human $\alpha_1$-antichymotrypsin (hACT) in *Nicotiana benthamiana*leaves. The proteins were expressed alone, co-expressed with tomato cystatin SlCYS8 (bApro + SlCYS8, hACT + SlCYS8), or fused along with a Gly-rich flexible peptide linker to the C-terminal end of the tomato inhibitor (SlCYS8/hACT) [29]. (**a**) The two recombinant proteins were immunodetected using appropriate primary and secondary antibodies [29, 30] following 16 % (w/v) Tricine-SDS-PAGE (bApro) or 12 % (w/v) SDS-PAGE (hACT) in reducing conditions and electrotransfer onto a nitrocellulose membrane. *Numbers* indicate the mass of commercial protein markers (kDa). (**b**) Relative levels of aprotinin and hACT expressed along with SlCYS8 were estimated by densitometric analysis of the immunoblot signals, compared to the proteins expressed alone (*Grey area*; arbitrary control value of 1.0). (**c**) hACT fused to SlCYS8 was quantified by an ELISA procedure, and compared to hACT expressed alone (*Grey area*; arbitrary value of 1.0) (adapted from ref. 29). Each bar on panels **b** and **c** is the mean of three independent (biological replicate) values ± SE

structural features of each fusion partner, in such a way as to maximize the distance and/or to minimize interactions between their functionally relevant loops or domains. Peptide linkers that have various structural properties should be assessed empirically for every recombinant protein/companion inhibitor combination, to identify a linker allowing for the translational fusion to remain stable *in planta* [29]. Table 1 lists a number of presumably stable, non-cleavable peptide linkers used to separate fusion protein partners in *N.benthamiana*leaves. Figure 2 illustrates the differential impact of freely expressed tomato cystatin SlCYS8 on steady-state levels of bovine aprotinin and hACT co-expressed in leaves of *N. benthamiana*, and the relevance of using SlCYS8 as an N-terminal fusion partner to achieve high-level expression of the human serpin *inplanta*.

### 3.2.2 Estimation of Protein Yield Improvement

The overall scheme for assessing the potential of a companion inhibitor fusion partner in increasing recombinant protein yield in *N.benthamiana*leaves consists of, as described above for freely co-expressed inhibitors (Subheading 3.1): (1) infiltrating *N.benthamiana*leaves with agrobacterial suspensions harboring the appropriate transgene-encoding vectors; (2) recovering leaf proteins after a 6-day incubation phase for heterologous expression; and (3) assessing the impact of companion inhibitor expression on accumulation and integrity of the recombinant protein. Step-by-step procedures detailed in Subheadings 3.1.1, 3.1.2 and 3.1.3 can be followed as described, except for the use of a single bacterial line harboring the inhibitor–recombinant protein fusion sequence (**step 4** of Subheading 3.1.1), and the use of appropriate [e.g. anti-hACT or anti-aprotinin] antibodies for recombinant protein detection (**steps 3** and **4** of Subheading 3.1.3).

**Table 1**
**Examples of non-cleavable peptide linkers used to separate protein partners of translational fusions transiently expressed in leavesof *Nicotiana benthamiana***

| Type | Sequence | References |
|---|---|---|
| Flexible (Gly-based) | Ser–$(Gly–Gly–Gly–Gly–Ser)_2$–Gly | [29] |
| | $(Gly–Gly–Gly–Gly–Ser)_3$ | [37] |
| | $(Gly–Gly–Gly–Ser)_3$ | [38] |
| | Gly–Gly–Gly–Ser–Gly–Asn–Ser | [39] |
| | Ser–Gly–Pro–Ser | [40] |
| | Gly–Pro–Gly–Pro | [40, 41] |
| Rigid | $(Glu–Ala–Ala–Ala–Lys)_3$ | [29, 42] |

## 4 Notes

1. *N. benthamiana* is used both as a convenient system for testing the stabilization of poorly stable proteins, and as a widely adopted expression host in plant molecular pharming [21]. The companion inhibitor approach works for different plants amenable to agroinfiltration, as observed notably with different cultivars of tobacco, *Nicotiana excelsior* and related species (unpublished data). It also works with stably transformed plants, such as potato and tobacco engineered to express inhibitors active against target proteases of different mechanistic classes (*see* refs. 16, 18).
2. Recombinant proteins targeted to the cell secretory pathway are taken here as an example, given the practical relevance of many secreted proteins considered for human disease treatment or diagnostics. The companion inhibitor approach may also be used to stabilize proteins in alternative cell compartments, as illustrated with a cytosol-targeted glutathione reductase in leaves of a rice cystatin-expressing tobacco line [18], a cytosol/nucleus-targeted form of hACT in leaves of a tomato cathepsin D inhibitor-expressing potato line [16], or an SlCYS8–hACT translational fusion in the cytosol of *N. benthamiana* leaf cells [29].
3. Other agrobacterial strains may be used for transient protein expression in *N. benthamiana* leaves, including strains LBA4404, C58C1, EHA105, AGL0, and GV3101.
4. Different inhibitors have been tested to prevent recombinant protein degradation in plant systems, including the Ser-type inhibitors bovine aprotinin and soybean Bowman-Birk inhibitor [14, 15], the Kunitz inhibitor of Asp and Ser proteases tomato cathepsin D inhibitor [14, 16, 17], and Cys-type inhibitors of the plant cystatin family such as rice cystatin I, tomato cystatin SlCYS8 and tomato cystatin SlCYS9 [17–19]. The choice of an effective companion inhibitor remains essentially empirical at this stage, given our still limited knowledge about the specificity of plant proteases and the identity of protease-susceptible sites in most recombinant proteins. Logical schemes are described in refs. 17, 20 to perform a basic characterization of a host plant's endogenous protease activities, to predict the eventual stability of a recombinant protein in a given plant system, and to identify a potentially effective companion inhibitor for protein protection.
5. A syringe agroinfiltration procedure may also be used to test the companion inhibitors [16, 17]. Detailed protocols for *N. benthamiana* leaf syringe agroinfiltration are given in refs. 23, 24.

6. Shorter, e.g. 4- or 5-day incubation periods may be preferable in those cases where very high level expression or toxicity of the recombinant protein induce early necrosis of leaf tissues.
7. A simple, widely applicable quantitative immunodetection approach is suggested here as a first step to quickly generate basic comparative data on yield and integrity of the recombinant protein expressed alone or along with the candidate companion inhibitor. Complementary analytical tools should be used in parallel, such as for instance an ELISA assay for a more reliable quantitation of the protein, or an in vitro enzymatic assay to confirm its functional integrity.

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# Part III

## Extraction and Scale-Up

# Chapter 9

# Production of Recombinant Cholera Toxin B Subunit in *Nicotiana benthamiana* Using GENEWARE® Tobacco Mosaic Virus Vector

**Lauren Moore, Krystal Hamorsky, and Nobuyuki Matoba**

## Abstract

Here, we describe a method to produce a recombinant cholera toxin B subunit in *Nicotiana benthamiana* plants (CTBp) using the GENEWARE® tobacco mosaic virus vector system. Infectious transcripts of the vector RNA are generated *in vitro* and inoculated on *N. benthamiana* seedlings. After 11 days, CTBp is extracted in a simple tris buffer at room temperature. No protease inhibitor is required. The leaf homogenate is treated with mild heat and a pH shift to selectively precipitate host-derived proteins. CTBp is purified to >95 % homogeneity by two-step chromatography using immobilized metal affinity and ceramic hydroxyapatite resins. This procedure yields on average 400 mg of low-endotoxin CTBp from 1 kg of fresh leaf material.

**Key words** Plant-made pharmaceutical, *Nicotiana benthamiana*, Plant virus vector, Ceramic hydroxyapatite chromatography, Cholera toxin B subunit

## 1 Introduction

Cholera toxin B subunit (CTB) is a 55-kDa homopentameric, non-toxic, GM1-ganglioside-binding subunit of the holotoxin. Due to potent mucosal immunogenicity, the protein induces robust systemic and mucosal antibody responses upon oral administration. These antibodies are capable of neutralizing cholera holotoxin [1], the virulence factor responsible for the severe diarrhea symptom of cholera. Hence, a recombinant CTB produced by bacterial fermentation is used as a component of the internationally licensed oral cholera vaccine Dukoral®. In addition to mucosal immunostimulatory effects, a number of studies have shown that CTB elicits anti-inflammatory and regulatory T-cell responses, suggesting that the protein may be utilized for the development of immunotherapeutics against allergies and autoimmune diseases (reviewed in: [2, 3]).

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Plant-based expression systems provide an alternative biomanufacturing platform for recombinant proteins, with potential advantages in cost-effectiveness and scalability [4]. We have developed an expression and purification scheme to produce a recombinant CTB containing an Asn4 → Ser mutation and a C-terminal Ser-Glu-Lys-Asp-Glu-Leu (SEKDEL) extension in *Nicotiana benthamiana* plants (CTBp) utilizing the GENEWARE® tobacco mosaic virus (TMV) vector expression system. The Asn4 → Ser mutation eliminates eukaryote-specific *N*-glycosylation at Asn4 upon expression in plant cells, while the KDEL endoplasmic reticulum retention signal increases recombinant protein accumulation. These amino acid sequence modifications do not affect CTB's GM1-ganglioside binding affinity, physicochemical stability, or oral immunogenicity [5].

The GENEWARE®expression system takes advantage of the functions of fully infectious TMV, including cell-to-cell and systemic movement activities that are mediated by movement protein (MP) and coat protein (CP), respectively. GENEWARE®also exploits the strength and duration of the viral subgenomic promoter, and its ability to reprogram the translational priorities of the plant host cells so that virus-encoded proteins are synthesized at high levels, similar to TMV CP [6, 7].

We have developed a simple extraction and two-step purification scheme to successfully produce high-purity CTBp from fresh leaf material of *N. benthamiana* plants within 11 days of inoculation. After extraction, a series of mechanical processes with mild heat treatment (55 °C) and pH adjustment are employed to remove host-derived impurities and thereby simplify downstream purification. Immobilized metal affinity chromatography (IMAC) and ceramic hydroxyapatite (CHT) chromatography are used to further purify CTBp. CTB is known to bind to immobilized $Ni^{2+}$ ions through internal histidine resides [8]; therefore CTBp can be purified to high purity using IMAC. CHT is a multimodal resin that utilizes cation exchange and metal affinity, and is known to offer unique selectivity and often separates biomolecules that appear homogenous using other chromatographic methods.

The expression and purification scheme described herein allows for an easy and efficient way to manufacture CTBp with low endotoxin contamination, which may facilitate the research and development of mucosal vaccines and immunotherapeutics.

## 2 Materials

Prepare all solutions using ultrapure MilliQ water (MilliQ Synthesis, Millipore, 18.2 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise).

### 2.1 RNA Transcription

1. GENEWARE®vector DNA plasmid (Kentucky Bioprocessing, Inc.) containing the coding sequence for CTBp with rice α-amylase signal peptide (obtained via standard molecular biology/subcloning procedures using *Pac* I/*Avr* II restriction sites based on pNM156 [5]).
2. mMessage mMachine® Kit (Ambion).
3. 0.7 % standard agarose gel, and materials for gel electrophoresis.

### 2.2 N. benthamiana Inoculation

1. *N. benthamiana* plants seeded in 4 in. pots.
2. Diurnal growth chamber with constant humidity (23 °C, >50 % humidity, 16 h day/8 h night).
3. Frog Eye Soup (FES): 112 mM glycine, 60 mM potassium phosphate dibasic, 22 mM sodium pyrophosphate decahydrate, 1 % bentonite, 1 % celite. Add approximately 250 mL water to a 1 L graduated cylinder or glass beaker. Weigh 2.52 g glycine, 3.136 g potassium phosphate dibasic, 3.0 g sodium pyrophosphate decahydrate and transfer to the cylinder. Stir to dissolve. Add water to a final volume of 300 mL then add 3 g bentonite and 3 g celite. Store FES at 4 °C (*see* **Note 1**).

### 2.3 CTBp Extraction

1. Extraction buffer: 20 mM Tris–HCl, 500 mM sodium chloride, 20 mM ascorbic acid, 10 mM sodium metabisulfite, pH 5.0. Weigh 2.42 g tris(hydroxymethyl)aminomethane, 29.22 g of sodium chloride, 3.52 g of ascorbic acid, 1.9 g of sodium metabisulfite and transfer to a cylinder containing 900 mL of water (*see* **Note 2**). Stir to dissolve. Adjust the pH to 5.0 (*see* **Note 3**) using 1 M NaOH. Bring the volume to 1 L with water.
2. Industrial blender.
3. Cheese cloth.
4. Miracloth.
5. 250 mL Centrifuge Bottles.
6. Water bath set at 55 °C.
7. 10 N NaOH.
8. Centrifuge.
9. 0.22 μM Bottle top filter unit.

### 2.4 Purification

1. IMAC Buffer A: 20 mM Tris–HCl, 500 mM sodium chloride, pH 8.0. A stock solution of 0.1 M tris(hydroxymethyl)aminomethane is made by using 12.1 g/L and a stock solution of 0.1 M hydrochloric acid is made by diluting concentrated hydrochloric acid. Mix 100 mL of 0.1 M tris(hydroxymethyl) aminomethane and 54.6 mL of 0.1 M hydrochloric acid. Bring the volume to 400 mL with water to make 50 mM tris buffer.

Adjust the pH to 8.0 using 1 M NaOH. Weigh 29.22 g of sodium chloride and transfer it to the cylinder containing 400 mL of 50 mM tris buffer. Add water to a volume of 900 mL. Stir to dissolve. Mix and adjust pH if needed using 1 M HCl. Add water to a volume of 1 L.

2. IMAC Buffer B: 20 mM Tris–HCl, 500 mM sodium chloride, 150 mM imidazole, pH 8.0. A stock solution of 0.1 M tris(hydroxymethyl)aminomethane is made by using 12.1 g/L and a stock solution of 0.1 M hydrochloric acid is made by diluting concentrated hydrochloric acid. Mix 100 mL of 0.1 M tris(hydroxymethyl)aminomethane and 54.6 mL of 0.1 M hydrochloric acid. Add water to 400 mL to make 50 mM tris buffer. Adjust the pH to 8.0 using 1 M NaOH. Weigh 29.22 g of sodium chloride and 10.2 g of imidazole and transfer them to the cylinder containing 400 mL of 50 mM tris buffer. Add water to a volume of 900 mL. Stir to dissolve. Mix and adjust pH using 1 M HCl if needed. Add water to 1 L.
3. CHT Buffer A: 10 mM Tris–HCl, 5 mM sodium phosphate, pH 8.0. A stock solution of 0.1 M tris(hydroxymethyl)aminomethane is made by using 12.1 g/L and a stock solution of 0.1 M hydrochloric acid is made by diluting concentrated hydrochloric acid. Mix 100 mL of 0.1 M tris(hydroxymethyl) aminomethane and 54.6 mL of 0.1 M hydrochloric acid. Add water to 400 mL to make 50 mM tris buffer. Adjust the pH to 8.0 using 1 M NaOH. A stock solution of 0.1 M sodium phosphate monobasic is made using 13.8 g/L and a stock solution of 0.1 M sodium phosphate dibasic (heptahydrate) is made using 26.8 g/L. Mix 5.3 mL of sodium phosphate monobasic and 94.7 mL of sodium phosphate dibasic. Add water to 200 mL to make 50 mM phosphate buffer. Adjust the pH to 8.0 using 1 M HCl. In a cylinder, mix 200 mL of 50 mM Tris–HCl with 100 mL of 50 mM phosphate buffer. Add water to a volume of 900 mL. Stir to dissolve. Mix and adjust pH if needed using 1 M NaOH. Add water to 1 L.
4. CHT Buffer B: 10 mM Tris–HCl, 250 mM sodium phosphate, pH 8.0. A stock solution of 0.1 M tris(hydroxymethyl)aminomethane is made by using 12.1 g/L and a stock solution of 0.1 M hydrochloric acid is made by diluting concentrated hydrochloric acid. Mix 100 mL of 0.1 M tris(hydroxymethyl) aminomethane and 54.6 mL of 0.1 M hydrochloric acid. Add water to 400 mL to make 50 mM tris buffer. Adjust the pH to 8.0 using 1 M NaOH. A stock solution of 1 M sodium phosphate monobasic is made using 138 g/L and a stock solution of 1 M sodium phosphate dibasic (heptahydrate) is made using 268 g/L. Mix 13.25 mL of sodium phosphate monobasic and 236.75 mL of sodium phosphate dibasic. Add water to a volume

of 500 mL to make 0.5 M phosphate buffer. Adjust pH to 8.0 using 1 M HCl. In a cylinder, mix 200 mL of 50 mM Tris–HCl with 500 mL of 0.5 M phosphate buffer. Add water to a volume of 900 mL. Mix and adjust pH if needed using 1 M NaOH. Add water to 1 L.

5. ATKApurifier 100 liquid chromatography system (General Electric Company), or similar.
6. Talon® Superflow Metal Affinity Resin (Clontech).
7. CHT™ Ceramic Hydroxyapatite, Type I, 40 μM (Bio-Rad).
8. XK 26/20 column (GE Healthcare Life Sciences).
9. XK 16/20 column (GE Healthcare Life Sciences).
10. 15 % Tris-Glycine gels (Lonza) and materials for SDS-PAGE.

### 2.5 Formulation

1. Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium.
2. Amicon Ultra centrifugal filter, 30 K (Millipore).
3. 15 % Tris-Glycine gels (Lonza) and materials for reducing and non-reducing SDS-PAGE.

## 3 Methods

### 3.1 Capped Transcription Reaction Assembly

1. Synthesize 7-methyl guanosine capped RNA in vitro using the mMessage mMachine kit, according to the manufacturer's directions. For capped transcription reaction assembly, use 1 μg GENEWARE® vector DNA plasmid (not linearized), without [α-32P]UTP tracer, and incubate the reaction for 2 h at 37 °C. Add 1 μL of TURBO DNase, mix well and incubate 15 min at 37 °C.
2. Run a standard 0.7 % agarose DNA gel to check the transcript. A band should be visible at approximately 10,000 bp.

### 3.2 Inoculation of N. benthamiana Plants with GENEWARE® Transcripts

1. For each plant to be infected, create 100 μL of inoculum containing 0.1 μL of transcript (obtained from Subheading 3.1) and 99.9 μL of FES (*see* **Note 1**).
2. Inoculate two leaves (*see* **Note 4**) per plant by pipetting 25 μL of inoculum at two different locations per leaf (Fig. 1a).
3. While wearing gloves, hand rub the inoculum into the leaves (Fig. 1b).
4. Keep plants in a growth chamber at 23 °C and >50 % humidity, with a 16 h daytime/8 h nighttime schedule, for 11 days. Water plants immediately after infection and then every other day with tap water.

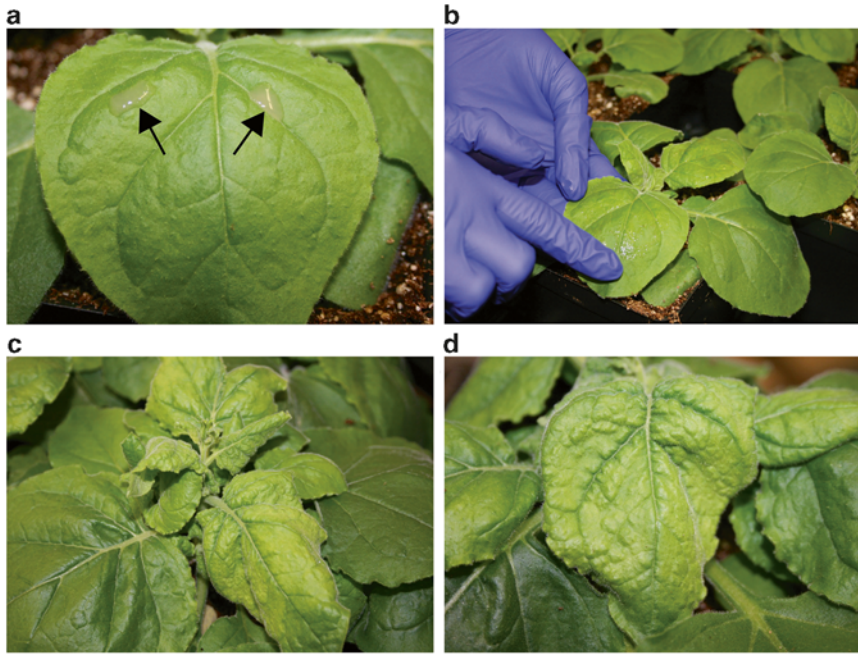


**Fig. 1** Vector inoculation and infection of *N. benthamiana* plants with GENEWARE® transcripts. (**a**) Inoculation of the third and fourth leave down from the apical meristem. A hundred microliters of inoculum contains 0.1 μL of transcript and 99.9 μL of FES. An aliquot of 25 μL is pipetted (*black arrows*) in two different locations per leaf. (**b**) The inoculum is hand rubbed into the leaves. (**c** and **d**) TMV infection is manifested by the mosaic, crinkled phenotype

### *3.3 Extraction of CTBp from N. benthamiana Plants*

The extraction procedure is performed at room temperature.

1. At 11 days post inoculation, harvest the infected leaves (*see* **Note 5**; Fig. 1c, d) expressing CTBp by cutting the leaves at the base of the stem and measure the total mass.
2. Immediately (*see* **Note 6**) add 2 mL of extraction buffer for every 1 g of leaf tissue, and mix in an industrial blender until the mixture is homogenous.
3. To remove the majority of the plant tissue, filter the extract through two layers of cheese cloth on top of one layer of miracloth. Collect the supernatant ("green juice") in sterile 250 mL centrifuge bottles.
4. In the centrifuge bottles, heat the extract for 25 min at 55 °C in a water bath (*see* **Note 7**) to allow host-derived proteins and starches to fall out of solution.
5. Centrifuge at 15,000 × *g* for 15 min at 4 °C.
6. Pour supernatant into a clean beaker. While mixing, pH the extract up to pH 8.0 by using 10 N sodium hydroxide. This pH shift facilitates the precipitation of host-derived impurities.

7. Centrifuge at 15,000 × *g* for 15 min at 4 °C.
8. Pass the supernatant through a 0.2 μM bottle top filter using a house vacuum.

### 3.4 Purification of CTBp from Clarified Extract

The purification procedures are performed at room temperature and CTBp purified product is stored at 4 °C until use.

#### 3.4.1 IMAC

1. Pack the Talon Superflow Metal Affinity Resin in an XK 26/20 column to a 10 mL bed volume on an AKTA purifier.
2. Equilibrate the column with 10 column volumes (CV) of IMAC buffer A at 10 mL/min.
3. Load the clarified extract at a flow rate of 5.0 mL/min followed by a 10 CV wash with IMAC buffer A at 10 mL/min.
4. Elute proteins using a single-step gradient to 100 % IMAC buffer B for 5 CV at 10 mL/min. Collect 10 mL fractions.
5. Analyze each fraction for CTBp purity by SDS-PAGE using 15 % Tris-Glycine gels.
6. Combine pure CTBp-containing fractions for CHT purification (*see* **Note 8** and Fig. 2a).

#### 3.4.2 CHT

1. Pack the CHT Hydroxyapatite, Type I, 40 μm resin in an XK 16/20 column to a 10 mL bed volume on an AKTA purifier.
2. Equilibrate the column with 10 CV of CHT buffer A at 10 mL/min.
3. Load the CTBp IMAC elution at a flow rate of 5.0 mL/min followed by a 10 CV wash with CHT buffer A at 10 mL/min.
4. Elute proteins using a gradient from 0 to 100 % CHT buffer B over 20 CV at 10 mL/min. Collect 10 mL fractions.
5. Analyze each fraction for CTBp purity by SDS-PAGE using 15 % Tris-Glycine gels (Fig. 2a).
6. Combine pure CTBp-containing fractions.

### 3.5 Formulation of CTBp

1. Ultrafiltrate and diafiltrate the CTBp (combined fractions from CHT purification in Subheading 3.4.2) into sterile Dulbecco's PBS (DPBS) using Amicon Ultra-15 30,000 MWCO centrifugal devices according to the manufacturer's instructions.
2. To determine the concentration of CTBp solution, measure the absorbance at 280 nm by UV-Vis spectroscopy. Use DPBS as a blank. Divide the absorbance value by CTBp's theoretical extinction coefficient at 280 nm of 0.7660 $(mg/mL)^{-1} cm^{-1}$.
3. Analyze the purity and pentamer formation of purified CTBp by use of an overloaded (5 μg) Coomassie-stained sodium

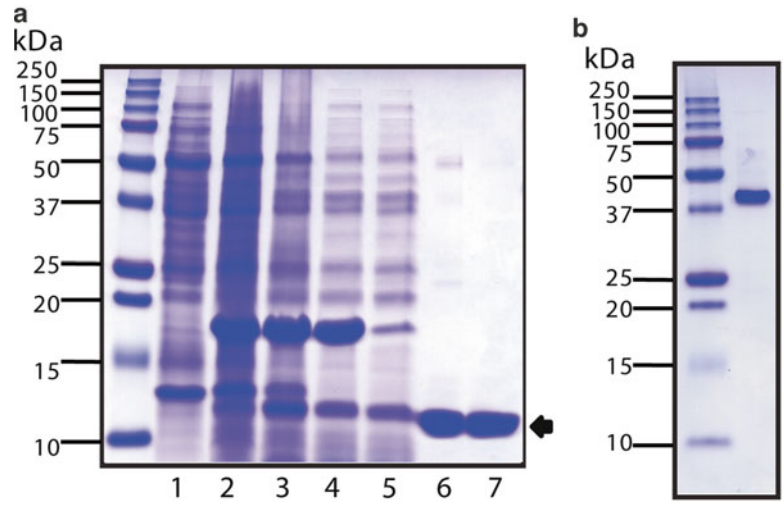


**Fig. 2** Expression, clarification, and purification of CTBp. (**a**) Reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of crude extracts and purified CTBp. Lane 1: Non-infiltrated leaf extract (extracted the same way as CTBp in Subheading 3.3 to create non-infiltrated green juice). Lane 2: CTBp expressing leaf extract ("green juice"). Lane 3: CTBp extract after heating at 55 °C for 25 min. Lane 4: CTBp extract after pH adjustment up to pH 8.0. Lane 5: CTBp after centrifugation and filtration (IMAC load). Lane 6: Five micrograms of CTBp purified by IMAC only. Lane 7: Five micrograms of CTBp purified by IMAC plus CHT. CTBp monomer is shown at 12.3 kDa, indicated by a *black arrow*, and was purified to >95 % purity after IMAC only as well as IMAC plus CHT. A major band at ~18 kDa in Lanes 2–4 corresponds to TMV CP. (**b**) Non-heat non-reducing SDS-PAGE analysis of 5 μg of CTBp purified by IMAC plus CHT. After both purification steps, CTBp was purified to >95 % purity while retaining pentamer formation (the protein migrates faster than the theoretical molecular size of approximately 60 kDa under the non-denaturing conditions)

dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-heat non-reducing conditions using 15 % Tris-Glycine gels (*see* **Note 9**; Fig. 2a, b).

## 4 Notes

1. FES has settlement during storage. Mix well immediately prior to use to make the solution homogeneous.
2. Buffers with ascorbic acid and sodium metabisulfite are made immediately prior to use to prevent oxidation of the ascorbic acid and sodium metabisulfite.
3. The pH of the extraction buffer is prepared outside the typical buffering range of Tris–HCl. This is to incorporate the pH transition step prior to IMAC purification (*see* Subheading 3.3,

**step 6**), which allows for the precipitation of starches and thereby aids in the downstream column purification process. We have found that during the short period of extraction the pH does not change drastically. The "green juice" ends up with a pH of approximately 5.3.

4. The two leaves chosen for inoculation are the third and fourth leaves down from the apical meristem.
5. Inoculated leaves are distinguishable by the mosaic, crinkled phenotype.
6. Once extracted, we have found that it is best to clarify and purify immediately. Leaving the extract at 4 °C overnight or freezing at −20 °C results in a significant loss of CTBp.
7. Start timing the 25 min when the extract reaches 55 °C. Generally, it takes approximately 20 min for the extract to reach 55 °C.
8. CTBp is purified to >95 % purity after IMAC purification (*see* Fig. 2a). The additional CHT step is performed to remove residual small molecules from the plant extract.
9. We routinely obtain approximately 400 mg of purified CTBp from 1 kg of fresh leaf material using the procedure described here. In addition to SDS-PAGE analysis, GM1-ganglioside-binding affinity can be confirmed using GM1-ganglioside capture enzyme-linked immunosorbent assay or surface plasmon resonance [5].

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# Chapter 10

# Total Soluble Protein Extraction for Improved Proteomic Analysis of Transgenic Rice Plant Roots

**Manish L. Raorane, Joan O. Narciso, and Ajay Kohli**

## Abstract

With the advent of high-throughput platforms, proteomics has become a powerful tool to search for plant gene products of agronomic relevance. Protein extractions using multistep protocols have been shown to be effective to achieve better proteome profiles than simple, single-step extractions. These protocols are generally efficient for above ground tissues such as leaves. However, each step leads to loss of some amount of proteins. Additionally, compounds such as proteases in the plant tissues lead to protein degradation. While protease inhibitor cocktails are available, these alone do not seem to suffice when roots are included in the plant sample. This is obvious given the lack of high molecular weight (HMW) proteins obtained from samples that include root tissue. For protein/proteome analysis of transgenic plant roots or of seedlings, which include root tissue, such pronounced protein degradation is especially undesirable. A facile protein extraction protocol is presented, which ensures that despite the inclusion of root tissues there is minimal loss in total protein components.

**Key words** Protein, Proteomics, Proteases, Root, Rice, Protocol

## 1 Introduction

Protein extraction from plant tissues is a crucial step in the study of plant recombinant proteins and proteomics, which is one of the rapidly growing fields of molecular biology [1]. Plants contain a wide range of proteins with diverse properties in terms of charge, susceptibility to proteolysis, ligand interactions, and subcellular protein localization. Specific extraction and purification protocols are required for enriching a set of proteins such as the cytoplasmic, organellar or membrane-bound proteins; yet extractions are complicated by the presence of a rigid cell wall and several interfering compounds such as proteases, pigments, tannins, and phenolics. A single protocol is therefore incapable of capturing the complete proteome [2, 3] and may even be incapable of capturing all of the recombinant proteins in a plant with multiple transgenes. Such plants may produce multi-subunit proteins of pharmaceutical,

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commercial, and industrial value; or multiple proteins required for metabolism, adaptation to various environments, and tolerance against both abiotic and biotic stresses. These may be expressed in the aerial parts of the plant or specifically in the roots, where degradation by proteases is especially pronounced; or in the case of analysis of seedlings, roots may be included in the tissue sample.

Issues with extraction can also affect estimated accumulation of recombinant proteins relative to total proteinif a large component of the proteome is not captured in the extraction. Incomplete extraction is particularly concerning for plants that contain multiple transgenes, as protein-level analyses may be required to appreciate the result of interactive gene products, whereby one gene product may feed into the action of another. Moreover, transcript abundance does not always reflect protein abundance, due to variations in posttranslational modifications, stability, and turnover rates. Thus, it is important to use protein extraction methods that will present as real a picture as possible of the amount of transgenic proteins in relation to each other and to other endogenous proteins.

Various procedures have been established for the extraction of nuclear, chloroplast, cell wall, and plasma membrane proteins. Some commonly used methods include Tris-buffer extraction, phenol extraction coupled with ammonium acetate precipitation, and trichloroacetic acid (TCA)-acetone precipitation. Each of these has its advantages and limitations, depending on factors such as the type of plant tissues, desired protein yield, and downstream analysis of the extracted proteins. The TCA-acetone procedure effectively denatures proteases, but does not remove other interfering substances such as carbohydrates and polyphenols [4]. In addition, the difficulty in resolubilizing the protein pellet can cause problems for running 2-dimensional gel electrophoresis (2D-GE) or subsequent proteomics. In contrast, phenol extraction [5] is the preferred method for 2D-GE: it generally results in better spot resolution and minimal streaking on the gels, in addition to extracting a higher number of proteins [6]. The major drawbacks in the phenol extraction method are its toxicity and that it is time-consuming [7]. The third method, which requires minimum extraction steps, is the Tris-buffer extraction buffer with protease inhibitor cocktail (PIC). However, its simplicity is marred by the fact that no generic protease inhibitors are available [8]. Here, we present a method of protein extraction based on Tris-buffer extraction with PIC, but including a heat-treatment step to remove a larger number of proteases. This method, which is relatively fast and nontoxic, can extract soluble, unbound proteins that are suitable for 2D-GE and proteomics.

The method was developed for extraction from plant roots, which have been shown to exude proteases unique in reaction kinetics and stability. Root proteases are proposed to play an

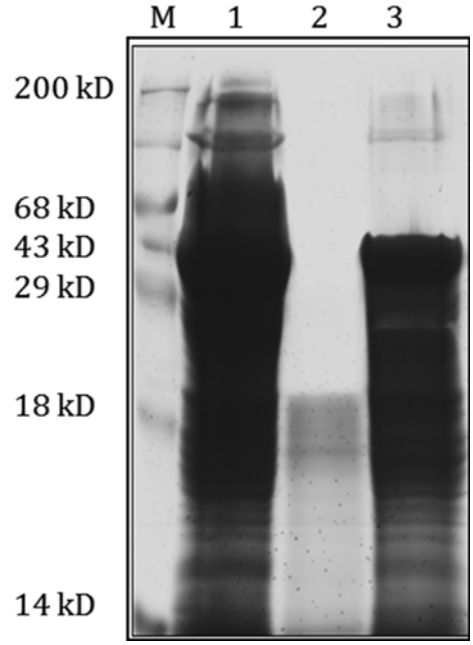


**Fig. 1** 1D-GE profiles of protein extractions from rice leaves (1), roots (2), and both leaf and root together, without boiling (3). All extractions contained 6 μL PIC. Relative lack of HMW proteins in both lanes 2 and 3 as compared to those in lane 1 implicates a protein degradation component present in roots. *M* molecular weight marker

important role in the cyclic transformation of nitrogen within plant roots and/or at the root-soil interface, and the levels of these proteases could be species or cultivar specific [9]. They also play a crucial role in plant nitrogen uptake and further assist in imparting drought tolerance to the plants [10]. We noticed that inclusion of roots resulted in a lack of high molecular weight (HMW) proteins extracted from rice plants (Fig. 1). While extraction from rice leaves gave a reasonable spread of proteins from low to high molecular weight (Fig. 1, lane 1), extraction from roots of the same plant (lane 2), or from leaves and roots together (lane 3), resulted in few HMW proteins. These findings implicate a protein degradation component present in roots. Such protein degradation was not seen when leaf, flower, or seed protein was separately extracted; however, lower amounts of HMW proteins were obtained when root extract was added to these protein samples 5–8 min before loading the gel, or when these tissues were combined with roots before the extraction procedure. Hence it was obvious that roots contribute to the degradation of HMW proteins. This degradation of HMW proteins was observed despite the addition of PIC in the extraction buffer.

Our data further suggested that proteases (and not phenolics) are indeed largely responsible for this degradation, but they are not

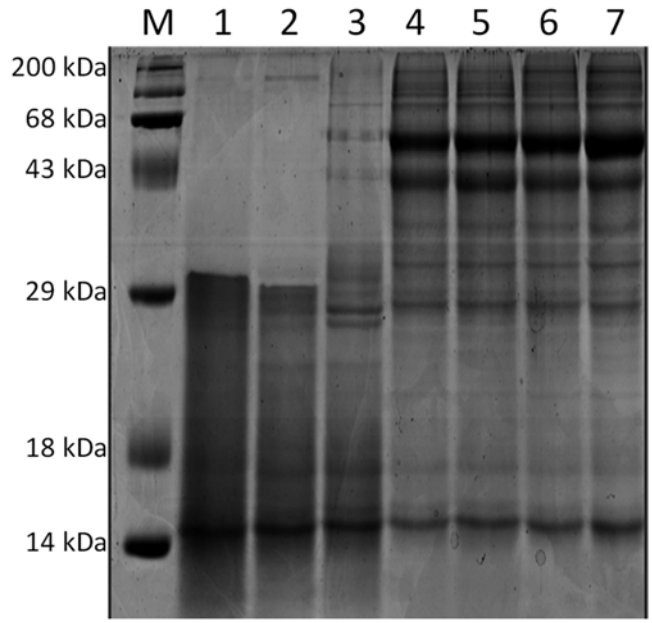


**Fig. 2** Leaf + root protein extract without PVPP but with 6 μL PIC (1); with PVPP and 6 μL PIC (2); with PVPP, 6 μL PIC, and 5 min boiling after adding sample buffer (3); with 8 min boiling before extraction and 6 μL PIC (4); with 8 min boiling before extraction, 6 μL PIC, and 5 min boiling after adding sample buffer (5); with 8 min boiling before extraction and 10 μL PIC (6); and with 8 min boiling before extraction and 15 μL PIC (7). Any further boiling or PIC did not improve results substantially (data not shown)

adequately controlled by PIC (Fig. 2). Few HMW proteins were observed in extraction from roots and leaves together in the presence of PIC (Fig. 2, lane 1), or in the presence of PIC and polyvinylpyrrolidone (PVPP), which is used to remove phenolics (lane 2). However, improvements were observed when a boiling step was added, which would denature proteases (lane 3). Boiling the crude homogenate before extraction was highly effective at improving accumulation of HMW proteins (lanes 4–7), and this effect increased with additional amounts of PIC (lanes 6 and 7).

Based on these results, a simple and efficient protocol is presented for extracting high-quality total soluble protein from roots or roots and leaves together. The method uses Tris buffer and heat treatment of the extract, and clearly results in improved quality and quantity of HMW proteins without affecting the electrophoretic resolution. Using this method we have obtained much improved 1D and 2D-GE results, in terms of number and intensity of protein band/spots (Raorane and Kohli, unpublished results). The success of this method may depend on the identities of the proteins of interest as well as their final use. For example, this method may be useful to study the intact primary structure using 1D or 2D-GE coupled

with immunoblot assays or even high-throughput proteomic applications. However, it may not be suitable for enzyme assays that require biologically active protein, which may be sensitive to the boiling step, or for studying protein oligomers, which may denature into individual components during boiling. Further, while Tris buffer (pH 8.0) provides a very favorable pH (physiological) for most proteins, it may not be suitable for proteins whose function or activity is optimal at a different pH.

## 2 Materials

All solutions and reagents must be prepared using ultrapure water unless otherwise indicated.

### 2.1 Protein Extraction

1. Protein extraction buffer (PEB): 50 mM Tris–HCl pH 8.0, 10 mM NaCl, 1 % sodium dodecyl sulfate (SDS), 0.1 mM dithiothreitol (DTT), 0.5 % β-mercaptoethanol. Dissolve 0.60 g of Tris in 80 mL of water in a 250 mL of glass beaker using a magnetic stirrer and adjust the pH to 8.0 with 1 N HCl. Add 0.058 g of NaCl, 1 g of SDS, 1.54 mg of DTT, and 500 μL of β-mercaptoethanol (*see* **Note 1**). Dissolve by mixing. Make up the volume to 100 mL with sterile distilled water. Store the buffer at 4 °C.
2. 0.1 M phenylmethanesulfonylfluoride (PMSF). Weigh 17.4 mg of PMSF in a 1.5 or 2 mL tube and add 1.0 mL of isopropyl alcohol. Mix by inverting.
3. Protease inhibitor cocktail (PIC) for plant cell and tissue extracts (Sigma): For the list of ingredients in the cocktail and their functions, *see* **Note 2**. This reagent is supplied in DMSO. Aliquot 100 μL/tube and store at –20 °C.
4. Mortar and pestle.
5. Liquid nitrogen.
6. 14 mL polypropylene round bottom tubes.
7. Burner or 95 °C water bath.
8. Shaker.
9. Centrifuge.
10. Bradford assay kit.
11. Spectrophotometer.

### 2.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Resolving Gel Buffer: 4× Tris–Cl/SDS, pH 8.8.
   Dissolve 91.0 g of Tris in 300 mL of distilled water in a 1 L glass beaker on a magnetic stirrer and adjust the pH to 8.8 with 1 N HCl. Slowly add 2 g of SDS and dissolve by mixing.

Make up the volume to 500 mL with sterile distilled water. Store at 4 °C.

2. Stacking Gel Buffer: 4× Tris–Cl/SDS, pH 6.8.
   Dissolve 6.05 g of Tris in 40 mL of distilled water in a 250 mL glass beaker on a magnetic stirrer and adjust the pH to 6.8 with 1 N HCl. Slowly add 0.4 g of SDS and dissolve by mixing (*see* **Note 3**). Make up the volume to 100 mL with sterile distilled water. Store at 4 °C.
3. 10× SDS-PAGE running buffer: Dissolve 30.3 g of Tris and 144.1 g of glycine (Amresco, Solon, OH) in approx. 600 mL of distilled water in a 1 L glass beaker using a magnetic stirrer. Slowly add 10 g of SDS (*see* **Note 3**) and dissolve by mixing. Make up the volume to 500 mL with sterile distilled water. Store at 4 °C.
4. 6× SDS sample buffer:
   Take 3.7 mL of 4× Tris–Cl/SDS, pH 6.8 to a 20 mL beaker. Add 3 mL of glycerol, 1 g of SDS, 3 mL of 2-mercaptoethanol (Sigma, St. Louis, MO), and 6 mg of bromophenol blue (Sigma, St. Louis, MO). Mix the components using the magnetic stirrer. Make up the volume to 10 mL with sterile distilled water. Aliquot the sample buffer into 1.5 mL microfuge tubes (Eppendorf, Germany) (1.0 mL/tube) and store at −20 °C.
5. Ammonium Persulfate (BioRad, Bio-Rad Laboratories, Hercules, CA): 10 % (w/v) solution in sterile distilled water. Store at −20 °C.
6. *N*,*N*,*N*,*N*′-tetramethyl-ethylenediamine (TEMED) (Sigma, St. Louis, MO). Store at 4 °C.
7. Acrylamide/bis-acrylamide 30 % (19:1).
8. Sterile distilled water.
9. Conical flask.
10. 7.2 cm × 10 cm × 1.5 mm gel cassette.
11. Equipment for polyacrylamide gel electrophoresis.
12. Prestained protein standard ladder.

### 2.3 Gel Staining

1. Coomassie Blue staining solution:
   Dissolve 0.5 g of Coomassie Brilliant Blue R250 (Sigma, St. Louis, MO) in 800 mL of methanol in a 2 L glass beaker and add 140 mL of acetic acid. Make up volume to 2 L by sterile distilled water.
2. Destain solution I:
   Mix 400 mL of methanol and 70 mL of acetic acid in a 1 L glass beaker and make up the volume to 1 L by sterile distilled water.

3. Destain solution II:
   Mix 50 mL of methanol and 70 mL of acetic acid in a 1 L glass beaker and make up the volume to 1 L by sterile distilled water.
4. Gel densitometer.

## 3 Method

### *3.1 Protein Extraction (Perform All the Steps on Ice or at 4 °C Unless Otherwise Stated)*

1. Pulverize the plant root or seedling tissues in a mortar and pestle using liquid nitrogen (*see* **Note 4**). A fine powder must be obtained for efficient protein extraction.
2. Transfer each powdered tissue sample into a separate 14 mL polypropylene round bottom tube. Add 6.0 mL of PEB for every 1.4 g of ground plant tissue (*see* **Note 5**), then add 1.2 μL of 0.1 M PMSF (*see* **Note 6**). Cap the tubes and mix the samples well by inverting.
3. Heat the samples in boiling water in a large beaker on a burner for 8 min, or 10 min at 95 °C in a water bath (*see* **Note 7**).
4. Transfer the tubes to ice, add 15 μL PIC, cap the tubes tightly, and place them horizontally in ice on a shaker at medium speed for 2 h.
5. Centrifuge the tubes at 12,000 × *g* for 15 min at 4 °C (*see* **Note 8**). Transfer the supernatant to a new tube.
6. Use the supernatant for protein quantification using a Bradford assay kit and spectrophotometer following the protocol outlined by the manufacturer, or store the protein samples at –20 °C until further use (*see* **Note 9**).

### *3.2 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE): (Some of the Substances and Solutions Are Hazardous. Wear Lab Coat, Appropriate Gloves, and Safety Glasses throughout the Protocol.)*

1. To make the resolving gel, combine 3.75 mL of resolving buffer, 5 mL of Acrylamide/Bis-acrylamide 30 % (19:1) (*see* **Note 10**), and 6.25 mL of sterile distilled water in a 50 mL of conical flask and mix the solution thoroughly. Add 75 μL of 10 % ammonium persulfate and 20 μL of TEMED and mix by swirling.
2. Cast the gel using a 7.2 cm × 10 cm × 1.5 mm gel cassette. Allow 1.5 cm of space at the top of the cassette for stacking the gel and gently overlay with water. Allow the gel to polymerize for 20 min at room temperature without disturbing the cassette.
3. To make the stacking gel, combine 1.25 mL of stacking buffer, 0.65 mL of Acrylamide/Bis-acrylamide 30 % (19:1) (*see* **Note 10**), and 3.05 mL of sterile distilled water in a 50 mL of conical flask and mix the solution thoroughly. Add 50 μL of 10 % Ammonium persulfate and 15 μL of TEMED to the solution and mix by swirling.

4. Cast the gel above the resolving gel. Insert a 10 well gel comb immediately into the stacking gel, without introducing air bubbles. Allow the gel to polymerize for 20 min at room temperature without disturbing the cassette (*see* **Note 11**).
5. Aliquot the protein samples into 20–30 μg portions and mix with an appropriate volume of 6× sample buffer. Do not mix the sample buffer with the prestained protein standard ladder. Load the protein standard (10 μL per well) and the protein samples (30 μg per well).
6. Electrophorese the gel at 120 V at room temperature using 1× SDS-PAGE running buffer until the dye front reaches the bottom of the gel (*see* **Note 12**).
7. After electrophoresis, remove the gel cassettes from the tank; pry the gel plates open. Transfer the gel carefully to a container with Coomassie Brilliant Blue staining solution. Allow the gel to stain overnight.
8. Replace the staining solution with the destain solution I and incubate the gel for 30 min.
9. Remove destain solution I and incubate the gel with destaining solution II until the background is clear.
10. Wash the gel with sterile distilled water and document the image using an appropriate gel densitometer.

## 4 Notes

1. β-mercaptoethanol may cause respiratory tract, skin, and eye irritation. Add β-mercaptoethanol immediately before use under a fume hood, heeding the prescribed local safety rules for its use.
2. Contents of the PIC (Sigma): 4-(2-Aminoethyl) benzenesulfonyl fluoride (AEBSF) inhibits serine proteases, such as trypsin and chymotrypsin; 1,10-Phenanthroline inhibits metalloproteases; Pepstatin A inhibits acid proteases, such as pepsin (human or porcine), renin, cathepsin D, chymosin (bovine rennin), and protease B (*Aspergillus niger*); Leupeptin inhibits both serine and cysteine proteases, such as calpain, trypsin, papain, and cathepsin B; Bestatin inhibits aminopeptidases, such as leucine aminopeptidase and alanyl aminopeptidase; E-64 inhibits cysteine proteases, such as calpain, papain, cathepsin B, and cathepsin L.
3. Carefully add SDS as last component, as this creates bubbles.
4. Liquid nitrogen may cause cold burns. Handle carefully and wear safety glasses and gloves.

5. SDS precipitates at 4 °C. The PEB should be warmed prior to use to dissolve the SDS.
6. Mix thoroughly until the crystals dissolve. PMSF is a cytotoxic chemical and degrades rapidly in aqueous solution.
7. Boiling will cause build-up of latent heat. Remove the tubes from the water bath and place them at room temperature for 2 min to allow release of the latent heat.
8. The refrigerated centrifuge must be turned on prior to centrifugation. This is to ensure that the temperature inside the centrifuge has cooled down to 4 °C before spinning the protein samples.
9. For our experiments, 1 mL of Bradford reagent was added to 20 μL of protein sample. The sample was mixed thoroughly by vortexing, and was then allowed to stand for 15 min before the absorbance reading using the spectrophotometer.
10. Acrylamide is a neurotoxin when it is not in the polymerized form. Wear appropriate gloves while handling acrylamide solutions.
11. If the gel is not used within the day, it can be stored overnight in a humid chamber at 4 °C with a wet paper towel or plastic wrap covering the cassettes. Longer storage times will cause the gel to dry despite these measures to retain humidity.
12. This 1-dimensional gel with the dimensions specified above requires a running time of about 1.5 h.

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# Chapter 11

# Temporary Immersion Bioreactors for the Contained Production of Recombinant Proteins in Transplastomic Plants

**Sherwin Barretto, Franck Michoux, and Peter J. Nixon**

## Abstract

Despite the largely maternal inheritance of plastid genomes, the risk of transgene dissemination from transplastomic plants can limit the scope for field cultivation. There is a need for a cost-effective, scalable process to grow large quantities of transplastomic plant biomass for biosynthesis of biopharmaceuticals and other high-value heterologous proteins. Temporary immersion culture is a means of achieving this under fully contained conditions. This method describes the organogenesis of transplastomic *Nicotiana tabacum* callus in RITA® temporary immersion bioreactors to produce rootless leafy biomass, and subsequent total soluble protein extraction, SDS-PAGE, and Western immunoblot analysis of heterologous protein expression. This method can be used for propagation of plastid or nuclear transformants, though is especially suitable for transplastomic biomass, as organogenesis leads to greater expression and accumulation of transplastomic proteins due to increases in chloroplast number and size.

**Key words** Temporary immersion bioreactors, Transgene containment, Transplastomic, Heterologous protein expression, Micropropagation, Morphogenesis

## 1 Introduction

The main reasons for using plants to express biopharmaceuticals and other recombinant proteins include a low requirement for technological input, immense scale-up potential, and low maintenance costs. As "molecular farming" host platforms are becoming more pervasive in both research and industry, a number of challenges must be addressed. Despite the economic and technical benefits of molecular farming, public and regulatory concerns with genetically modified organism (GMO) containment are hindering its widespread implementation, especially in Europe. The European Union (EU) is considered to have the strictest legislation regarding GMOs and GMO-derived products [1]. The EU regulatory approach is highly precautionary and follows all "what if" scenarios, as codified in a number of European Directives on GMOs: Directive

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90/219/EEC on the contained use of GMOs (amended by Directive 98/81/EEC, and more recently 2009/41/EC) and Directive 90/220/EEC (replaced by Directive 2001/18/EC) on the deliberate release of GMOs into the environment [1].

The likelihood of transgene dissemination can be reduced through physical containment of plant growth, such as greenhouse cultivation or in vitro culture. Containment also provides greater product consistency and maintenance of quality standards as compared with field cultivation. One approach to contained cultivation is the use of Temporary Immersion Bioreactors (TIBs), involving periodic immersion of biomass in liquid media. Temporary immersion (TI) culture is amenable to scaled-out automated micropropagation of large quantities of rootless leafy biomass under standardized conditions [2, 3]. There are several technical benefits, such as enhanced growth and morphogenesis through avoiding permanent submersion, adequate transfer of oxygen and nutrients, reduced shear, and the possibility of sequential medium changes [4, 5]. Moreover, the infrastructure, energy, and maintenance requirements are low compared with conventional expression platforms such as bacteria, yeast, and mammalian cells. The potential of this method has recently been tested with transplastomic and nuclear transformants, using pneumatic medium supply in RITA® bioreactors. The complete method described in this chapter was used for the recombinant expression of GFP as a reference and TetC [6, 7], OspA, and YFP-OspA [8] as potential vaccine antigens in transplastomic tobacco, *Nicotiana tabacum* (Patent Application WO2011/030083).

## 2 Materials

### 2.1 In Vitro Micropropagation

#### 2.1.1 Culture Media

To exclude microbial contamination and confirm the presence of the transplastomic construct (which contains *aadA* for resistance to spectinomycin) in transformant plants, 500 mg/L spectinomycin antibiotic is added (omitted for wild type). Additionally 1 mg/L PPM biocide agent (supplied by Plant Cell Technology, Washington DC, USA) is included as a safeguard against bacterial and fungal contamination (added before autoclaving). For all media, add components together before adding ultrapure water (*see* **Note 1**). Titrate the solutions to pH 5.8 using dilute NaOH and autoclave at 120 °C for 20 min. Spectinomycin must be filter sterilized and added after autoclaving as it is thermally labile. For solid media, add spectinomycin after the temperature cools to below 50 °C, before pouring into culture vessels or plates. All actions after autoclaving, including addition of spectinomycin and media transfers to culture vessels, must be undertaken in a laminar flow hood and aseptic practice must be followed. All media can be stored for up to 3 months at 4 °C. All culture vessels (except disposable Petri dishes), such as Magenta vessels, Erlenmeyer flasks, and RITA® bioreactors, must be autoclaved at 120 °C for 20 min, beforehand.

1. Germination Medium: 4.4 g/L Murashige and Skoog (MS) basal medium, 3 % (w/v) sucrose, 1 mg/L PPM biocide agent (Plant Cell Technology, Washington DC, USA), 8 g/L agar, pH 5.8, with and without 500 mg/L spectinomycin. Pour media into Magenta culture vessels while still liquid, after autoclaving and addition of spectinomycin, to a depth of approximately 2 cm, and allow to solidify before replacing the lid.
2. Callus Induction Medium (CIM): 4.4 g/L MS basal medium, 3 % (w/v) sucrose, 1 mg/L 1-naphthaleneacetic acid (NAA), 0.1 mg/L kinetin, 1 mg/L PPM, pH 5.8, with and without 500 mg/L spectinomycin. For callus proliferation on plates, add 8 g/L agar. After autoclaving and addition of spectinomycin, pour media into standard Petri plates while still liquid, and allow to solidify before replacing lids. To maintain sterile conditions, plates should be sealed with parafilm.
3. Shoot Regeneration Medium: 4.4 g/L MS basal medium, 3 % (w/v) sucrose, 0.1 μM thidiazuron (TDZ) (*see* **Note 2**), 1 mg/L PPM, pH 5.8, with and without 500 mg/L spectinomycin.

### 2.1.2 Bioreactor Components

1. Autoclaved RITA® temporary immersion bioreactors (Vitropic, France).
2. Hydroponics air pump.
3. Airline manifold with taps (required for pneumatic immersion of multiple bioreactors).
4. Programmable timer switch.
5. Flexible tubing.
6. 0.2 μm sterile air filters.
7. One-way valve and "T" or "Y"-type 3-way connectors (*see* **Note 3**).
8. Gas flow meter (optional).

### 2.1.3 Other

1. Indoor air-conditioned plant cultivation facility set to 25 °C, under a 16 h photoperiod, with a light irradiance of approximately 45–120 μmol/$m^2$ s.
2. Seeds of tobacco transformants.
3. 100 % ethanol.
4. Commercial-grade bleach (3–8 % sodium hypochlorite) with 0.1 % (v/v) Tween-20.
5. Autoclaved ultrapure water.
6. Magenta vessels (approx. 350 mL).
7. Sterile scalpel.
8. Autoclaved Erlenmeyer flasks sealed with aluminum foil.
9. Vacuum pump.

10. Disposable sterile filter system such as Nalgene 0.2 μm PES filter units (Thermo Scientific).
11. Orbital shaker.
12. Weighing balance.
13. 80 °C oven.
14. 50 mL conical tubes (such as Falcon tubes).

### 2.2 Determination and Semi-quantification of Protein Expression

#### 2.2.1 Total Soluble Protein (TSP) Extraction

1. Pestle and mortar.
2. Liquid nitrogen.
3. Spatula.
4. Tongs.
5. Cryogenic microcentrifuge tubes.
6. Microcentrifuge.
7. Protein extraction buffer (PEB): 50 mM HEPES–KOH (pH 7.5), 2 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM potassium acetate, 5 mM magnesium acetate, and 2 tablets of complete protease inhibitor EDTA-free cocktail (Roche Applied Sciences, Germany) per 50 mL buffer (*see* **Note 4**).

#### 2.2.2 Total Soluble Protein Quantification

1. Bradford Assay Reagent.
2. Bovine serum albumin (BSA) as a quantification standard.
3. Cuvettes.
4. Spectrophotometer.

#### 2.2.3 SDS-PAGE

1. 5× solubilization buffer: 300 mM Tris–HCl (pH 6.8), 10 % (w/v) sodium dodecyl sulfate (SDS), 50 % (w/v) glycerol, 0.1 % (w/v) bromophenol blue, and 10 % (w/v) β-mercaptoethanol (*see* **Note 5**).
2. Bio-Rad mini-gel electrophoresis system (Bio-RAd Laboratories, USA) or similar.
3. 10 % or 12 % polyacrylamide minigels (*see* **Note 6**).
4. SDS-PAGE running buffer: 25 mM Tris–HCl (pH 8.3), 190 mM glycine, 0.1 % (w/v) SDS.
5. Precision Plus Protein™ Dual Color Standards (Bio-Rad) or similar.
6. Target protein standards of known concentration (for quantitative Western blotting).
7. Coomassie-blue buffer or SYPRO® Orange Protein Gel Stain (Life Technologies) (5000× dilution of concentrate in 10 % (w/v) acetic acid).
8. Standard gel imager (visualization of Coomassie) or CCD digital imager (visualization of SYPRO® Orange).

*2.2.4 Western Immunoblotting*

1. Bio-Rad Mini Trans-Blot® Electrophoretic Transfer Cell or similar "wet" electroblotting system.
2. 0.2 μm nitrocellulose membrane.
3. Transfer buffer: 4.2 g $NaHCO_3$, 1.5 g $Na_2CO_3$, 1 L methanol, 4 L ultrapure water (store at 4 °C).
4. Suitable plastic containers for storing membranes.
5. Tris-buffered saline (TBS).
6. TBS-Tween (0.1 % Tween 20).
7. Dried skimmed milk powder.
8. Relevant primary antibody against protein of interest diluted in TBS.
9. Relevant horseradish peroxidase (HRP)-conjugated secondary antibody diluted in TBS.

*2.2.5 Enhanced Chemiluminescence (ECL) Detection*

1. ECL kit.
2. Standard office transparent plastic wallet.
3. CCD imaging system capable of chemiluminescent detection (e.g., Fujifilm LAS-3000 imager) or darkroom with X-ray film developer. If the latter is used, high-quality X-ray film for ECL applications and X-ray film cassettes are also needed.
4. Densitometric image analysis software, such as ImageJ from National Institutes of Health (NIH) (free and open source, available to download from http://imagej.nih.gov/ij/).
5. Tweezers.
6. Scalpel blade.
7. Paper towels.

## 3 Methods

All procedures must be carried out at room temperature unless otherwise specified.

### *3.1 In Vitro Micropropagation*

This protocol describes the in vitro micropropagation of transplastomic tobacco, *Nicotiana tabacum*, culminating in organogenesis in TIBs. This protocol may also be adapted for nuclear transformants. All inoculation and subculture steps must be undertaken in a laminar flow hood under aseptic conditions (Fig. 1).

*3.1.1 In Vitro Germination of Sterile Seedling*

1. Surface-sterilize seeds in ethanol for 1 min, then in commercial-grade 5 % bleach with 0.1 % (v/v) Tween-20 for 15 min, and wash 3 times for 5 min in autoclaved ultrapure $H_2O$.
2. Using a sterile scalpel blade or suitable inoculation loop, transfer seeds to autoclaved Magenta vessels containing germination media.

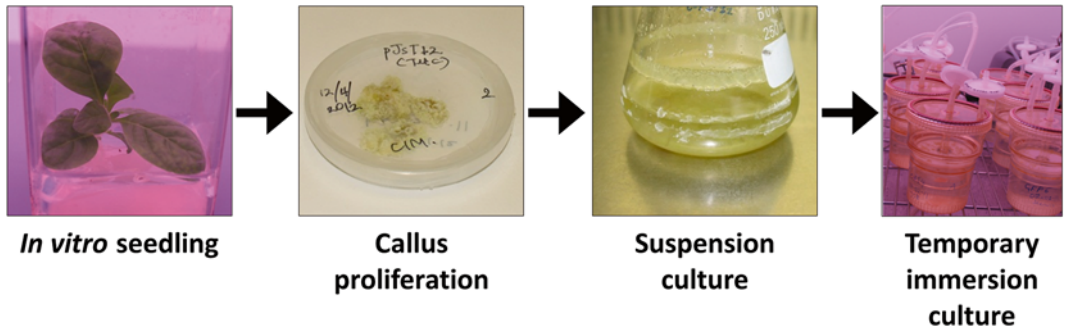


**Fig. 1** Micropropagation of transplastomic tobacco

3. Allow seedlings to germinate and grow for 3–4 weeks to provide donor material for generation of callus germplasm.

*3.1.2 Callus Induction and Proliferation*

1. Cut sections from vascularized leaves (approximately 1.5 $cm^2$) with a sterile scalpel and plate on culture plates containing CIM, abaxial side down.
2. After 2–4 weeks, plate primary calli to new plates containing CIM for further growth for 2–3 weeks (*see* **Note 7**). Callus isolated from the original functional leaf tissue rapidly proliferates in fresh medium. Repeat 2–3 times if necessary.

*3.1.3 Liquid Suspension Cultures*

1. Inoculate autoclaved Erlenmeyer flasks with small pieces of friable callus and an appropriate volume of liquid CIM (*see* **Note 8**). Place cultures on orbital shakers and shake at 140 rpm. In suspension culture, calli form fine uniform aggregates. Allow to grow for 2–3 weeks.
2. Subculture at least once by adding more CIM and pouring into new flasks. Replenishment of media and dilution of waste products extend the duration of the "exponential phase" and allow generation of large quantities of fine aggregates for inoculation of multiple TIBs.

*3.1.4 Temporary Immersion Regeneration of Shoots from Callus*

1. Unpack the sterile filter system in a laminar flow hood. Connect the filter system to the vacuum pump. Filter callus suspensions from the suspension growth medium. If necessary, pool together two or more flask cultures to obtain enough inocula for multiple TIB cultures.
2. Place a weighing balance inside the laminar flow hood. Add 0.5 g of fine suspension aggregates into each TIB and then pour 300 mL of shoot regeneration medium. Replicate cultures should be set up for each transformant or environmental condition being investigated, either as duplicates or triplicates.
3. Connect the RITA® TIBs to the air pump and manifold using the following setup. For each manifold outlet, a makeshift

"pressure relief" valve should be made by connecting a short length of tubing to the stem of a "T"-connector. Make a moderately tight knot in the tubing. The air pump and manifold should be connected to the one-way solenoid valve, which should be connected to the "pressure relief" valve. The tubing downstream should be connected to two or three RITA® TIBs, splitting the air stream with "T" or "Y" adapters. The air flow should give moderate bubbling during periodic immersion. This should be achieved through turning the manifold tap 50° and a moderately tight knot. If a gas flow meter is available (optional), avoid air flow rates of greater than 300 mL/min (*see* **Note 9**). Set the timer to provide 4 min of pneumatic immersion every 8 h. In the RITA® system, temporary immersion is performed by applying an air over-pressure that pushes the medium from a lower compartment to the upper compartment, which contains the biomass.

4. At the end of the 40-day culture period, shoot biomass is harvested from the RITA® bioreactors. Shoot biomass should be photographed, and visual observations such as physiological differences between growth regimes, hyperhydricity (vitrification), and the presence of undifferentiated callus should be noted. Subsequently, total biomass should be weighed for "fresh biomass" determination.
5. For further immunoassay analysis, excise small representative samples of leaves and freeze at −80 °C (*see* **Note 10**). Other notable tissue morphologies, such as undifferentiated callus, primordia, or hyperhydric (vitrified) leaves, may be sampled for comparative analysis.
6. To determine the dry weight:fresh weight ratio, a large quantity of harvested biomass should be weighed, then dried at 80 °C for 48 h in pre-weighed plastic containers (such as Magenta vessels), and weighed again. For actual dry biomass determination, the total fresh weight should be multiplied by this ratio (Figs. 2 and 3).

### *3.2 Determination and Semi-quantification of Heterologous Protein Expression Through Temporary Immersion Morphogenesis*

#### *3.2.1 Total Soluble Protein (TSP) Extraction*

1. Grind frozen plant samples into a fine powder with a pestle and mortar, with liquid nitrogen, and transfer the powder into cryogenic microcentrifuge tubes (*see* **Note 11**).
2. Lysates should be weighed, and 100 µL protein extraction buffer (PEB) should be added per 100 mg biomass (*see* **Note 12**).
3. Centrifuge lysate-buffer mixtures at 18,000×*g* (or maximum speed) for 30 min and pipette the supernatants into new microcentrifuge tubes. Repeat this step, avoiding the pellet each time. TSP extractions may be stored at −80 °C before further processing.
4. Quantify the total soluble protein using the Bradford assay [9] according to the manufacturer's instructions, using an absorbance measurement at 595 nm (*see* **Note 13**).

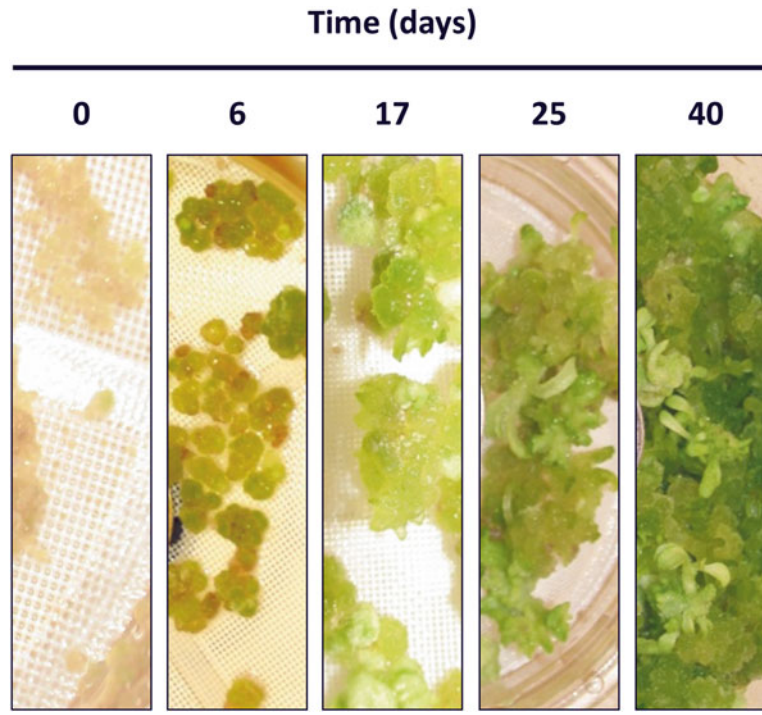


**Fig. 2** Visual demonstration of organogenesis in TIB

**Fig. 3** Development of leaves and shoots in TIB after 40-day culture

*3.2.2 SDS-PAGE*

1. Mix TSP extract samples with 5× solubilization buffer, and boil at 100 °C for 10 min to denature the proteins. Solubilized samples may be stored at −20 °C or −80 °C.
2. Spin solubilized samples for 1 min prior to loading. Load gels with approximately 10 μg of protein per well (*see* **Note 14**).

Run gels at room temperature at 100–120 V for approximately 3 h. For Western immunoblotting, gels must be run in duplicate, with one gel for immunoblot transfer and another to be stained to confirm equal protein loading.

3. Coomassie-blue or Sypro Orange stains may be used to visualize the separated proteins. Minigels must be immersed in staining solution in suitable sealed plastic boxes, and placed on a shaker.

*3.2.3 Western Immunoblotting and Enhanced Chemiluminescence (ECL) Detection*

1. "Wet" transfer of proteins to a 0.2 μm nitrocellulose membrane can be conducted with the Bio-Rad Mini Trans-Blot® Electrophoretic Transfer Cell, according to the manufacturers' instructions (*see* **Note 15**).
2. Block the membrane in 5 % (w/v) dried skimmed milk in TBS-Tween for 1 h, then incubate with the primary antibody (in TBS-Tween) for 1 h at room temperature or overnight at 4 °C. Wash the membrane 3×10 min in TBS-Tween. Incubate the membrane in secondary antibody in TBS-Tween for 1 h at room temperature. Wash 4×10 min in TBS-Tween and then once in TBS without Tween.
3. Conduct Enhanced Chemiluminescence (ECL) detection. Detection may be undertaken using a CCD digital imaging system or traditional X-ray film, depending on preference or availability.
4. For quantitative analysis of the target protein, densitometric software can be used (*see* **Note 16**) (Fig. 4).

## 4 Notes

1. If plant growth regulators or antibiotic is available in solid form, concentrated aqueous stock solutions must be prepared, in ultrapure water (unless otherwise stated). Alternatively, readymade stock solutions may be commercially available. The appropriate volume of stock solution must be added to media for dilution to the required concentration. Recommended stock concentrations: 1 mg/mL NAA, 1 mg/mL kinetin, 10 mM TDZ, 100 mg/mL spectinomycin. It is recommended to prepare fresh stocks just before media preparation, although aliquots may be stored long term at −20 °C.
2. TDZ (molecular weight 220.3 g/mol) is very insoluble. A stock solution of 10 mM TDZ may be prepared by dissolving 22 mg of TDZ in a few drops of 1 N NaOH or KOH, before adding water to make 10 mL. Alternatively, TDZ may be dissolved in DMSO.
3. Ensure that plenty of flexible tubing, air filters, and 3-way adapters are available well before setting up the rig and inoculation.

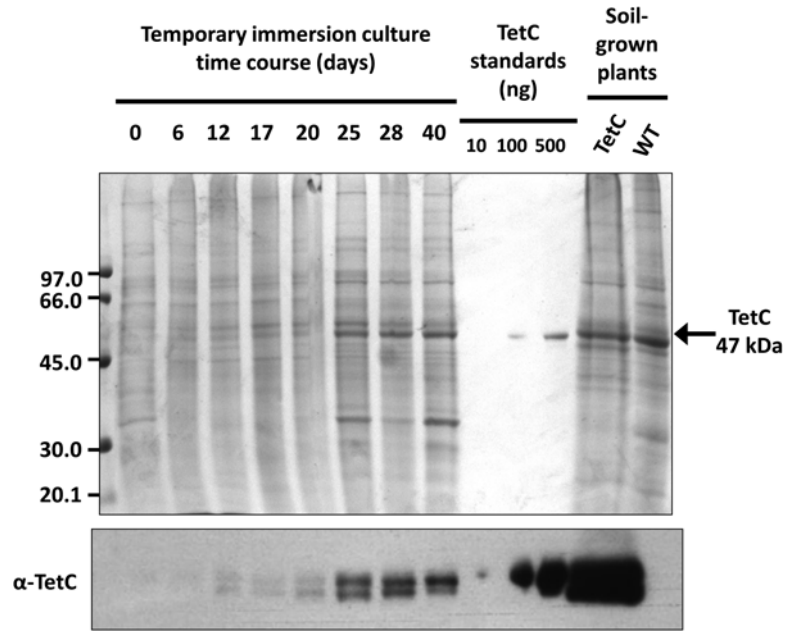


**Fig. 4** Evaluation of differential TetC expression in *N. tabacum* cv. Petit Havana during temporary immersion culture by SDS-PAGE Coomassie-stained gel (*upper image*) and Western immunoblot (*lower image*). Biomass from in vitro culture was taken at various points over a 40-day temporary immersion culture period; soil-grown plants were grown for 40 days. Each lane is loaded with 8 μg of TSP. Primary antibody: 1:3000 rabbit anti-TetC. Secondary antibody: 1:10,000 HRP-conjugated anti-rabbit IgG

Tubing with a 7 mm internal diameter is recommended, as this is compatible with most standard 3-way adapters and air filters.

4. Protein extraction buffer, without DTT or protease inhibitors, may be filter-sterilized and stored long term at 4 °C. DTT is highly unstable in the presence of air, and must be added to protein extraction buffer just before use. Protein extraction buffer containing protease inhibitors is stable for approximately 1 week at 4 °C.
5. Solubilization buffer, without β-mercaptoethanol, may be stored at room temperature. β-mercaptoethanol must be added just before use as it is unstable in the presence of air.
6. We make gels with an acrylamide/bis-acrylamide mixing ratio of 37.5:1, and 6 M urea. The addition of urea is optional, though it is considered to give sharper bands.
7. Look out for primary calli on the cut edges of leaf sections. When plating the calli onto new plates, avoid excessive scraping or shearing. Select friable calli for subculture as this has the greatest

regeneration potential, and avoid dense, compact calli. Friable callus is soft, loosely associated, and can be separated easily.

8. As a rule-of-thumb, 250 mL Erlenmeyer flasks should be loaded with 100 mL of medium, and 500 mL flasks with 200 mL of medium. This is to ensure adequate mixing and oxygen transfer during liquid culture.
9. Ideally, this setup should be undertaken in advance. After attaching inoculated TIBs, test the system by briefly applying air flow and observing suspension of media. Excessive bubbling or a long time to reach full suspension ($\geq$1 min) is indicative of high or low air flow, respectively, so adjust air flow accordingly by turning the manifold taps. Avoid excessive air flow as inocula are susceptible to shear damage.
10. From each culture, healthy shoots, ideally taken from several meristemoid clusters (for reliable representation of the biomass population), should be stored in 50 mL conical tubes or other containers suitable for cryo-storage.
11. A makeshift "spoon" for pouring small volumes of liquid nitrogen can be made with tongs and a 50 mL conical tube. The mortar and pestle should be pre-cooled by pouring liquid nitrogen. Grinding actions should be strong and brisk, involving quick sweeping circular motions of the pestle. The lysate should not be allowed to thaw at any point. If necessary, pour additional liquid nitrogen to maintain a cool temperature. The powder should be scooped with a pre-cooled spatula and loaded into pre-cooled cryo microcentrifuge tubes. The tubes should be temporarily placed in a dewar of liquid nitrogen to prevent thawing while awaiting the next step. Safety precautions for handling liquid nitrogen must be followed throughout, including the use of eye/face protection, gloves, and good ventilation.
12. Care must be taken to minimize thawing of lysates and associated protein degradation, before suspension in PEB. The PEB must be added and mixed so that it contacts the solid powder before the powder defrosts (the PEB contains protease inhibitors to minimize proteolysis). Avoid adding excessive buffer, as this will result in unnecessary dilution of TSP. In our experience, adding 100 μL of buffer per 100 mg lysate should give a TSP concentration of 0.7–1.2 μg/μL.
13. A dilution series of known concentrations of bovine serum albumin (BSA) should be used as a standard. Abs (595 nm) should be correlated against BSA concentration to generate the extinction coefficient against which the protein concentrations of the plant extracts can be calculated. A sample of PEB alone should be used as a blank.

14. Aim to load between 7 and 12 μg of protein per well. For detection of poorly expressed proteins, loading of higher amounts is recommended.
15. Alternative "semi-dry" or "dry" electroblotting systems such as the iBlot® system (Life Technologies) can be used for convenience and speed, but transfer quality may be compromised. For quantitative Western blotting purposes, "wet" transfer is preferred.
16. For quantitative densitometric analysis, overexposures should never be used.

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# Chapter 12

# Plant Cell-Based Recombinant Antibody Manufacturing with a 200 L Orbitally Shaken Disposable Bioreactor

**Nicole Raven, Stefan Schillberg, and Stefan Rasche**

## Abstract

Tobacco BY-2 cells are an attractive platform for the manufacture of a variety of biopharmaceutical proteins, including antibodies. Here, we describe the scaled-up cultivation of human IgG–secreting BY-2 cells in a 200 L orbitally shaken disposable bioreactor, resulting in cell growth and recombinant protein yields that are proportionately comparable with those obtained from cultivations in 500 mL shake flasks. Furthermore, we present an efficient downstream process for antibody recovery from the viscous spent culture medium using expanded bed adsorption (EBA) chromatography.

**Key words** Antibody purification, Expanded bed chromatography, *Nicotiana tabacum* Bright Yellow 2 (BY-2), Plant cell suspension culture, Scaled-up manufacture, Single-use technology

## 1 Introduction

The suitability of different plant-based production hosts for the manufacture of valuable pharmaceutical and industrial proteins such as enzymes, antibodies, vaccine candidates, and other biotherapeutics has been well described [1–3]. Plant-based production systems include intact plants [4, 5], plant tissues and organs [6, 7], simple plants in containment [8, 9], and cell suspension cultures [10, 11]. Tobacco-based cell suspension cultures such as NT-1 and BY-2 [12] are often chosen as production hosts because they can be easily transformed and show favorable growth characteristics and high productivity [13]. Recombinant protein production according to good manufacturing practice (GMP) is made possible by the contained cultivation of plant cell suspension cultures in bioreactor systems with rigid process control [14]. In recent years, disposable bioreactor systems have become well accepted as an alternative to stainless steel bioreactors for mammalian cell cultures by the biomanufacturing industry, mainly due to their cost-effectiveness, flexibility, and safety [15].

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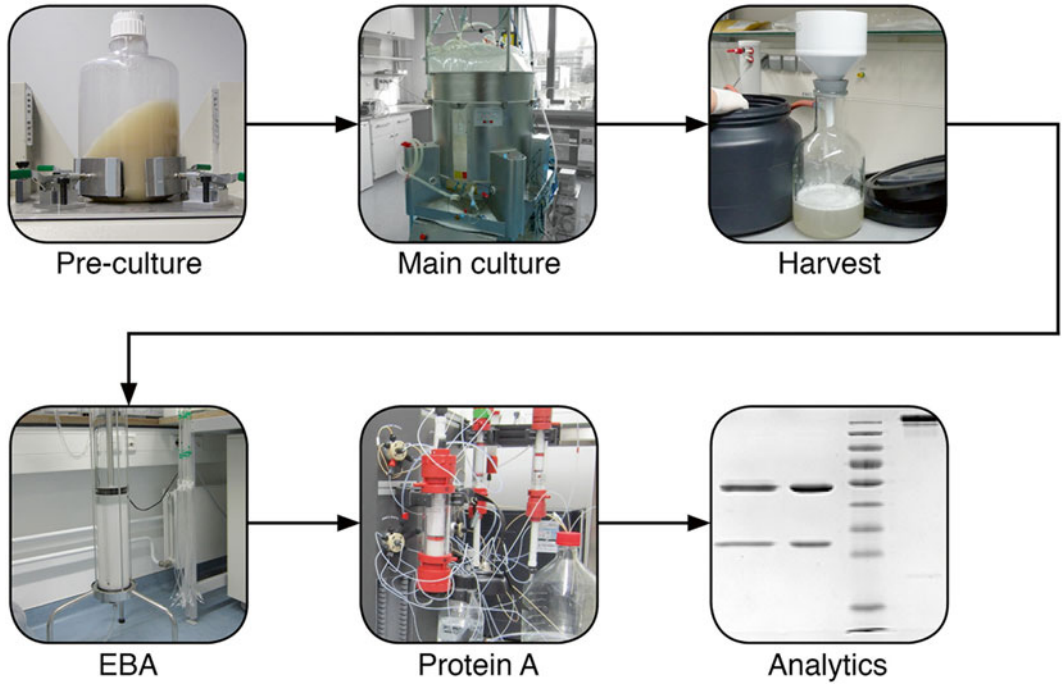


**Fig. 1** Upstream and downstream process steps of 100 L scale antibody manufacture from BY-2 suspension culture. EBA is expanded bed adsorption chromatography; Protein A refers to M12 antibody capture using staphylococcal protein A, which binds IgG molecules by their Fc region

Plant cell cultures have been successfully cultivated in various disposable bioreactor systems such as wave-mixed bioreactors or stirred-tank reactors [16–18]. Orbitally shaken bioreactor systems are one of the most recent trends in disposable cultivation technology. The simple bag geometry of orbitally shaken bioreactor systems and their predictable liquid distribution characteristics account for straightforward scalability [19] and facilitate the characterization of bioprocessing capabilities. Here, we describe the cultivation of human M12 antibody-secreting BY-2 suspension cells in a disposable orbital shaker at a working volume of 100 L, a 200-fold greater scale compared to routine shake flask cultivation. After cell removal by vacuum filtration, we applied expanded bed adsorption (EBA) chromatography upstream of the protein A chromatography as a novel strategy for efficient antibody recovery from the viscous spent medium. The overall antibody recovery of this manufacturing process (*see* Fig. 1) ranged between 75 and 85 %, and product purity of >95 % was typically achieved.

## 2 Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when discarding waste materials.

### 2.1 Plant Cultivation

1. MSN medium: For each liter of medium, weigh 4.3 g Murashige & Skoog (MS) medium, 30 g sucrose, 10 g potassium nitrate, 0.2 g potassium dihydrogen phosphate, and 0.1 g myo-inositol, then transfer the components into an appropriate beaker or vessel. Add ultrapure water to 95 % of the final volume and dissolve the components using a magnetic stirrer. Add 0.2 mg/L 2,4-dichlorophenoxyacetic acid (*see* **Note 1**) and 1 mg/L thiamine–HCl (*see* **Note 2**). Without raising the pH above the desired value, carefully adjust the pH to 5.8 using 1 M potassium hydroxide and fill with ultrapure water to the final volume. Medium sterilization is achieved either by heat sterilization in an autoclave at 121 °C and 100 kPa (15 Psi) for 35 min or by filter sterilization using Sartopore 2 sterile MidiCap filters (Sartorius Stedim AG, Goettingen, Germany) for media volumes of up to 20 L or Sartopore 2 150 sterile capsule filters (Sartorius Stedim AG) for larger media volumes. The smaller filter size is operated at a flow rate of 150 mL/min while the flow rate for the larger filter size is adjusted to 1.5 L/min. The medium is stored at room temperature until usage.
2. Pre-culture cultivation vessels: Tobacco BY-2 suspension cells are routinely cultivated in 250 mL wide neck glass Erlenmeyer shake flasks. A 5 L pre-culture used as inoculum for a 100 L batch cultivation is cultivated in a 20 L polycarbonate carboy vessel.
3. Incubator shaker with a shaking diameter ($d_0$) of 5 cm (e.g., ISF1-X incubator shaker, Kuhner AG, Birsfelden, Switzerland): This incubator is used for the cultivation of 100–500 mL BY-2 shake flask cultures.
4. Incubator shaker with a shaking diameter ($d_0$) of 7 cm (e.g., SR200-X shaker, Kuhner AG): This shaker is used for the cultivation of BY-2 cells in a 20-L Nalgene polycarbonate carboy vessel.
5. SB200-X 200 L orbitally shaken bioreactor system: The SB200-X bioreactor system (Kuhner AG) is equipped with a 350-L disposable Cultibag ORB 200 L (Sartorius Stedim AG). The maximal nominal working volume of the bioreactor system is 200 L.

### 2.2 Culture Broth Clarification

1. Miracloth (Merck-Millipore, Darmstadt, Germany).
2. Nutsche filter with a diameter of at least 17 cm.

3. 15 L gas washing bottle.
4. Membrane vacuum pump with a capacity of 1.7 m/h.

### 2.3 Expanded Bed Adsorption (EBA) Chromatography

1. Streamline BPG 100/950 glass chromatography column (10 cm internal diameter × 95 cm height; GE Healthcare, Munich, Germany) with flow distribution provided by a perforated plate fitted with a 23 μm mesh at the inlet.
2. Masterflex L/S with Easy-LoadII pump head (Cole-Parmer via VWR International GmbH, Darmstadt, Germany).
3. Streamline SP adsorbent (GE Healthcare): 2 L Streamline SP adsorbent is packed into the Streamline BPG 100/950 glass chromatography column, resulting in a packed mode bed height of 31 cm.
4. Analogue pressure gauge with a measuring range of 0–6 bar (Anderson Instruments Company, INC., Fultonville, NY).
5. High salt elution buffer: 50 mM sodium phosphate, pH 8.0, 500 mM NaCl. For each L, weigh 19.0 g tri-sodium phosphate docecahydrate and 29.2 g NaCl. Transfer the components into an appropriate vessel and add ultrapure water to 95 % of the final volume. Dissolve by stirring on a magnetic stirrer. Without raising the pH above the desired value, carefully adjust the pH to 8.0 using 1–10 M NaOH and add the required amount of ultrapure water to reach a final volume of 1 L or equivalent. The buffer is stored at room temperature.

### 2.4 Preparative Protein A Chromatography

1. XK 50/20 column (GE Healthcare).
2. ÄKTAexplorer 10 (GE Healthcare).
3. Protein A sorbent: 150 mL MabSelect SuRe™ (GE Healthcare).
4. Equilibration and wash buffer: Phosphate buffered saline (PBS), pH 7.4. For each liter, weigh 8.0 g sodium chloride, 0.2 g potassium chloride, 2.9 g sodium dihydrogen phosphate·12 $H_2O$, and 0.2 g potassium phosphate. Dissolve the components in 900 mL ultrapure water by stirring on a magnetic stirrer and carefully adjust the pH to 7.5 using 1–10 M NaOH. Fill to a final volume of 1 L with ultrapure water, run the buffer through a 0.22 μm filter, and store at room temperature.
5. Elution buffer: 100 mM glycine, pH 2.8. Dissolve 7.5 g glycine in 900 mL ultrapure water by stirring on a magnetic stirrer; carefully adjust the pH to 2.8 using 1–10 M HCl without lowering the pH below the desired value. Fill to a final volume of 1 L with ultrapure water, run the buffer through a 0.22 μm filter, and store at room temperature.

### 2.5 Desalting

1. Pre-packed HiPrepTM 26/10 desalting column (Sephadex G-25, GE Healthcare).
2. ÄKTAexplorer 100 (GE Healthcare).

3. M12 antibody storage buffer: 10 mM sodium acetate, pH 4.7, 5 mM EDTA. We find the preparation of a 10× stock solution to be the most convenient. For each liter of 10× stock solution, weigh 8.2 g sodium acetate and 37.2 g EDTA·2 $H_2O$ and transfer into a suitable vessel. Add 900 mL of ultrapure water and dissolve by stirring on a magnetic stirrer. Without lowering the pH below the desired value, carefully adjust the pH to 4.7 using acetic acid. Fill to a final volume of 1 L with ultrapure water. The 10× stock solution is stored at room temperature. To prepare the M12 antibody storage buffer, take 100 mL from the 10× stock solution and dilute in 900 mL ultrapure water. Mix by stirring on a magnetic stirrer. Check the pH value and re-adjust to 4.7 if necessary. Run the buffer through a 0.22 µm filter and store at room temperature.

### 2.6 Storage of Purified M12 Antibody

1. Acrodisc 32 mm sterile syringe filter with 0.2 µm Supor membrane (Pall via VWR International GmbH).
2. 150-mL FlexBoy Bag with luer locks (Sartorius Stedim AG).

### 2.7 Antibody Analytics

1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE): XCell SureLock Mini-Cell protein gel electrophoresis system using Novex NuPAGE 4–12 % Bis-Tris protein gels and NuPAGE MES SDS running buffer (all Life Technologies, now Fisher Scientific).
2. Coomassie Brilliant Blue staining of SDS-polyacrylamide gels according to Fairbanks et al. [20]: Fairbanks A solution: 0.05 % (w/v) Coomassie Brilliant Blue R-250, 25 % (v/v) 2-propanol, 10 % (v/v) acetic acid; Fairbanks B solution: 0.05 % (w/v) Coomassie Brilliant Blue R-250, 10 % (v/v) acetic acid; Fairbanks C solution: 0.002 % (w/v) Coomassie Brilliant Blue R-250, 10 % (v/v) acetic acid; Fairbanks D solution: 10 % (v/v) acetic acid; Whatman paper.
3. Analytical protein A chromatography: YMC EcoPlus 5/125 glass chromatography column (YMC Europe GmbH, Dinslaken, Germany) packed with 0.3 mL MabSelect SuRe™ and Äktaexplorer 10 (both GE Healthcare). Running buffer: PBS, pH 7.4 (*see* **item 4** in Subheading 2.4).

## 3 Methods

### 3.1 Tobacco BY-2 Pre-culture Cultivation

1. Routinely cultivate 100-mL aliquots of transgenic BY-2 suspension cells in 250 mL glass Erlenmeyer shake flasks in the dark on an incubator shaker ($d_0 = 5$ cm) at 180 rpm and 26 °C (*see* **Note 3**).

2. Inoculate 5 L MSN medium inside a 20 L Nalgene polycarbonate carboy vessel with 250 mL of 7-day-old M12 antibody-producing BY-2 suspension cells (from shake flask cultivation) using sterile techniques.
3. Insert and fix the 20 L Nalgene vessel to an incubator shaker ($d_0 = 7$ cm) and incubate the culture at 26 °C shaking at 180 rpm for 7 days.

### 3.2 100 L Scaled-Up Tobacco BY-2 Cultivation in an SB200-X Orbital Shaker

1. Insert the 350-L disposable bag into the SB200-X (*see* Fig. 2) and connect the supply and exhaust air filters to the bag (*see* **Note 4**).
2. Fill the bag with air by gassing and adjust the position of the bag in the SB200-X bioreactor if necessary (*see* **Note 4**).
3. Connect the tubing of one bag with a Sartopore 2 150 sterile capsule using MPC connectors under sterile conditions. Then, sterilize 100 L of freshly prepared MSN medium by pumping it through the filter at a flow rate of 1.5 L/min directly into the 350 L bag (*see* **Note 5**).
4. The medium is incubated at 26 °C and 80 rpm for 24 h to confirm the sterility of the bioreactor system (*see* **Note 6**).

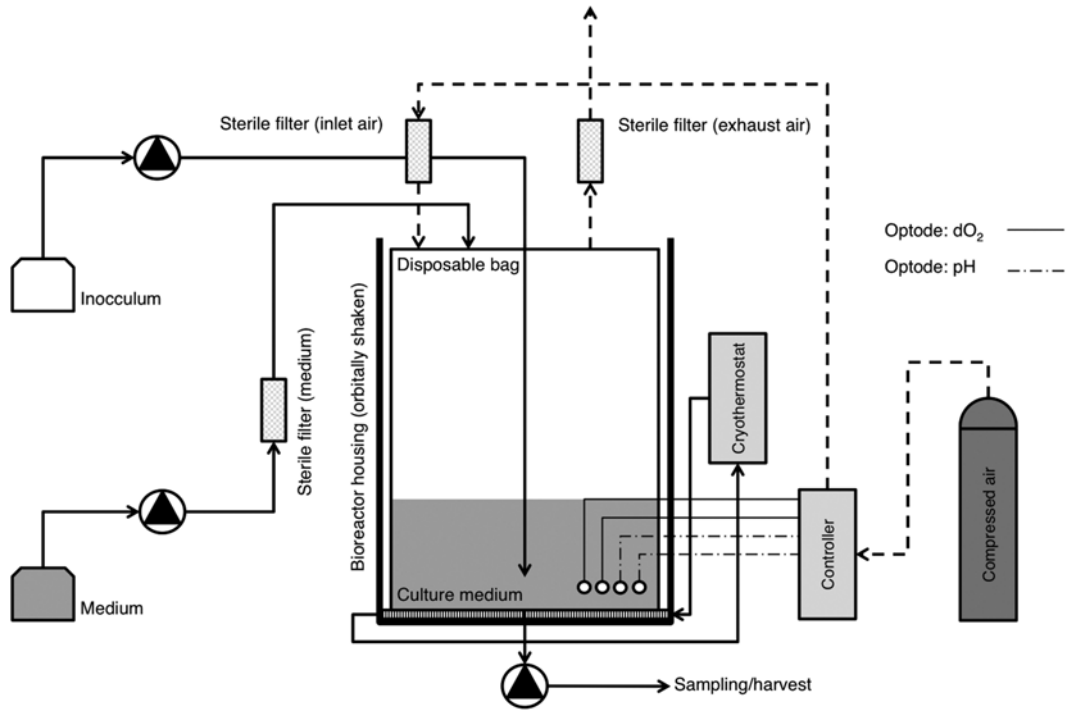


**Fig. 2** Process diagram of 100 L scale plant cell-based recombinant antibody manufacture

5. Place the 5 L pre-culture from the Nalgene vessel into the SB200-X bioreactor system using MPC connections that are attached to both cultivation vessels.
6. Cultivate for 5–7 days at 26 °C and 80 rpm (*see* **Note 7**).

### 3.3 Harvest and BY-2 Cell Removal

1. Harvest the culture broth by opening the harvest tubing of the 350-L disposable bag (*see* Fig. 2) and transferring into an appropriate container (*see* **Note 8**).
2. Prepare the vacuum filtration unit to separate the BY-2 cells from the spent medium by placing two layers of miracloth onto the sieve bottom of the Nutsche filter, then mount the Nutsche filter to the gas washing bottle and connect the gas washing bottle to a vacuum pump. Switch on the pump and pour the harvested culture broth into the Nutsche filter. The BY-2 cells are retained in the Nutsche filter while the spent medium containing the M12 antibody is collected in the gas washing bottle (*see* **Note 9**).
3. Take 20 L from the clarified spent medium and dilute 1:3 with ultrapure water to attain a conductivity of less than 12 mS.

### 3.4 Expanded Bed Adsorption (EBA) for M12 Antibody Capture

1. Fill the streamline BPG 100/950 glass chromatography column to approximately 1/3 full with deionized water via the bottom valve. Then, reverse the flow direction of the pump to suck out any air that might be trapped underneath the endpiece net. Leave approximately 5 cm of water in the column.
2. Suspend 2 L of Streamline SP adsorbent in PBS to a slurry of approximately 50 %, then quickly pour the slurry into the column and let the resin settle. The resulting bed height should be approximately 31 cm.
3. Fill the column to the rim with deionized water and insert the top adapter without trapping air under the adapter net. Lower the adapter carefully until the gasket is submerged in water. Fill the remaining space with deionized water, then close the column lid, and connect the column to a second masterflex pump that serves as a hydraulic drive to lower the adapter (Fig. 3a).
4. Set all valves to up-flow conditions according to Fig. 3a.
5. Equilibrate the resin with ultrapure water at a flow rate of 550 cm/h, resulting in a stable height of $H/H_0 = 2.0$ (*see* **Note 10**).
6. Pump 60 L of the diluted spent medium through the bed at 183–260 cm/h, resulting in a bed height that does not exceed 80 cm. Avoid further expansion of the bed height by reducing the flow rate if necessary.

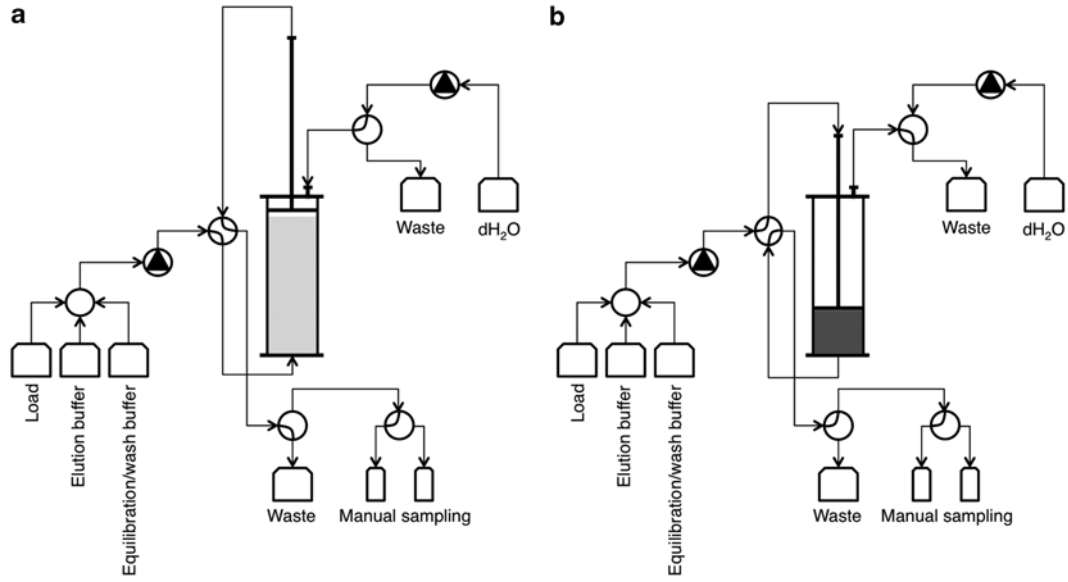


**Fig. 3** Process diagram of expanded bed adsorption (EBA) chromatography: (**a**) shows the expanded bed mode used during equilibration, sample loading, and washing, (**b**) shows the packed bed mode used for elution of the antibody

7. Take samples every 10–20 L and determine the M12 concentration in the flow through (e.g., by analytical protein A chromatography) to monitor the capacity of the resin.
8. Wash with 20 L (equivalent to 10 column volumes, CVs) of ultrapure water at 183 cm/h.
9. Enable packed bed mode by setting all valves to down-flow conditions according to Fig. 3b and pump deionized water at 183 cm/h until a stable packed bed is reached. Lower the top adapter by pumping deionized water in the housing between adaptor and lid. Leave approximately 0.5–1 cm space between the packed bed and the adapter.
10. Bound M12 antibody is eluted using high salt elution buffer that is applied in packed bed mode at 183 cm/h. Collect elution fractions of 1-L portions.
11. The antibody content in the elution fractions is analyzed by analytical protein A chromatography (*see* **Note 11**). The pooled antibody-containing elution fractions comprise a volume of approximately 12 L. Insoluble contaminants are removed by pumping the pooled fractions through a filtration unit consisting of a SUPERCAP 100 PDH4 depth filtration module (Pall, Crailsheim, Germany) connected to a Sartopore 2 150 sterile capsule filter (Sartorius Stedim AG). The clarified solution is further processed in a subsequent round of preparative protein A chromatography.

### 3.5 Preparative Protein A Affinity Chromatography

1. Pour 150 mL of MabSelect SuRe™ into an XK 50/20 column and pack the column according to the manufacturer's instructions.
2. Connect the packed column to the ÄKTAexplorer 100.
3. Equilibrate the column with 3 CVs of glycine, pH 2.8, followed by 10 CVs of PBS, pH 7.4, at 60 cm/h.
4. Load the pooled EBA eluate onto the column at a flow rate of 60 cm/h.
5. Wash the column with 5 CVs of PBS at a flow rate of 60 cm/h.
6. Elute the bound proteins using 5 CVs of 100 mM glycine, pH 2.8, in 20-mL fractions (*see* **Note 12**).
7. Determine the M12 antibody content in the elution fractions by analytical protein A chromatography.
8. Pool the M12-containing elution fractions and use for desalting.

### 3.6 Desalting

1. Connect a pre-packed HiPrepTM 26/10 desalting column to the ÄKTAexplorer 100.
2. Equilibrate the column with 5 CVs of M12 storage buffer.
3. Pass 15-mL aliquots of the elution fractions containing M12 over the column (*see* **Note 13**).
4. Filter the purified antibody through an Acrodisc 32 mm sterile syringe filter with 0.2 μm Supor membrane that is connected to a 150 mL FlexBoy Bag with a luer lock. The bag is stored at 4 °C. Save a small aliquot to determine the antibody concentration and the final recovery.

### 3.7 Antibody Analytics: SDS PAGE and Coomassie Brilliant Blue Staining

1. Perform an SDS PAGE with aliquots of the purified antibody according to the manufacturer's instructions.
2. Place the SDS-polyacrylamide gel into a box and cover it with Fairbanks A solution.
3. Close the box with a lid and heat the solution in a microwave until it begins to boil.
4. Incubate the box at room temperature for 5 min with gentle agitation.
5. Discard the Fairbanks A solution and wash the SDS-polyacrylamide gel shortly with deionized $H_2O$.
6. Cover the SDS-polyacrylamide gel with Fairbanks B solution and repeat **steps 3** and **4**.
7. Cover the SDS-polyacrylamide gel with Fairbanks C solution and repeat **steps 3** and **4**.
8. Cover the SDS-polyacrylamide gel with Fairbanks D solution, add a piece of Whatman paper to remove the remaining dye and repeat **steps 3** and **4**.

9. Discard the Fairbanks D solution and remove the Whatman paper. Add fresh Fairbanks D solution and a new piece of Whatman paper and repeat **steps 3** and **4**.

### *3.8 Antibody Analytics: Analytical Protein A Chromatography*

1. Pour 0.3 mL MabSelect SuRe™ into a YMC EcoPlus 5/125 glass chromatography column and pack the column according to the manufacturer's instructions.
2. Connect the packed column to the ÄKTAexplorer 10.
3. Equilibrate the column with 10 CVs of PBS, pH 7.4, at 611 cm/h.
4. Load the sample onto the column at a flow rate of 92 cm/h.
5. Wash the column with five CVs of PBS at a flow rate of 611 cm/h.
6. Elute the bound proteins using 20 CV of 100 mM glycine, pH 2.8.
7. Determine the antibody content in the elution fractions by comparing the peak area of the M12 elution peak with an M12 standard calibration curve ranging from 4 to 500 µg/mL.

## 4 Notes

1. Prepare a 2 mg/mL stock solution of 2,4-dichlorophenoxyacetic acid by adding 100 mg 2,4-dichlorophenoxyacetic acid to 50 mL dimethyl sulfoxide (DMSO). Dissolve by stirring on a magnetic stirrer. Aliquot the solution in 1 mL portions and store at 4 °C. Add 100 µL stock solution to 1 L MS medium.
2. Prepare a 4 mg/mL stock solution of thiamine-HCl by adding 200 mg thiamine-HCl to 50 mL ultrapure water. Dissolve by stirring on a magnetic stirrer. Aliquot the solution in 1 mL portions and store at 4 °C. Add 250 µL stock solution to 1 L MS medium.
3. Weekly passaging of the cultures is performed, and fresh cultures are inoculated using 3–5 % (v/v) of a 7-day-old pre-culture.
4. The manual B071_030 "How to fit the disposable bag into SB200-X" provides an excellent illustrated guidance through all of the steps. Please contact Kuhner AG for a copy (http://kuhner.com).
5. Ensure that the exhaust filter is open at all times.
6. Confirm the absence of microbial contamination by microscopic evaluation of a medium sample from the 350 L disposable bag. We use 32-fold magnification with a Leitz Diavert phase contrast inverted microscope (Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany).

7. The duration of the cultivation is dependent on the time point at which the cells become oxygen-limited. The dissolved oxygen tension (DOT) is continuously recorded during the cultivation process. Once the DOT drops to 0 %, harvesting should be initiated within the next 24 h.
8. We use 25-L rain barrels.
9. The separated BY-2 cell material is transferred into an autoclaving bag and inactivated by heat sterilization in an autoclave at 121 °C and 100 kPa (15 Psi) for 35 min.
10. The column inlet pressure is constantly measured using an analogue pressure gauge and kept below 100 kPa.
11. Alternatively, M12 antibody quantitation can be determined by sandwich ELISA as described by Kirchhoff et al. [21].
12. Protein elution is monitored by measuring absorbance at 280 nm.
13. The volume will slightly increase (~20 %) during desalting to avoid any loss of protein.

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# Chapter 13

# Liquid-Liquid Phase Separation of Oil Bodies from Seeds

**Cory L. Nykiforuk**

## Abstract

Fundamentally, oil bodies are discrete storage organelles found in oilseeds, comprising a hydrophobic triacylglycerol core surrounded by a half-unit phospholipid membrane and an outer shell of specialized proteins known as oleosins. Oil bodies possess a number of attributes that were exploited by SemBioSys Genetics to isolate highly enriched fractions of oil bodies through liquid-liquid phase separation for a number of commercial applications. The current chapter provides a general guide for the isolation of oil bodies from Arabidopsis and/or safflower seed, from which protocols can be refined for different oilseed sources. For SemBioSys Genetic's recombinant technology, therapeutic proteins were covalently attached to oleosins or fused in-frame with ligands which bound oil bodies, facilitating their recovery to high levels of purity during "upstream processing" of transformed seed. Core to this technology was oil body isolation consisting of simple manipulation including homogenization of seeds to free the oil bodies, followed by the removal of insoluble fractions, and phase separation to recover the oil bodies. During oil body enrichment (an increase in oil body content concomitant with removal of impurities), a number of options and tips are provided to aid researchers in the manipulation and monitoring of these robust organelles.

**Key words** Oil body, Liquid phase separation, Oilseeds, Recombinant oil body, Targeted oil body

## 1 Introduction

Amongst cultivated oilseed crops and a number of oleaginous organisms, lipids are stored within discrete intracellular organelles called oil bodies [1]. Oil bodies have also been commonly referred to as lipid bodies, lipid droplets, oil globules, spherosomes, and oleosomes [2], but for the purposes of this chapter will be referred to herein exclusively as oil bodies. An oil body is composed of a neutral lipid core surrounded by a monolayer of phospholipids embedded with integral oil body proteins, called oleosins, caleosin, and steroleosins [2–4], derived through a budding process off the smooth endoplasmic reticulum concomitant with triacylglycerol (TAGs) bioassembly during seed maturation [5, 6]. Oleosins are anchored to the surface of oil bodies by a highly conserved hydrophobic domain, flanked by amphipathic N- and C-terminal domains [3, 6–8] that confer steric hindrance and a net electronegative

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repulsion [7], and thereby prevent coalescence during desiccation or exposure to freezing [9, 10]. Aside from exploiting oilseeds for the production of modified oils and biofuels [11–19], by virtue of these properties, oil bodies have been utilized for a number of applications including cosmeceuticals, nutraceuticals [20, 21], and pharmaceuticals [22–27]. For recombinant protein expression, seeds have been genetically engineered to express proteins which were covalently targeted to oil bodies as oleosin fusions or expressed as fusion proteins which associate or target oil bodies through affinity capture [22, 24–27]. Other naturally occurring lipoproteins have also been exploited in similar manners [28–31]. As naturally derived emulsions, oil bodies are easily separated under aqueous extraction conditions, using robust methodology, and subsequently enriched and isolated using liquid phase separation [32].

Oil bodies typically range in diameter between 0.5 and 2.0 μm [33, 34]. The size of an oil body is inversely related to the oleosin content [9, 35], but following their isolation, oil body size converts to a fairly uniform distribution dependent upon the species and processing parameters used. Aside from species-specific characteristics, a number of factors should be taken into consideration regarding isolation, including the quality of the seed, composition of the oils, dryness of the seed, and thickness of the seed coat or size of seed. The current methodology can be used to isolate non-transgenic (wild-type) oil bodies, transgenic oil bodies (covalent association with oleosin), or targeted oil bodies (the recombinant protein associates with the oil body through an oil body ligand) from "typical" (*see* **Note 1**) oilseeds or tissues. For the purposes of this chapter, we have limited the selection to non-transgenic *Arabidopsis thaliana* and *Carthamus tinctorius* (safflower) seeds, which represented the model and commercial oilseed platforms utilized at SemBioSys Genetics, respectively, for ease of examples and discussion. For more detailed discussions on recombinant expression in Arabidopsis and/or safflower and recovery of recombinant proteins post-oil body isolation, refer to references herein [22–27].

## 2 Materials

Oilseed material should be of the highest quality possible (*see* **Note 2**). Unless otherwise noted, solutions should be prepared with ultrapure water (Milli-Q water Type 1, http://www.millipore.com/) and chemicals and reagents should be of the highest grade possible. At small scale the manipulation of oil bodies can be conducted by hand (scooping or decanting) using equipment and containers (common glassware; beakers) found in most wet laboratories, whereas at larger volumes (kg of seed) peristaltic pumps (e.g., Watson Marlow 700 series) with stainless steel tri-clamps and hosing along with carboys (50–100 L) and stainless steel tanks

equipped with butterfly valves are typically required. It is also advisable to conduct operations at large scale using platform weighing scales to determine buffer ratios, fraction weights, etc. For the purposes of this chapter, the use of large scale equipment will be mentioned, but safe operation and handling will be deferred to the manufacture's operational manuals.

### 2.1 Seed Pretreatment

1. Distilled water ($dH_2O$).
2. 70 % ethanol (for Arabidopsis).
3. 50 % bleach (sodium hypochlorite; for Arabidopsis).
4. Razor blade and tweezers (for safflower at small scale).

### 2.2 Seed Homogenization

1. Seed homogenization equipment. Depending upon the scale of seed from which oil bodies are to be isolated, typical equipment can include mortars and pestles (including microfuge tubes and pestles; Corning Axygen®), handheld polytrons (Kinematica AG PT6100, Kinematica Inc.), and Warring blenders; to more sophisticated large scale mills (Fryma MZ130 Colloid Mil; Romaco Fryma Koruma) and homogenizers (IKA homogenizer).
2. One of the following standard extraction buffers:

   Extraction buffer A (EB-a): 20–50 mM Tris–HCl pH 8.0, 0.5 M NaCl, with or without 0.4 M sucrose.

   Extraction buffer B (EB-b): 20–50 mM $Na_2HPO_4$–$NaH_2PO_4$, pH 8.0, 0.5 M NaCl.

   Extraction buffer C (EB-c): 20–50 mM $NaH_2CO_3$ pH 8.0.

   Oil body extraction buffers can be prepared as stock solutions, filtered (0.22 μm), and stored at 4 °C, or used immediately after filtration.

### 2.3 Removal of Solids

1. Cheesecloth (for smaller scale), or filtration system with equivalent pore size (for large scale) to remove solids prior to phase separation.

### 2.4 Phase Separation

1. Fixed angle or swinging bucket centrifuge (small scale), or stationary two- or three-phase decanter or disc stacked centrifuge (large scale) for phase separation.
2. Buffer-compatible vessels suitable for desired scale (e.g., centrifuge tube, carboy).
3. Mixing apparatus suitable for desired scale (e.g., vortex, stir bar, impeller).
4. Standard wash buffers:

   Wash buffer A1 (WB-a1): 20–50 mM Tris–HCl, pH 8.0, 0.5 M NaCl, 0.4 M sucrose.

   Final Wash buffer A2 (WB-a2): 20–50 mM Tris–HCl, pH 8.0.

Wash buffer B1 (WB-b1): 20–50 mM $Na_2HPO_4$, pH 8.0, 0.5 M NaCl.

Final Wash buffer B2 (WB-b2): 20–50 mM $Na_2HPO_4$, pH 8.0.

Wash buffer C (WB-c): 20–50 mM $NaH_2CO_3$, pH 8.0.

High stringency wash buffer D (WB-d): 20–50 mM Tris–HCl, pH 8.0, 1–8 M Urea, 0.5 M NaCl with or without 0.4 M sucrose (*see* **Note 3**).

5. Standard formulation buffers:

Formulation buffer A (FB-a): 20–50 mM Tris–HCl pH 8.0. Add Proclin 300 0.2 % v/v after recovery of final oil body phase (OB3 or LP3) to control bacterial growth into final.

Formulation buffer B (FB-b): 20–50 mM $Na_2HPO_4$ pH 8.0. Add Proclin 300 0.2 % v/v after recovery of final oil body phase (OB3 or LP3) to control bacterial growth into final.

Formulation buffer C (FB-c): 20–50 mM $NaH_2CO_3$ pH 8.0. Add Proclin 300 0.2 % v/v after recovery of final oil body phase (OB3 or LP3) to control bacterial growth into final.

Buffers can be prepared as stock solutions, filtered (0.22 μm) and stored at 4 °C, or used immediately after filtration. Note that formulation buffers are identical to final wash buffers, but a broad spectrum antimicrobial (Proclin 300) is added for storage.

### 2.5 Oil Body Analytics

Materials commonly used in the analytics of isolated oil bodies are common SDS-PAGE gel systems, particle size analyzers, pH meters, and conductivity meters. Specific materials discussed here include:

1. 15 % SDS-PAGE gel.
2. 1/10 (v/v) 50 mM Tris–HCl, pH 8.0, with 2 % SDS.
3. 1 M Dithiothreitol (DTT).
4. Polyvinylidene difluoride (PVDF) membrane (e.g., Immobilon-P; Millipore Corporation, Bedford, MA, USA).
5. Anti-oleosin antibody.
6. Particle size analyzer (e.g., Mastersizer 3000, Malvern Instruments Ltd.).
7. Bicinchoninic acid (BCA) protein assay (e.g., Pierce, Rockford, IL, USA).
8. Microbial Count Test Agar (MCTA); 1.5 % w/v pancreatic digested casein, 0.5 % w/v peptone, 0.5 % w/v NaCl, 0.5 % w/v polysorbate 80, 0.07 % w/v lecithin, 1.5 % w/v agar, suspended in deionized water and adjusted to pH 7.3 with NaOH (at 25 °C).
9. 1:1 w/v hexane.
10. Nitrogen gas.

## 3 Methods

The isolation or enrichment of oil bodies is partly a function of tissue from which it is prepared, buffers used, number of washes performed, and scale of isolation. In general, the isolation of oil bodies consists of five steps; (1) homogenization/grinding of plant seeds, (2) removal of solids from the homogenate, (3) separation of the oil body phase from the aqueous phase, (4) washing of the oil body phase to increase purity, and (5) formulation of the washed oil body preparation (emulsion). Throughout and upon completion, the analysis of retained and generated fractions should be performed to determine the level of purity and integrity of oil body. The unit of operations and standard protocols used to achieve oil body isolation by liquid-liquid phase separation at lab scale (<25 g) and pilot scale (>kg) are outlined in Table 1 as a quick reference guide.

### 3.1 Seed Pretreatment

1. Seed surface disinfectant methods include (1) washes of seeds in 70 % ethanol, then 50 % bleach, followed by numerous washes with sterile distilled water are typical for *A. thaliana* seed; or alternatively (2) hot distilled (80 °C) deionized water to remove surface particulates and reduce microbial load in larger scale safflower seed preparations (*see* **Note 4**).
2. For small scale preparations (<5 g), safflower seeds can be dehulled with a sterile razor blade and tweezers. Cut the safflower seed with a sharp razor, longitudinally along the fused pericarp and through the embryo. Remove the embryo from each half of the seed using tweezers or the edge of the razor.

### 3.2 Seed Homogenization

In general, improved grinding/homogenization of oilseed tissue will increase the overall recovery of oil bodies. The homogenization of oilseed tissue should result in a slurry of uniform consistency.

1. Combine seeds with extraction buffer. The choice of extraction buffer is dependent upon the scale of the isolation as a function of cost (*see* **Note 5**, and *see* Table 1). Typical extraction buffers include: (EB-a) 50 mM Tris–HCl, pH 8.0, 0.5 M NaCl, with or without 0.4 M sucrose; (EB-b) 20 mM $Na_2HPO_4$–$NaH_2PO_4$, pH 8.0, 0.5 M NaCl; (EB-c) 20–50 mM $NaH_2CO_3$, pH 8.0. Additional excipients can be added to the buffer systems to facilitate extractions (*see* **Note 6**), but are not typically required for oil body isolations from wild-type (non-transgenic) Arabidopsis or safflower seed. The pH and conductivity of the extraction buffer can also be adjusted to influence the electrostatic interaction of contaminants with oil bodies or influence the solubilization of storage proteins from different seed sources, but these should be determined empirically

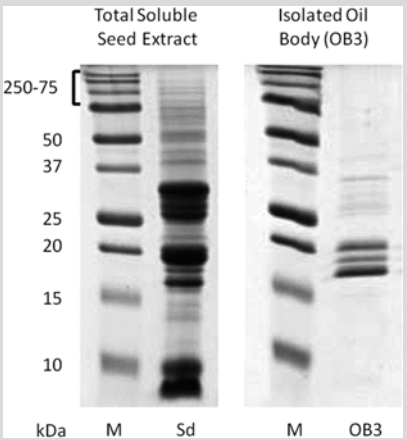
**Table 1**
**Quick reference guide to oil body isolation at various scales. The same methodology is used for wild-type (wt) non-transgenic oilseeds and recombinant oilseeds utilizing SemBioSys technology with direct covalent linkage to oleosin (recombinant OBs) or noncovalent association (targeted OBs)**

| Step | Substep | Rough scale | | | | Fractions generated[a] | Retained fraction[a] |
|---|---|---|---|---|---|---|---|
| | | <1 g (small) | 3–5 g | >25 g | >kg (large) | | |
| Seed selection and pretreatment | Seed selection | *See* **Note 1** | | | | N/A | |
| | Pretreatment | Disinfect (dependent upon quality of seed and/or purpose of oil bodies as per **Notes 2** and **5**) | | | | Intact seed | Intact seed |
| | | Dehull (if thick pericarp present as in Safflower) | | Dehulling not necessary; ground hull and large particulates removed following tissue homogenization | | | |
| | | Imbibe (optional, depending upon the seed source, e.g., flax can be soaked and washed multiple times to reduce mucilage content) but should perform a time course study to determine imbibition and germination occurs [36] | | | | | |
| Tissue Homogenization | Volume (buffer:seed) | 3:1 (v/w) | | 4:1 (v/w) or higher if analysis favorable | | N/A | |
| | Extraction buffer (EB)[b] | EBa | EBa OR EBb | EBa, EBb, or EBc | EBb or EBc | N/A | |
| | Homogenization | Directly in microfuge tube with pestle (<300 mg), handheld glass homogenizer (1–2 g; safflower seed must be de-hulled), mortar and pestle (>5 g), handheld polytron directly in falcon tube (>5 g) | | Handheld polytron, Warring blender | IKA Fryma colloid mill (set grinding gap) In-line homogenizer optional (*see* **Note 9**) | Seed slurry | Seed slurry |
| Removal of solids particulates | Filtration | Proceed directly to centrifugation to separate pellet (P) from undernatant (UND) and oil body (OB) fat pad | Cheesecloth[c] | | RC30 basket centrifuge | Filtered homogenate (HOM) or cake (CAKE; retentate from RC30 basket centrifugation) | HOM |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Tissue separation/oil body washes | First centrifugation | Rotating centrifugation 10,000 × *g* (10 min) or equivalent RCF | | | Two-phase (decanter; SB7, Westphalia) or three-phase (disc stacked; SA1 or SA7, Westphalia) centrifugation | At small scale Pellet (P1), undernatant (UND1) and oil body phase (OB1). At large scale sludge (SL1), heavy phase (HP1) and light phase (LP1) | OB1 at small scale, LP1 at large scale |
| | Wash of OB1 or LP1 | The oil body phase can be removed directly with an autoclaved toothpick or spatula depending upon scale and access OR carefully scrape along the outside edge of the fat pad from the side of the container to gently dislodge and float fat pad in buffer and transfer the fat pad to fresh wash buffer already dispensed in a sterile container | | | Recovery of the LP1 into sterile container (*see* **Note 11**) | | |
| | Wash buffer (WB).[d] Use the same volume as original extraction buffer volume | WB-a1. For high stringency wash use WB-d (or variation as per **Note 3**) | WB-a1<br>OR<br>WB-b1. For high stringency wash use WB-d (or variation as per **Note 3**) | WB-a1, WB-b1, or WB-c | WB-b1 or WB-c | | |
| | Second centrifugation | Rotating centrifugation 10,000 × *g* (10 min) or equivalent RCF | | | Two-phase (decanter; SB7) or three-phase (disc stacked; SA1 or SA7) centrifugation | At small scale Pellet (P2), undernatant (UND2) and oil body phase (OB2). At large scale sludge (SL2), heavy phase (HP2) and light phase (LP2) | OB2 at small scale, LP2 at large scale |
| | Wash of OB2 or LP2 | The oil body phase can be removed directly with an autoclaved toothpick or spatula depending upon scale and access OR carefully scrap along the outside edge of the fat pad from the side of the container to gently dislodge and float fat pad in buffer and transfer the fat pad to fresh wash buffer | | | Recovery of the LP2 into sterile container | | |
| | Wash buffer (WB). Use the same volume as original extraction buffer volume | WB-a2 | WB-a2<br>OR<br>WB-b2 | WB-a2, WB-b2, or WB-c | WB-b2, or WB-c | | |
| | Third centrifugation | Rotating centrifugation 10,000 × *g* (10 min) or equivalent RCF | | | Two-phase (decanter; SB7) or three-phase (disc stacked; SA1 or SA7) centrifugation | At small scale Pellet (P3; typically no longer visible or very small), undernatant (UND3; should appear relatively translucent or clear) and oil body phase (OB3). At large scale sludge (SL3), heavy phase (HP3) and light phase (LP3) | OB3 at small scale, LP3 at large scale |
| | Recovery of OB3 or LP3 | The oil body phase can be removed directly with an autoclaved toothpick or spatula depending upon scale and access OR carefully scrap along the outside edge of the fat pad from the side of the container to gently dislodge and float fat pad in buffer and transfer the fat pad to fresh formulation buffer (FB1, FB2, FB3) | | | Recovery of the LP3 into sterile container | | |

(continued)

**Table 1**
**(continued)**

| Step | Substep | Rough scale | | | | Fractions generated[a] | Retained fraction[a] |
|---|---|---|---|---|---|---|---|
| | | <1 g (small) | 3–5 g | >25 g | >kg (large) | | |
| Formulation | Formulation buffer | At this point dispersal of the OB3 into FB-a, FB-b or FB-c can be concentrated into reduced volumes. Typically use a final volume equal to original seed weight (i.e., if OBs extracted from 100 mg seed would use 100 µl of formulation buffer). If the water content is considered too high at this point, removal can be performed by additional rounds of centrifugation and decanting/siphoning of undernatant | | | LP3 is typically not further diluted but ProClin 300 is added as a preservative | OB3 at small scale, LP3 at large scale | OB3 at small scale, LP3 at large scale preserved or non-preserved |
| Analytical | Identity<br>Purity<br>Characterization | In-process samples from each retained fraction can be analyzed for pH, microbial load, protein content, and purity of oleosins as a marker for oil body isolation, oil content, wet weight, dry weight, and oil body sizing | | | |  | |

[a]Fractions generated are classified by scale. For preparations <25 g the separation of phases can be performed by rotating centrifugation resulting in fractions corresponding to oil body phase (OB), undernatant (UND), and insoluble pellet (P). At larger scales > kg, the use of commercial equipment separates the components corresponding to large particulate and solids as cake (CAKE) and homogenate (HOM) during initial filtration in RC-30 basket centrifuge, followed by separation into the phases of insoluble small particulates (filtrate from basket centrifugation) as sludge (SL), undernatant or heavy phase (HP), and oil body phase or light phase (LP)
[b]Refer to Subheading 2.2, **items 2–4** for extraction buffers (EBs)
[c]The cheesecloth can be squeezed to recover more homogenate
[d]Refer to Subheading 2.3, **items 1–5** for Wash buffers (WBs)

in concert with the quality of the seed by design of experiment (matrix of conditions against oil body yield and purity). It should also be noted that as oil body purity increases, the overall net charge will approach zero at approximately pH 5.5, resulting in clumping and aggregation of oil bodies [7, 21]. Therefore, if pH adjustments are considered useful, long-term exposure near the pI of oil bodies or multiple exposures of the oil bodies to pH extremes through the pI of oil bodies should be avoided to prevent stripping of oleosins, which will exacerbate coalescence and lead to clumping.
Use a seed to buffer ratio of 1:3 (w/w) or 1:4 (w/w) as indicated in Table 1. The buffer ratios can be adjusted depending upon the seed quality (refer to **Note 2**). Alternatively, if purity is consistently poor, the proportion of buffer can be increased to promote better solubilization of host cellular proteins, carbohydrates, etc. Once again, the ratio of seed to buffer should be determined empirically as it relates to overall yield and purity of the final oil body, especially with regard to the first separation following homogenization.

2. After adding buffer to the seed (small scale) or co-mixing of the seed and buffer (large scale; using a hopper for the seed and a pump for the buffer), grind/homogenize the tissue until the embryos have been fully "dispersed" in the solution (*see* **Note 7**). At this point the mixture will appear "milky" throughout.
3. For small scale homogenates proceed to the removal of solids (Table 1 and Subheading 3.3); however, for large scale preparations further homogenization of the seed slurry can be performed using an in-line homogenizer (e.g., IKA homogenizer) to further reduce the size of particulates beyond the setting of the mill (*see* **Note 8**).

### 3.3 Removal of Solids

Particulates and large insoluble debris should be removed from homogenates prior to phase separations.

1. For small scale homogenates, the removal of solids can be performed directly during centrifugation (Subheading 3.4). For mid-scale homogenates, solids can be removed with multilayered cheesecloth (cheesecloth folded over 3 times for eight layers total). For large scale preparations, ground seed hulls and other large particulate matter can be removed from the seed slurry by filtration using a basket centrifuge (e.g., RC-30 basket centrifuge, Rousselet-Robatel) containing a cheesecloth pore size-equivalent filtration bag at a speed of $335 \times g$ (*see* **Note 9**). Collect the filtrate for subsequent separation of the lighter oil body phase (Light phase; LP) from the heavier aqueous phase (Heavy phase; HP).

### 3.4 Phase Separation

The inherent properties of oil bodies lend themselves to separation within aqueous phases. The simplest modality involves centrifugation at lab scale or partitioning of the light phase at large scale. The ultimate goal is to generate enriched oil body devoid of contaminants (OB or LP as per Table 1).

For the separation of the oil bodies, stationary two-phase or three-phase centrifugation (decanter or disc stacked; ~8000 × *g*) at large scale or rotating centrifugation using fixed angle or swinging buckets for smaller scale (up to 10,000 × *g*) are suitable. Retention of oil bodies (homogenates and/or oil bodies) can be performed in suitable buffer-compatible vessels depending upon scale (centrifuge tube to carboy) with appropriately scaled mixing (vortex, stir bar, impeller), but with care to avoid unnecessary shear and mechanical stress or excessive heat. For small scale the use of sterile consumables is preferred with nonconsumables sterilized prior to use by autoclaving. For large scale operations, stainless steel equipment can be pre- and post-sterilized by washing sequentially with 70 % ethanol, 1 M NaOH with 2.5 m/L Foam Force LP (EcoLab), hot (80 °C) deionized water, 20 mM acetic, and rinsed with hot (80 °C) deionized water. Pre-cleaning of large scale equipment is recommended. Post-cleaning of large scale equipment should be conducted in concert with disassembly and final rinsing of components with hot deionized water and allowed to dry prior to re-assembly. Together these extra precautions will help control microbial contamination.

1. At lab scale, initial centrifugation at 10,000 × *g* for 10 min will result in a fat pad (OB1) residing in the uppermost layer. Below the fat pad are an observable undernatant (UND) and typically an insoluble pellet (PEL). For large scale processing, the oil bodies will elute from the decanter/disc stacked centrifuge as the light phase (LP1). Separation using decanter/disc stacked centrifuges is dependent upon the feed rate and ring dam (also referred to as paring ring) size and will need to be determined through testing (*see* **Note 10**). The determination of these settings is obtained by measuring the LP and HP phases along with analytical determinations to track oil body separation toward the LP (the target amount of LP should be >70 %). The first phase separation can be considered paramount because recovery at this point will factor heavily in the overall recovery levels, and should be monitored accordingly. In addition, the buffer conditions during homogenization and primary separation will in part dictate the overall recovery of oil bodies during the first phase separation, and therefore should be determined empirically.
2. At small scale, the initial fat pad (OB1) can be recovered by simplistic means (with autoclaved toothpick or spatula) directly, floated as an intact fat pad, or recovered as the light phase (LP)

following disc stacked/decanter centrifugation (decanted light phase (LP) in two-phase separation or three-phase separation).

3. Wash the recovered oil body phase (OB1 or LP1) twice in standard (WB-a1, WB-a2, WB-b1, WB-b2, WB-c) or stringent (WB-d) wash buffer (according to scale, *see* Table 1) to remove contaminants (*see* **Note 3**).
   Ultimately, the sequential washing and enrichment of oil bodies (OB fat pad or recovered LP) are performed to reduce the level of contaminants and endogenous components. Therefore, additional washes and recovery of the oil body phase can be performed if purity is still not considered high enough, bearing in mind that losses will accrue. In addition, losses are reduced by increasing scale and reducing edge effects (losses through hold up volumes in the decanter/centrifuges; *see* **Note 12**). The consistency of the final oil body phase (OB3 or LP3) will depend upon the amount of remaining buffer, and can be determined (1 – dry weight/wet weight × 100 % = percent buffer). For dry weight, a predetermined weight of oil bodies is dried in an oven at 80 °C until a stable dry weight is obtained. At small scale the removal of excess buffer can be performed by centrifugation and decanting of the undernatant (or syringe through the oil body); however at large scale the adjustment of the ring dam and feed rate will dictate oil body concentration.
4. For small scale, transfer OB3 to formulation buffer (FB-a, FB-b, or FB-c). For large scale, add ProClin 300 preservative to LP3.

### 3.5 Oil Body Analytics

1. During processing of oil bodies, small aliquots should be retained from each fraction (OB, UND, and pellet at small scale, or LP, HP, and SL at large scale) for final analysis and optimization efforts. The identity of oil bodies, and their recombinant payload, is easily determined by the presence of oleosin in SDS-PAGE gels (Table 1) or Western blotting. However, additional analysis should be performed including particle size analysis, pH, and conductivity.
   Weigh each fraction using tared receptacles (e.g., microcentrifuge tube, blue capped falcon tube, container, etc.). For SDS-PAGE, solubilize fractions by boiling in 1/10 (v/v) 50 mM Tris–HCl, pH 8.0 with 2 % SDS. For reducing conditions, add 1 M DTT to a final concentration of >5 mM just prior to boiling samples. Separate on 15 % SDS-PAGE gel, standardized across fractions. For Western analysis, blot separated proteins to polyvinylidene difluoride membrane and probe with anti-oleosin [36] (*see* **Note 11**). Protein content can also be assayed from SDS solubilized fractions using a bicinchoninic acid (BCA) protein assay in the absence of reductant.

2. Microbial growth should be assessed and remain under 100 cfu/g. Plate aliquots of known weight in volumes ranging from 50 to 200 μl in triplicate on microbial count test agar (MCTA) and incubate at ~35 °C for 2–3 days.
3. Free oil can also be assessed by hexane washes. Briefly, an aliquot of oil bodies (200–300 μl) of known weight is washed twice with hexane (1:1 w/v). The oil bodies will remain intact and free oil will partition to the organic phase after centrifugation ($555 \times g$ for 2 min). The upper organic phases are collected by pipetting, without disturbing the interphase, into preweighed microcentrifuge tubes. After pooling of the organic phases, the samples are dried under nitrogen at 42 °C and free oil assessed gravimetrically. Free oil content should not exceed 1.0 % by weight of the total oil content (HIP extracted weight).

## 4 Notes

1. A typical oil body in the context of this chapter refers to those isolated from common agronomic species including rapeseed (*Brassica* spp.), soybean (*Glycine max*), sunflower (*Helianthus annuus*), cottonseed (*Gossypium* spp.), castor (*Ricinus communis*), safflower (*Carthamus tinctorius*), mustard (*Brassica* spp. and *Sinapis alba*), coriander (*Coriandrum sativum*), squash (*Cucurbita maxima*), and linseed/flax (*Linum usitatissimum*). For flax the presence of mucin (mucilage) can complicate oil body extractions, which can be partially mitigated by sequential washes of the seed with distilled $H_2O$ prior to homogenization.
2. As with any process, the quality of the feedstock is paramount in generating high-quality product. For oil body isolation, seed quality is no exception to this rule. The key attributes to consider in quality is the physical appearance (color, size/weight), intactness of the seed coat (cracks in seed coat or emerging radicle suggest that the seed was wet and imbibition resulted in premature germination), and overall cleanliness with respect to soil, animal droppings, etc., oil content (proportion and composition), and biochemical composition (dryness and protein content). A quick physical inspection of the seed may provide an indication of whether disinfection should be performed or whether a different seed lot should be chosen. If there are no alternative sources of seed, the size and weight can indicate incomplete seed filling (smaller embryos within thick seed hull for Safflower). Oil content should also be determined through HIP extraction of total lipids [26]. In the case of poor seed fill or low oil content, smaller homogenization and washing volumes can be adjusted accordingly, but typically the oil body preparation will be disproportionally unclean.

3. High stringency washes with a chaotrope, such as Urea, can increase the purity of the recovered oil bodies. The stringency of the wash will increase with increased molarity of urea in the buffer. Following the urea wash, subsequent washes in wash buffers (WB-a1, WB-a2, WB-b1, WB-b2, WB-c) will reduce and remove urea from the oil bodies. Contaminants can also be removed through washes with detergents, or increased salt and neutral lipids (*n*-hexane) [37], but will need to be removed to stabilize the oil body preparation.
4. If desired, seed pretreatments can be performed at all scales to remove contaminating particulate and microbial load. The necessity and stringency of the pretreatments is dependent upon what the isolated oil bodies are to be used for and the risk of microbial contaminants in subsequent analysis or preservation of the oil bodies. For surface sterilization of *A. thaliana* seeds (or equivalent, e.g., canola, without a seed hull), the use of NaOCl is recommended, whereas hot deionized water is more suitable for Safflower (or equivalent, e.g., sunflower, with a seed hull) at large scale.
5. For bench-scale preparations (<100 g) Tris or phosphate-based buffers are typically used, whereas sodium bicarbonate-based buffers can be used for large scale preparations. The key parameters are slightly alkaline pH and low salt/conductivity. For small scale preparations, sucrose up to 0.4 M is typically added to increase the density of the extraction buffer and/or form a more compact fat pad after centrifugation.
6. Depending upon the source of the seed (species and quality) additional excipients including but not limited to reductants (e.g., dithiothreitol), redox pairings (e.g., cysteine:cystine), non-ionic detergents (e.g., tween, Triton-X100, etc.) can be added to facilitate removal of contaminants from the surface of the oil body. Also, high stringency wash buffers are used for these purposes (*see* **Note 3**).
7. Large scale seeds are homogenized with a colloid mill (e.g., MZ130, Fryma Inc.) and testing of the homogenization method can be performed by assessing the amount of free oil (mechanical disruption of oil bodies). Adjustment of the grinding gap setting (of teeth) will also influence the homogenization of seeds and can be adjusted accordingly.
8. The use of in-line homogenizers can be replicated by recycling of the collected seed slurry from a colloid mill with the use of pumps. In this scenario, the grinding buffer container is placed downstream of the colloid mill and used as the source of grinding buffer. Seed is fed into the mill but continuously looped with a pump to recirculate the seed slurry/homogenate through the mill. Once the homogenate is considered

sufficient, the butterfly valve from the container is closed and a final volume of buffer is applied to flush mill.

9. Removal of solids using a basket centrifuge lined with cheesecloth equivalent filtration bag can be operated at 335 × *g* until the majority of liquid has been recovered from the homogenate. Additional liquid can be recovered (i.e., squeezed) from the remaining solid fraction by increasing the centrifuge to 755 × *g* for a period of time until the recovery of remaining liquid is complete
10. Removal of solids and aqueous fractions is performed using 2 phase separation (HASCO200 2-phase decantation centrifuge, NX 310-B, Alpha Laval, or SB-7, Westphalia) and/or 3 phase separation (SA-1 or SA-7, Westphalia). Critical parameters are the size of the ring dam (removable rings with a central circular opening varying in size) to regulate the separation of the aqueous phase from the oil body phase (~29–30 mm). Other factors are the feed rate (set by peristaltic pump) and temperature/viscosity of the feed. Refer to manufacture's manuals to adjust settings and change the ring dam.
11. Another manner in which to analyze oil bodies is to delipidate the OB or LP phase. These provide highly resolved bands on reduced SDS-PAGE gels. For this optional approach the fraction is delipidated in 70 % acetone, followed by centrifugation at 10,000 × *g* for 10 min; the pellet is washed in 100 % acetone and centrifuged again. The pellet is solubilized in 1 M KOH (original fraction volume) and heated gently at 42 °C. The solubilized protein can then be neutralized and solubilized in SDS-PAGE loading buffer with reductant prior to separation.
12. The separation and recovery has also been modified into a continuous process. In this instance the LP1 is mixed in-line with buffer for washing and subsequent separation in the next disc stacked centrifuge. This is repeated again using in-line mixers and pumps. For the purposes of these discussions the recovery of each phase is needed to optimize the recovery of oil bodies and therefore, continuous processing of oilseeds is not described but is figuratively outlined elsewhere [22].

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# Chapter 14

# Continuous Flow Separation of Hydrophobin Fusion Proteins from Plant Cell Culture Extract

**Lauri J. Reuter, Andrew J. Conley, and Jussi J. Joensuu**

## Abstract

Fusion to fungal hydrophobins has proven to be a useful tool to enhance accumulation and recovery of recombinant proteins in plants. Aqueous two-phase separation (ATPS) is an attractive system to capture hydrophobin fusion proteins from plant extracts. The process can simultaneously purify and concentrate target protein with minimal background. ATPS avoids the use of chromatographic column steps, can be carried out in a short time frame, and is amenable to industrial-scale protein purification. A drawback of performing ATPS in large volumes is the lengthy time required for phase separation; however, this can be avoided by incorporating continuous systems, which are often preferred by the processing industry. This method chapter illustrates the capture of GFP-HFBI hydrophobin fusion protein from BY-2 plant cell suspension extract using a semi-continuous ATPS method.

**Key words** Continuous aqueous two-phase system, ATPS, Hydrophobin, HFBI, Protein purification, Protein fusion

## 1 Introduction

### 1.1 Hydrophobins

Hydrophobins are small, surface-active fungal proteins with various functions in the fungal life cycle including adhesion, aerial growth, and coating of spores. Hydrophobins have a unique structure, in which part of the surface is occupied by conserved aliphatic side chains forming an exposed hydrophobic region called a "hydrophobic patch." The core of the protein is stabilized by eight conserved cysteine residues forming four intramolecular disulfide bridges [1, 2]. Exposure of the hydrophobic patch is responsible for the amphipathic nature of these proteins and makes them act in many ways like typical detergent molecules. Therefore, hydrophobins have many potential applications for coupling and absorbing proteins with nonbiological surfaces [3]. Because of their strong surface-active properties, hydrophobins are also capable of altering the hydrophobicity of their fusion partners, thus enabling purification using a surfactant based aqueous two-phase

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system (ATPS) [4]. In ATPS, a surfactant is added to a crude protein extract to trigger phase separation. While the majority of proteins remain in the aqueous phase, hydrophobins concentrate in the surfactant phase due to their interaction with the micellar surfactant structure. The hydrophobin fusion protein can be then recovered from the surfactant phase with nondenaturing organic solvents like isobutanol [5].

### 1.2 ATPS and Plants

For a successful ATPS, conditions must exist where the aqueous surfactant mixture forms a two-phase system. Nonionic surfactants (i.e., the surfactant molecule does not have a net charge in aqueous solution) that phase separate in a temperature-dependent manner have been most successful for hydrophobin purification. For practical reasons the temperature at which the phase separation occurs should be in the range of 5–30 °C [6]. Several such surfactants exist, one example being Triton X-114 [7, 8].

In addition to separation temperature there are several factors that influence the outcome of ATPS-based protein purification. These include reaction pH, ionic composition and strength and presence of solids and surfactant concentration [9]. Surfactant concentration has a major effect on protein purification parameters in ATPS. Typically, a higher surfactant concentration leads to better protein recovery but lower concentration of purified protein. Conversely, high protein concentrations can be achieved with low surfactant concentrations, but with poor recovery (Fig. 1a). Both surfactant concentration and reaction volume impact the time required for the phase separation (Fig. 1b). While a volume of 10 mL can separate within a few minutes (Fig. 1b), a larger ATPS volume of 20 L requires 90 min for separation [10]. ATPS is an attractive method for industrial-scale protein purification since it can simultaneously concentrate and purify the target protein. However, the long separation time required for the large batch volumes can limit the output and usefulness of this technique. Continuous protein phase separation can facilitate the handling of high volumes and improve the ATPS speed, and may be especially well suited for sensitive target molecules that require fast processing. Plants and plant cell culture are attractive hosts for expression of recombinant hydrophobin fusion proteins for two major reasons. First, it has been reported that hydrophobin fusions can increase the accumulation of their recombinant fusion partner through the formation of protein bodies [11, 12]. Second, there are few endogenous plant proteins that co-purify with hydrophobins when using the ATPS method, making the purification procedure efficient and effective [10, 11, 13]. This chapter illustrates the capture of GFP-HFBI hydrophobin fusion protein from tobacco BY-2 cell suspension extract using a continuous ATPS method.

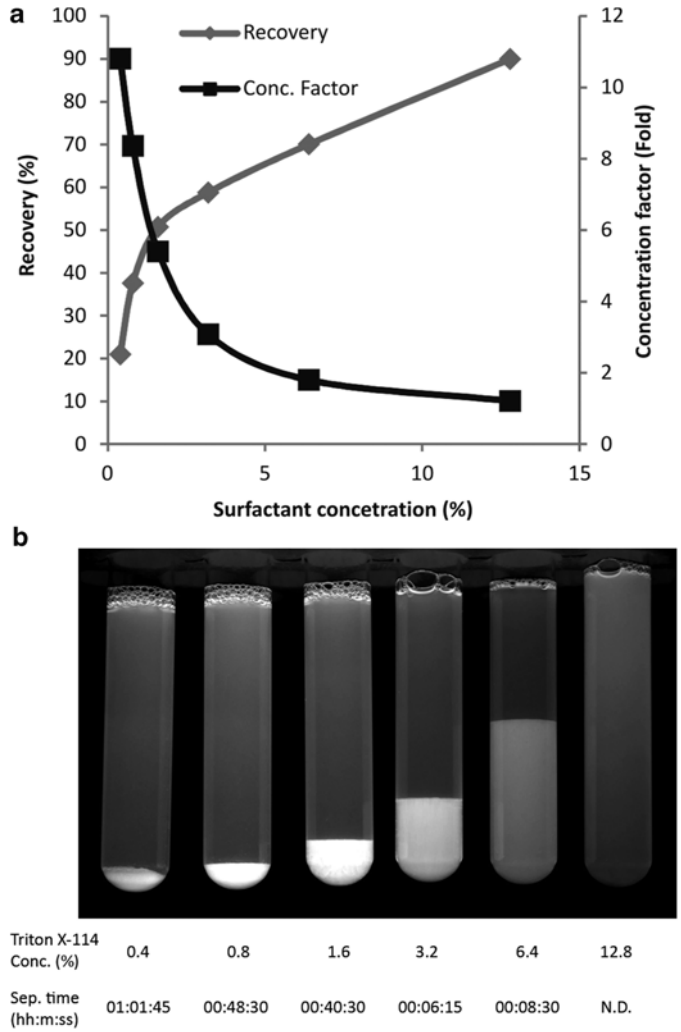


**Fig. 1** Influence of surfactant concentration on aqueous two-phase separation. (**a**) Effect of Triton-X114 surfactant concentration on recovery (*left y*-axis) and concentration (*right y*-axis) of GFP-HFBI protein from BY-2 suspension culture extract. Pre- and post-separation samples were quantified by fluorometry. (**b**) Effect of Triton-X114 concentration on phase separation time of GFP-HFBI protein. The ATPS experiment with various surfactant concentrations (0.4–12.8 %) was monitored under UV light by time-lapse photography. The phase separation was considered to be complete when no more GFP-HFBI movement toward the surfactant phase was observed

## 2 Materials

All solutions and equipment should be clean, sterile, and detergent-free. Water should be deionized, distilled, and autoclaved (conductivity <10 μS). Suitable eye protection, lab coat, and gloves should be used for chemical handling.

### 2.1 Protein Extraction from BY-2 Cells

1. Tobacco BY-2 cell suspension culture expressing a protein of interest fused to a hydrophobin HFBI tag. In this example BY-2 cells expressing GFP-HFBI fusion was used for easy visualization. For construct details and BY-2 transformation *see* refs. [10, 11] (*see* **Note 1**).
2. Buchner filtration funnel with suitable filter paper (Whatman GF/B), and filter flask with a vacuum source.
3. Freeze dryer.
4. Large ceramic mortar and pestle.
5. Protein extraction buffer: 1× phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, 1.8 mM $KH_2PO_4$ pH 7.4.), 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), 100 mM Na-ascorbate, 0.4 μM leupeptin.
6. Refrigerated centrifuge (e.g., Sorvall RC-5C).
7. Miracloth filter cloth (Millipore).

### 2.2 Continuous Flow Aqueous Two-Phase Separation (ATPS)

1. Beaker and a magnetic stirrer plate.
2. Separation funnel (100 mL volume).
3. Triton X-114 surfactant (Sigma-Aldrich).
4. 1-2 peristaltic pumps (e.g., BioRad Econo pump) with suitable tubing and connectors.
5. Isobutanol.
6. Laboratory pipettes with suitable tips for liquid handling, plastic test tubes.

### 2.3 SDS-PAGE Gel Analysis

1. Tris–HCl polyacrylamide Any kD Ready gel (BioRad).
2. SDS-PAGE running buffer: 25 mM Tris, 200 mM glycine, and 0.1 % (w/v) SDS.
3. MiniProtean III SDS-PAGE electrophoresis apparatus (BioRad).
4. 4× SDS-PAGE loading buffer: 240 mM Tris–HCl, pH 6.8, 40 % glycerol, 8 % (w/v) SDS, 0.1 % (w/v) bromophenol blue, and 5 % (v/v) 2-mercaptoethanol.
5. GelCode Blue Stain Reagent (Thermo Scientific).

## 3 Methods

### 3.1 Protein Extraction from BY-2 Cells

1. Harvest 400 mL of BY-2 suspension culture, at the peak of product accumulation, by vacuum filtration. Scrape the cells into a preweighed container and freeze-dry overnight. Weigh the cells after freeze-drying. You should get about 5 g of freeze-dried cells from 400 mL of culture (*see* **Note 1**).

2. Grind the freeze-dried material into a fine powder using a mortar and pestle at room temperature.
3. Set up a beaker on a magnetic stirrer plate and mix the cell powder with 30 volumes of protein extraction buffer (i.e., add 30 mL buffer per 1 g powder) for 5 min at room temperature (*see* **Note 2**).
4. Centrifuge the cell suspension for 15 min, 4000 × *g* at room temperature and collect the supernatant by pouring it through Miracloth filter cloth. You should be able to collect about 110 mL of protein extract.
5. Remove 75 μL of sample for SDS-PAGE and mix it with 25 μL of 4× SDS-PAGE loading buffer (Fig. 3, TSP).

### 3.2 Continuous Flow Aqueous Two-Phase Separation (ATPS)

1. Measure the volume of the remaining protein extract and weight 4 % (W/V) of Triton X-114 in a beaker. Set the beaker on a magnetic stirrer and add the protein extract. Premix solution for 5 min (*see* **Note 3**).
2. Prepare 1–2 peristaltic pumps with suitable tubing. *See* Fig. 2 for setup illustration.
3. Pour 40 mL of the protein extract-Triton X-114 mixture to a separation funnel and let the phases pre-separate for 10 min. Place the beaker with remaining solution back on mixer plate.
4. Set up the first peristaltic pump between the beaker and the separation funnel and start feeding the liquid at 1 mL/min flow rate to the separation funnel. Note that the protein extract should drain slowly at the top of the pre-separated phases. This can be achieved easily if the end of the tubing is touching the wall of separation funnel. To avoid disturbing the phase separation, do not let the solution drip from a distance (*see* **Note 4**).
5. Open the funnel tap very carefully and start draining the lower phase into a collection vessel (e.g., 50 mL plastic Falcon tube). A suitable flow rate is 0.6 mL/min. Alternatively, another peristaltic pump can be placed between the bottom of the separation funnel and the collection vessel. *See* Fig. 2b. Let the phase separation continue until the feed supply is empty.
6. When you have finished collecting the surfactant (lower) phase, sample 75 μL of the aqueous (upper) phase to be analyzed by SDS-PAGE and mix it with 25 μL of 4× SDS-PAGE loading buffer (Fig. 3, residue).
7. Mix the collected surfactant phase with equal volume of isobutanol in a 50 mL Falcon tube and mix by rolling gently for 5 min. Let the phases separate for 10 min and collect the bottom phase (*see* **Note 5**).
8. Take 75 μL of sample to be analyzed on SDS-PAGE and mix it with 25 μL of 4× SDS-PAGE loading buffer (Fig. 3, back extract continuous).

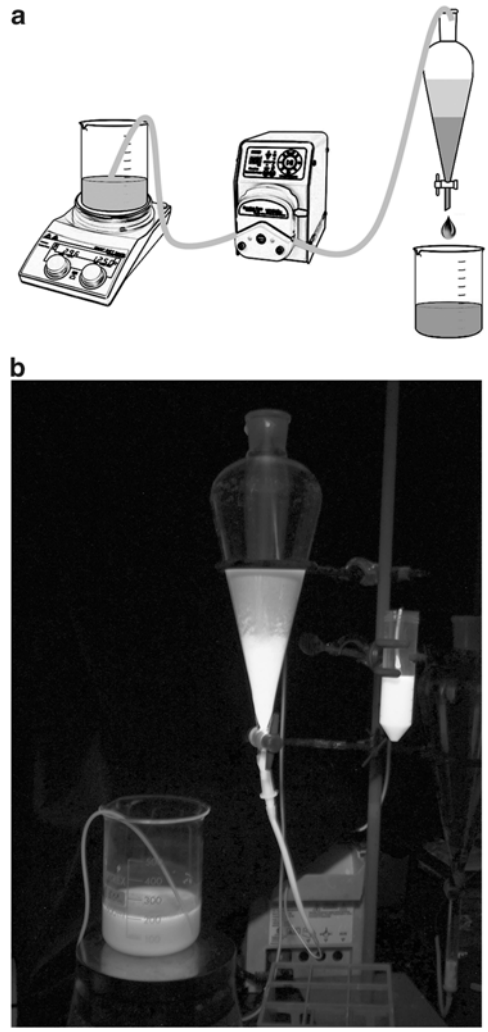


**Fig. 2** Experimental setup for continuous aqueous two-phase separation. (**a**) Schematic representation of required equipment: magnetic stirrer, peristaltic pump, and separation funnel. (**b**) Continuous ATPS of GFP-HFBI protein from BY-2 suspension culture extract. The purification setup was imaged under UV illumination

### *3.3 SDS-PAGE Gel Analysis*

1. Boil the three collected samples (75 μL + 25 μL of 4× SDS-PAGE loading buffer) for 5 min.
2. Prepare a polyacrylamide gel of appropriate percentage and load 5–10 μL of the samples per well.
3. Run the gel at 200 V until the dye front reaches the end of the gel.
4. Disassemble the running apparatus and desalt the gel twice for 15 min with $H_2O$.
5. Stain the gel overnight with GelCode Blue Stain Reagent.
6. Destain the gel with $H_2O$ for at least 1 h.

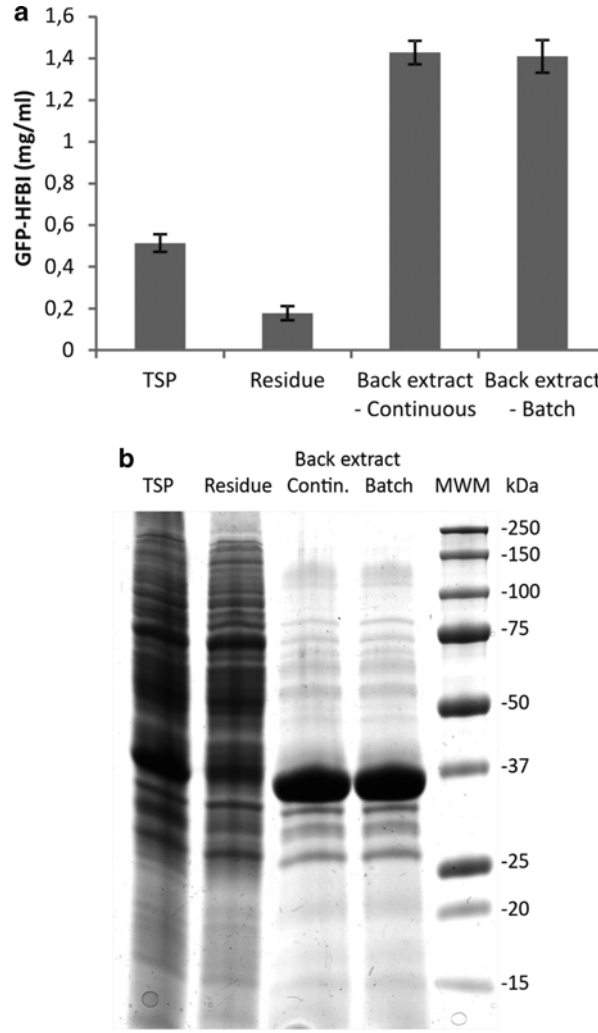


**Fig. 3** Continuous aqueous two-phase separation of the GFP-HFBI fusion protein from BY-2 suspension culture extract with 4 % Triton X-114 surfactant. GFP-HFBI purification was performed in continuous and batch mode and samples were analyzed by fluorometric quantification (**a**) and SDS-PAGE gel (**b**). GFP-HFBI fusion protein (37 kDa) was recovered in similar purity and concentration with the continuous process when compared to the batch ATPS separation. *TSP* total soluble protein, *MWM* molecular weight marker. Equal volumes (5 μL) of the TSP and ATPS phases were loaded onto the gel

## 4 Notes

1. The same protocol can be applied for hydrophobin fusion proteins expressed in stably or transiently transformed tobacco plants. For leaf tissue, use frozen material and homogenize either in a mortar with liquid nitrogen or by using a homogenizator with cold extraction buffer.

2. For labile target proteins it is preferable to do the protein extraction and following centrifugation at +4 °C.
3. For a successful phase separation the ambient working temperature should be above the cloud point of Triton X-114 (22 °C). The cloud point can be lowered by adding glycerol [8].
4. The feed flow rate can be increased to at least 2 mL/min with this setup. The drain rate for the surfactant phase should be about 60 % of the feed rate. An additional peristaltic pump can be connected to drain the aqueous top phase. To speed up separation of large volumes, several parallel separation vessels can be set up.
5. The isobutanol and surfactant are mixed gently and allowed to separate. The separation can be sped up by spinning for 5 min at 4000 × *g* in a tabletop centrifuge equipped with a swing-out rotor. The solution separates into two phases: the upper phase contains a mixture of isobutanol and surfactant, while the lower phase is an aqueous phase containing the hydrophobin fusion protein. The fusion protein is typically quite abundant relative to other plant proteins (Fig. 3), but this aqueous solution is saturated with isobutanol. The further use of the target protein may require removal of residual isobutanol. This can be achieved for example by dialysis, diafiltration, or using a gel filtration column such as the BioGel P-6 (BioRad).

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# Part IV

## Analysis

# Chapter 15

# Molecular Analyses of Transgenic Plants

**Kurniawan Rudi Trijatmiko, Felichi Mae Arines, Norman Oliva, Inez Hortense Slamet-Loedin, and Ajay Kohli**

## Abstract

One of the major challenges in plant molecular biology is to generate transgenic plants that express transgenes stably over generations. Here, we describe some routine methods to study transgene locus structure and to analyze transgene expression in plants: Southern hybridization using DIG chemiluminescent technology for characterization of transgenic locus, SYBR Green-based real-time RT-PCR to measure transgene transcript level, and protein immunoblot analysis to evaluate accumulation and stability of transgenic protein product in the target tissue.

**Key words** Transgene, Transformation, Gene expression, qRT-PCR, Southern blot, Western blot, Digoxigenin, Chemiluminescent, SYBR Green

## 1 Introduction

A large number of transgenic plants must typically be generated in order to obtain plants that express transgenes stably over generations. While the patented genome editing technologies TALEN and CRISPR can improve efficiency, these are not readily available. It is therefore a challenge to identify the limited number of transgenic plants, from among the thousands generated, that will express the transgene stably over generations [1]. Once transgenic plants are identified, a variety of analytical tools can be used to study the transgene and its products. Southern blot can be used to determine transgene copy number and characterize the transgenic locus; real-time quantitative RT-PCR can be used to analyze the transcript levels of the transgene; and protein immunoblot analysis, or Western blot, can be used to evaluate the accumulation of recombinant proteins.

Plant transformation methods, such as *Agrobacterium-mediated* transformation and direct gene transfer, can result in the integration of one or multiple intact or rearranged gene copies at one or multiple unlinked loci [2]. Whereas most single-

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copy integration events express the transgenic protein in a stable and predictable manner, multiple copies of transgene integrated at one locus in head-to-head or tail-to-tail arrangements generally result in silencing [1]. Transgenic events also need to be characterized for regulatory approval prior to commercial release of transgenic products. Approval may additionally require that the inserted DNA fragment be intact, that there are no DNA rearrangements at the integration site or elsewhere in the genome, and that there is no transfer of vector DNA such as an antibiotic resistance gene or origin of replication. DNA blot analysis, originally described by Southern [3], can provide definitive information about the transgenic locus structure (Fig. 1), show the absence of unintended plasmid backbone sequence, and demonstrate the stable heritability of inserted DNA sequences over a number of breeding generations.

For this purpose, digoxigenin (DIG)-labeled probes have been reported to be equivalent to radioactively labeled probes under optimized conditions for detection of single-copy transgenes in genomes of transformed plants [4, 5]. Advantages of using the DIG system include avoidance of using radioactive labels, ease of probe preparation, extended storage, and multiple use of the same probe [6].

Using the DIG system, genomic DNAs digested with restriction enzyme are separated by agarose gel electrophoresis, denaturated by alkali treatment, and transferred to nylon membrane. A probe labeled with DIG antigen is denaturated by heating and hybridized to the single-strand DNA fragments on the membrane. An alkaline phosphatase-conjugated antibody is bound to the DIG antigen on the membrane. Detection of alkaline phosphatase activity with a chemiluminescent substrate results in light emission that can be recorded by exposure to X-ray film (Fig. 2).

**Fig. 1** (continued) configuration, and (**c**) two copies in a head-to-tail configuration. The T-DNA is indicated by the *thick grey box*, with flanking restriction sites from the host genomic sequence also shown (*top*). Fragment sizes, which may bind to Probe 1 and/or Probe 2, are indicated (*bottom*) for DNA digested with *AscI*, *BamHI*, or *AvrII*. In all cases of intact T-DNA insertion at a single locus, without intervening genomic sequence, a single band is expected from *AscI* or *AvrII* digests using either Probe 1 or Probe 2. (**a**) In the case of single-copy T-DNA insertion, a single band is also expected from the *BamHI* digest using either probe. Note that actual sizes of bands from *BamHI* and *AvrII* digests would be variable, depending on the nearest restriction sites in the genome. (**b**) In the case of T-DNA inserted at a single locus in a head-to-head configuration, a *BamHI* digest would result in a single band using Probe 1 and two bands using Probe 2. Actual sizes of bands from *AvrII* and *BamHI* digests would be variable, depending on the nearest restriction site in the genome. (**c**) In the case of T-DNA inserted at a single locus in a head-to-tail configuration, a *BamHI* digest would result in two bands, using either Probe 1 or Probe 2. Actual sizes of bands from *AvrII* and *BamHI* digests would be variable, depending on the nearest restriction site in the genome

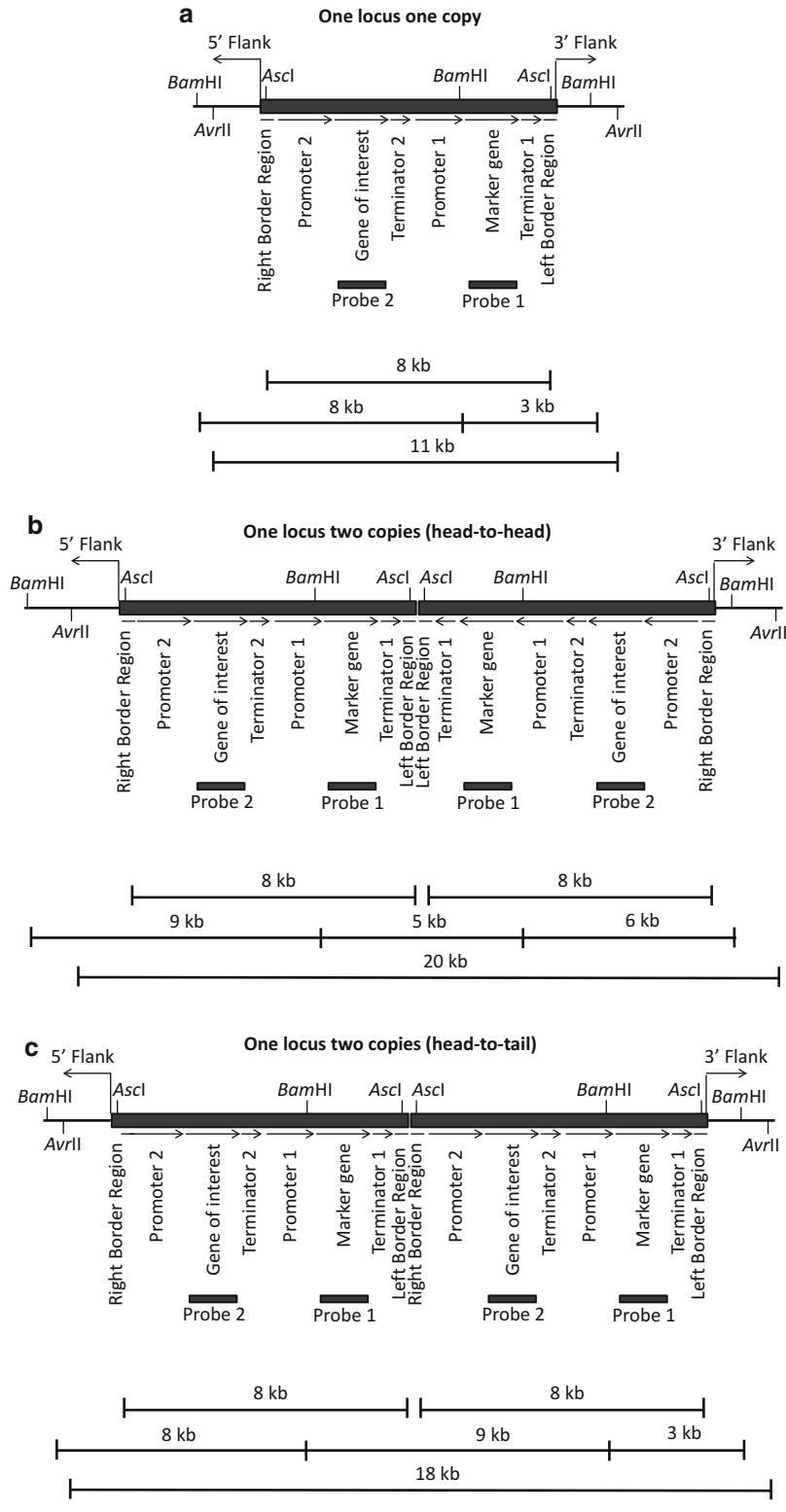


**Fig. 1** Schematic diagrams illustrating the expected bands in Southern blot using different enzyme/probe combinations in the case of T-DNA inserted at a single locus in (**a**) a single copy, (**b**) two copies in a head-to-head

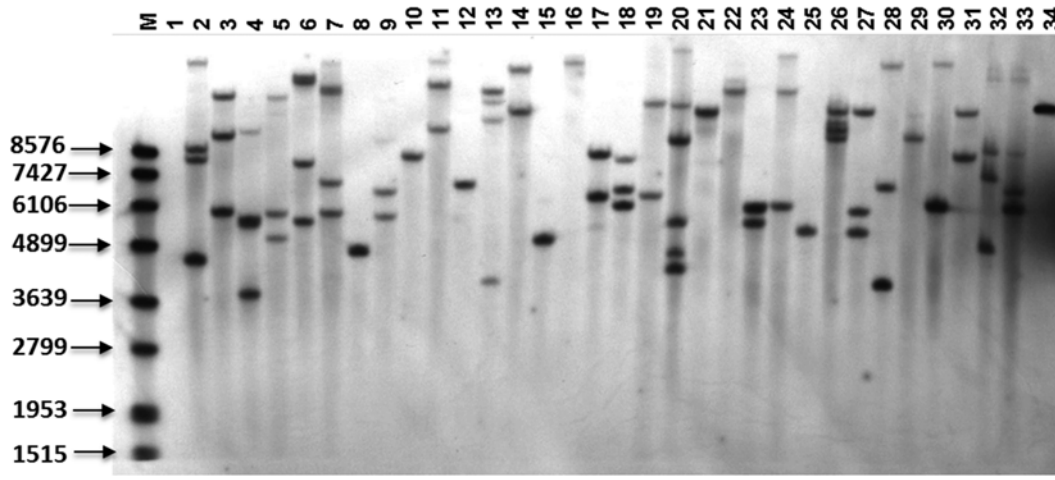


**Fig. 2** Southern blot analysis of DNA isolated from transgenic rice plants demonstrating different copy numbers. Ten micrograms of rice genomic DNA was digested with *Bam*HI (cuts once within the transgene) and hybridized to the DIG-labeled marker gene probe. *Lane M*: DIG-labeled DNA molecular weight marker, with sizes indicated in bp. *Lane 1*: DNA from an untransformed plant. *Lane 2–33*: DNA from different transgenic plant lines. Single bands in lanes 8, 10, 12, 15, 16, and 25 suggest a single transgene insertion; however, the blot would need to be reprobed with a probe that hybridizes on the opposite side of the *BamHI* site to be sure (*see* Fig. 1). *Lane 34*: 400 pg of *Bam*HI digested binary plasmid

The presence of a single-copy, intact transgene, as determined by Southern blot, can contribute to the successful expression of a transgene. However, it does not necessarily lead to sufficient gene expression. The level of transcript accumulation is another contributing factor and can be measured using real-time quantitative RT-PCR [7]. The availability of nonspecific double-stranded DNA (dsDNA) binding fluorophores, such as SYBR Green, and 96- or 384-well-plate compatible real-time PCR machines that can measure fluorescence at the end of each PCR cycle make it possible to perform qRT-PCR on hundreds of samples in parallel [8].

Generally two quantification types in real-time RT-PCR are possible: relative quantification and absolute quantification [9]. Relative quantitation measures changes in the expression of a target gene relative to an invariant control gene. Housekeeping genes (e.g., glyceraldehyde-3-phosphate dehydrogenase, ß-actin, 18S rRNA, etc.) that are not expected to change under the experimental conditions can serve as a convenient internal standard. Because the absolute quantity of the internal standard is not known, only relative changes can be determined by this method [10]. This may be adequate for most transgenic research projects because the fold change may be informative irrespective of the absolute value. The advantage of relative quantification is that the absolute values of the genes used for standards are not required.

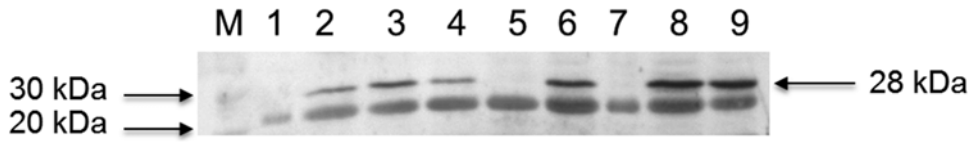


**Fig. 3** Western blot analysis of recombinant soybean ferritin protein in transgenic rice seeds. Fifty micrograms of total protein extracted from ten polished seeds of each plant was separated by SDS-PAGE and immunoblotted with the soybean ferritin rabbit polyclonal antibody. *Lane M*: Molecular weight marker. *Lane 1*: protein from an untransformed plant. The band at 22 kDa is the endogenous (native) ferritin protein that is also detected by the polyclonal antibody. *Lane 2, 3, 4, 6, 8, and 9*: protein from transgenic segregants of line 1. *Lane 5 and 7*: protein from a null segregant of line 1

While real-time RT-PCR can be used to measure transcripts, protein immunoblot analysis [11] is a powerful tool to evaluate the accumulation and stability of the transgenic protein in the target tissue. Proteins separated by electrophoresis are transferred to a solid support, such as nitrocellulose membrane. A primary antibody is bound to a specific antigen on the membrane, and this antibody is detected using alkaline phosphatase or horseradish peroxidase-linked secondary antibody. Detection of alkaline phosphatase or horseradish peroxidase activity with an appropriate colorimetric substrate produces a color on the membrane (Fig. 3).

## 2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials.

### *2.1 Southern Hybridization Using DIG Chemiluminescent Technology*

1. PCR DIG Probe Synthesis Kit (Roche) or similar.
2. Plasmid.
3. Transgene-specific primers. The primers should be designed to amplify part or full length of coding sequences. The size of the amplicon is ideally between 100 and 150 bp for qRT-PCR while for probe the amplicon could be between 200 and 1000 bp.
4. Thermal cycler.
5. Restriction enzymes (*see* **Note 11**) and appropriate buffers.
6. 3 M sodium acetate, pH 5.2. Add about 80 mL water to a glass beaker. Weigh 40.8 g sodium acetate trihydrate ($CH_3COONa\ 3H_2O$) and transfer to the glass beaker. Mix and adjust the pH with glacial acetic acid. Make up to 100 mL with water.
7. 100 % and 70 % ethanol.

8. Vacuum dryer.
9. 1 M Tris–Cl, pH 7.5. Add about 800 mL water to a glass beaker. Weigh 121.4 g Tris (hydroxymethyl) aminomethane and transfer to the glass beaker. Mix and adjust the pH with HCl. Make up to 1 L with water.
10. 0.5 M EDTA, pH 8.0. Add about 80 mL water to a glass beaker. Weigh 18.6 g EDTA and transfer to the glass beaker. Mix and adjust the pH with NaOH. Make up to 100 mL with water.
11. 1× TE buffer. Add about 800 mL water to a 1-L graduated cylinder. Add 10 mL 1 M Tris–Cl pH 7.5 and 2 mL 0.5 M EDTA pH 8.0. Mix on a stirrer. Make up to 1 L with water.
12. 6× Ficoll loading dye. Add about 30 mL water to a 50-mL polypropylene centrifuge tube. Add 7.5 g Ficoll 400, 3 mL 1 M Tris–Cl pH 7.5, 6 mL 0.5 M EDTA pH 8.0, 0.125 g Orange G. Make up to 50 mL with water. Incubate at 65 °C to dissolve. Make aliquots of 1 mL. Store at −20 °C.
13. UV-transilluminator.
14. Horizontal Electrophoresis Systems: gel size (W × L) 20 × 25 cm.
15. Standard combs, 36 teeth, 1.5 mm thickness of teeth.
16. Agarose (low electroendosmosis).
17. 50× TAE buffer: 2 M Tris–Acetate, 0.05 M EDTA, pH 8.3. Add about 800 mL water to a 1-L graduated cylinder. Weigh 242 g Tris base and transfer to the glass beaker. Add 57.1 mL of glacial acetic acid and 100 mL of 0.5 M EDTA (pH 8.0). Mix and adjust the pH with KOH. Make up to 1 L with water.
18. Digoxigenin-labeled DNA Molecular Weight Marker.
19. Depurination solution: 0.25 M HCl. Add about 800 mL water to a 1-L graduated cylinder. Add 20.8 mL concentrated HCl (12 N) to the cylinder inside a fume hood and mix on a stirrer placed inside the hood. Make up to 1 L with water.
20. Denaturation solution: 0.5 M NaOH, 1.5 M NaCl (*see* **Note 1**). Add about 400 mL water to a 500-mL graduated plastic cylinder. Weigh 10 g NaOH and 43.83 g NaCl and transfer to the cylinder. Mix until dissolved. Make up to 500 mL with water.
21. Neutralization solution: 1.5 M NaCl, 0.5 M Tris–HCl, pH 7.5. Add about 800 mL water to a glass beaker. Weigh 60.57 g Tris base and 87.66 g NaCl and transfer to the glass beaker. Mix and adjust the pH with HCl. Make up to 1 L with water.
22. Transfer tray.
23. Glass plate.
24. Rectangular plastic container with sealed lid (approximate dimension 16 cm × 25 cm × 7.5 cm).

25. Positively charged nylon membrane, ≥20 cm × 11 cm (e.g., Hybond-N+, GE Healthcare).
26. 3MM Chr sheets 46 × 57 cm (Whatman).
27. Paper towels.
28. 20× SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0. Add about 800 mL water to a glass beaker. Weigh 175.32 g NaCl and 88.23 g Tri–sodium citrate·$2H_2O$ and transfer to the glass beaker. Mix and adjust the pH with HCl (1 N). Make up to 1 L with water.
29. Hybridization Incubator.
30. Rotary shaker.
31. Hybridization buffer (e.g., DIG Easy Hyb, Roche).
32. Low stringency wash buffer: 2× SSC, 0.1 % sodium dodecyl sulfate (SDS). Add about 800 mL water to a 1-L graduated cylinder. Add 100 mL 20× SSC and 10 mL 10 % SDS. Mix on a stirrer. Make up to 1 L with water.
33. High stringency wash buffer: 0.1× SSC, 0.1 % SDS. Add about 800 mL water to a 1-L graduated cylinder. Add 5 mL 20× SSC and 10 mL 10 % SDS. Mix on a stirrer. Make up to 1 L with water.
34. Maleic Acid Buffer: 0.1 M Maleic acid, 0.15 M NaCl, pH 7.5. Add about 800 mL water to a glass beaker. Weigh 11.61 g Maleic acid and 8.77 g NaCl and transfer to the glass beaker. Mix and adjust pH with NaOH pellets (*see* **Note 2**). Make up to 1 L with water.
35. Washing buffer: 0.1 M Maleic acid, 0.15 M NaCl, pH 7.5; 0.3 % (v/v) Tween 20. Add 600 mL Maleic Acid Buffer to a glass beaker. Add 1.8 mL Tween 20. Mix well.
36. Blocking solution: 0.1 M Maleic acid, 0.15 M NaCl, pH 7.5; 1 % (w/v) blocking reagent. Add 250 mL Maleic Acid Buffer to a glass media bottle. Weigh 2.5 g blocking reagent (Roche) and transfer to the bottle. Place the bottle on an incubator at 68 °C and shake the bottle every 10 min until dissolved (*see* **Note 3**).
37. Antibody solution: 0.1 M Maleic acid, 0.15 M NaCl, pH 7.5; 1 % (w/v) blocking reagent; 75 mU/mL Anti-Digoxigenin-AP (Roche). Add 40 mL Blocking Solution to a 50-mL polypropylene tube. Add 4 μL Anti-Digoxigenin-AP (*see* **Note 4**). Mix well.
38. Detection Buffer: 0.1 M Tris–HCl, 0.1 M NaCl, pH 9.5. Add about 800 mL water to a glass beaker. Weigh 12.11 g Tris and 5.84 g NaCl and transfer to the glass beaker. Mix and adjust pH with HCl. Make up to 1 L with water.
39. CDP-Star reagent, ready-to-use (0.25 mM solution).

40. Bag sealer.
41. X-ray film.
42. 20.32 × 25.4 cm (8 × 10 in.) autoradiography cassette.
43. Developer and Replenisher solution (Kodak GBX).
44. Fixer and Replenisher solution (Kodak GBX).
45. Stripping Buffer: 0.2 M NaOH, 0.1 % SDS. Add about 800 mL water to a 1-L graduated cylinder. Weigh 8 g NaOH and transfer to the cylinder. Add 10 mL 10 % SDS. Mix on a stirrer. Make up to 1 L with water.

### 2.2 Relative Quantitative Reverse Transcriptase PCR

1. Total RNA extraction kit.
2. RNase-free DNAse I kit.
3. First-strand cDNA synthesis kit.
4. Thermal cycler with gradient function and reagents for conventional PCR.
5. Horizontal Electrophoresis Systems: gel size (W × L) 20 × 25 cm.
6. Standard combs, 36 teeth, 1.5 mm thickness of teeth.
7. Agarose (low electroendosmosis).
8. SYBR Green real-time PCR master mix.
9. Primers for target and reference genes (*see* **Note 25**).
10. 96-well plate.
11. Sealing foil.
12. Real-time PCR machine.
13. Spectrophotometer.

### 2.3 Western Blot

Wear appropriate gloves and work in a fume hood when using 2-mercaptoethanol, concentrated HCl, methanol, or acrylamide.

1. Gel cassette (10 cm × 10.5 cm) and comb (10 wells, 1.5 mm thickness, 4.8 mm width, 7.2 mm depth).
2. Whatman 3MM chromatography paper.
3. Nitrocellulose membrane (0.45 μm).
4. Transblot semidry cell.
5. Primary antibody.
6. Conjugated peroxidase-antibody.
7. 2× Sample buffer: 12.5 mM Tris–HCl, pH 6.8, 20 % glycerol, 2 % SDS, 0.001 % bromophenol blue, 2 % 2-mercapthoethanol. Add 56.75 mL of water to a glass beaker. Add 1.25 mL of 1 M Tris–HCl pH 6.8, 20 mL glycerol, 20 mL 10 % SDS, 2 mL 2-mercapthoethanol and mix on a stirrer (*see* **Note 5**). Transfer the solution into a glass media bottle. Weigh 1 mg of bromophenol blue and transfer it to the bottle. Mix by shaking.

8. 5× SDS electrophoresis buffer: 125 mM Tris base, 960 mM glycine, 0.5 % SDS, pH 8.3. Add about 800 mL of water to a glass beaker. Weigh 15.1 g of Tris base, 72 g glycine, 5 g SDS and transfer them to the beaker. Mix and adjust the pH with concentrated HCl. Make up to 1 L with water.
9. 10× TBS: 50 mM Tris base, 700 mM NaCl, pH 7.9. Add about 400 mL of water to a glass beaker. Weigh 6.05 g of Tris base, 40.91 g NaCl and transfer them to the beaker. Mix and adjust the pH with 2.7 mL of concentrated HCl. Make up to 500 mL with water.
10. TBST: 5 mM Tris base, 70 mM NaCl, pH 7.9, 0.05 % Tween 20. Add 900 mL of water to a 1-L graduated cylinder. Add 100 mL of 10× TBS and 0.5 mL of Tween 20. Mix on a stirrer.
11. Blocking solution: 5 mM Tris base, 70 mM NaCl, pH 7.9, 0.05 % Tween 20, 3 % bovine serum albumin (BSA). Add 30 mL of TBST to a 50-mL polypropylene tube. Weigh 900 mg of BSA and transfer it to the tube. Mix by inverting.
12. Color development solution: 0.05 % 4CN, 17 % methanol, 4.2 mM Tris base, 58.3 mM NaCl, pH 7.9, 0.015 % $H_2O_2$. Add 4 mL of methanol to a 15-mL polypropylene tube. Weigh 12 mg of 4-chloro-1-naphthol (4CN) and transfer it to the tube. Mix until dissolved. Add 20 mL of 1× TBS to a separate 50-mL polypropylene tube. Add 12 μL ice-cold 30 % $H_2O_2$ to the 50-mL tube. Transfer 4 mL 4CN solution from the 15-mL tube into the 50-mL tube (*see* **Note 6**). Mix well.
13. Anode buffer #1: 300 mM Tris base, 10 % methanol, pH 10.4. Add about 350 mL of water to a glass beaker. Weigh 18.1 g of Tris base and transfer to the glass beaker. Add 100 mL of methanol. Mix and adjust the pH to 10.4 with 10 N NaOH. Make up to 500 mL with water.
14. Anode buffer #2: 25 mM Tris base, 10 % methanol, pH 10.4. Add about 350 mL of water to a glass beaker. Weigh 1.51 g of Tris base and transfer to the glass beaker. Add 100 mL of methanol. Mix and adjust the pH to 10.4 with 10 N NaOH. Make up to 500 mL with water.
15. Cathode buffer: 25 mM Tris base, 40 mM Aminocaproic acid, 10 % methanol, pH 9.4. Add 100 mL of methanol to a 100-mL glass beaker. Weigh 2.62 g of Aminocaproic acid and transfer it to the glass beaker. Mix on a stirrer. Add about 350 mL of water to a separate 500-mL glass beaker. Weigh 1.51 g of Tris base and transfer to the 500-mL beaker. Add 100 mL of Aminocaproic acid dissolved in methanol to the 500-mL beaker. Mix and adjust the pH with 10 N NaOH. Make up to 500 mL with water.

16. Acrylamide mixture: 30 % acrylamide, 0.8 % bis-acrylamide. Add 100 mL of water to a glass beaker. Weigh 30 g of acrylamide and 0.8 g of bis-acrylamide, and transfer these to the glass beaker (*see* **Note 7**). Mix on a stirrer. Store in the dark at 4 °C.
17. 4× Tris/SDS pH 8.8: 1.5 M Tris–HCl, pH 8.8, 0.4 % SDS. Add 80 mL of water to a glass beaker. Weigh 18.2 g of Tris base and 0.4 g of SDS, and transfer these to the beaker. Mix and adjust the pH with concentrated HCl. Make up to 100 mL with water. Store at 4 °C.
18. 4× Tris/SDS pH 6.8: 0.1 M Tris–HCl, pH 6.8, 0.4 % SDS. Add 80 mL of water to a glass beaker. Weigh 6.05 g of Tris base and 0.4 g of SDS and transfer these to the beaker. Mix and adjust the pH with concentrated HCl. Make up to 100 mL with water. Store at 4 °C.
19. Ammonium persulfate: 10 % solution in water (*see* **Note 8**).
20. *N*,*N*,*N*,*N*′-tetramethyl-ethylenediamine (TEMED).

## 3 Methods

Carry out all procedures at room temperature unless otherwise specified.

### *3.1 Southern Hybridization Using DIG Chemiluminescent Technology*

1. Prepare a probe labeling reaction using transgene-specific primers and the PCR DIG Probe Synthesis Kit according to the manufacturer's instructions (*see* **Note 9**).
2. Run a portion (5 μL) of each reaction on an agarose gel, along with a DNA molecular weight marker. Examine the bands on the gel to check if the labeling reaction was successful (*see* **Note 10**).
3. Select an appropriate restriction enzyme for digestion of genomic DNA (*see* **Note 11**). Digest 10 μg of pure and intact high molecular weight DNA (*see* **Note 12**) in 150 μL final volume, using two to three units of restriction enzyme per ug of DNA. Incubate the tubes overnight at the temperature recommended by the manufacturer (most commonly 37 °C). Next day, take 5 μL of each sample and run in a gel to make sure DNA is digested well (*see* **Note 13**).
4. Add 0.1 volume of 3 M Na acetate, pH 5.2, to the digestion reaction and mix. Add 2.5 volumes of cold 100 % EtOH and mix. Incubate at −20 °C for 30 min to 2 h. Centrifuge at 13,000 × *g* for 10 min at room temperature. Remove the supernatant and add 500 μL of 70 % cold EtOH. Centrifuge at 13,000 × *g* for 10 min at room temperature. Remove the supernatant and centrifuge again for 2 min. Remove the remnant of supernatant using a 20-μL pipette and vacuum dry the pellet

for 2 min. Resuspend in 15 μL 0.1× TE and 3 μL 6× Ficoll loading dye (*see* **Note 14**). Incubate at 65 °C for 15 min, flicking the tube every 5 min to dissolve the DNA.

5. For positive control, digest 400 pg of the plasmid that was used for transformation, with the same restriction enzymes, for 3 h.
6. To prepare the gel, combine 392 mL of water with 4 g agarose, and heat in a microwave until dissolved. Add 8 mL 50× TAE and mix by stirring (*see* **Note 15**). When the bottle is cool enough to hold, pour the gel into the gel tray and insert the comb. Let the gel solidify for 1 h.
7. Fill the electrophoresis tank with fresh 1× TAE buffer. Place the entire tray into the tank and add 1× TAE buffer to a level approximately 2–3 mm above the gel surface. Remove the comb from the gel.
8. Load 18 μL of each sample and 50 ng of DIG-labeled DNA Molecular Weight Marker into the wells.
9. Run the gel at 30 V for 16 h. Next day in the morning, change the voltage to 15 V and run the gel for 5 additional hours.
10. Following electrophoresis, trim the gel to size for blotting (20 cm × 11 cm).
11. Submerge the gel in Depurination Solution and shake at 50 rpm for 10 min at room temperature. Rinse the gel with sterile, double distilled water for a few seconds.
12. Submerge the gel in Denaturation Solution for 15 min at room temperature, with gentle shaking, then rinse the gel with sterile, double distilled water for a few seconds. Repeat.
13. Submerge the gel in Neutralization Solution for 15 min at room temperature, and repeat.
14. Equilibrate the gel for at least 10 min in 20× SSC.
15. Cut the nylon membrane to 20 cm × 11 cm. Mark with a pencil in the top right: cultivar, construct, restriction enzyme, date. Soak the membrane for 5 min in 2× SSC.
16. Cut the Whatman 3MM paper to 25 cm × 30 cm and 20 cm × 11 cm, two sheets each.
17. To set up the blot (Fig. 4), fill a transfer tray with 1 L 20× SSC. Put in a glass plate and place the two pieces of 25 cm × 30 cm Whatman 3MM paper on top of it, with the 30 cm ends of the paper hanging off of the tray into the buffer. Soak the Whatman 3MM with 20× SSC. Roll a sterile pipette over the sandwich to remove all air bubbles.
18. Place the gel upside-down atop the soaked sheets of Whatman 3MM paper. Roll a sterile pipette over the sandwich to remove all air bubbles that formed between the gel and paper.

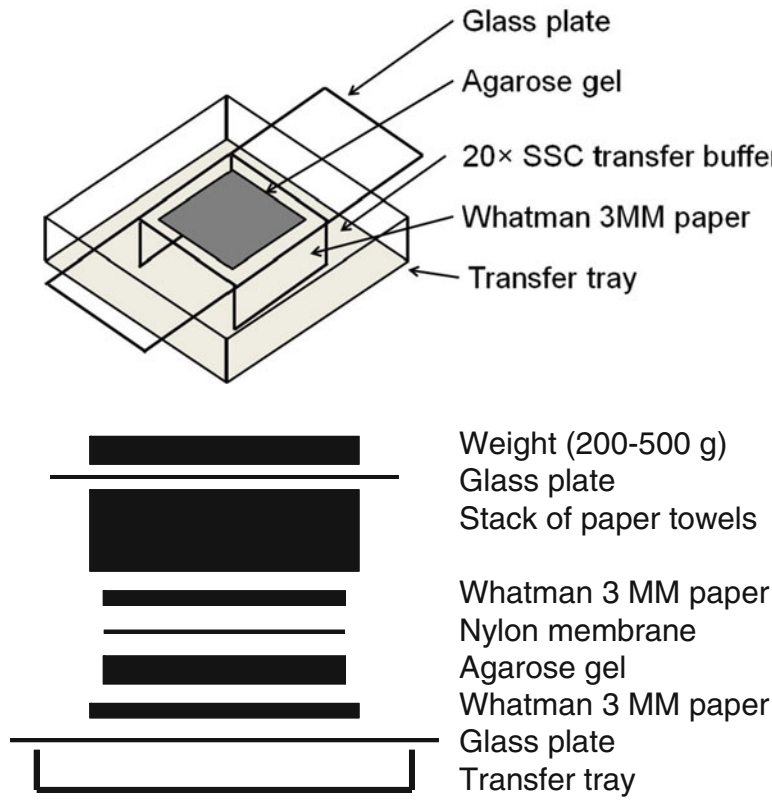


**Fig. 4** Setup for Southern blot transfer sandwich

19. Place the membrane over the gel, making sure that the loading wells are covered by the membrane. Use a pipette to eliminate air bubbles as above. Cut off any parts of the gel that are not covered by the membrane.
20. Wet the two pieces of 20 cm × 11 cm Whatman 3MM in 20× SSC and place them atop the membrane. Use a pipette to eliminate air bubbles as above.
21. Cover any parts of the Whatman 3MM paper atop the glass plate that are not covered by the gel and any open parts of the tray with plastic wrap. Complete the blot assembly by adding a stack of paper towels, a glass plate, and a 200–500 g weight. The finished blot transfer "sandwich" should look like Fig. 4.
22. Allow the blot to transfer overnight in Transfer Buffer (20× SSC). Next day, carefully take the membrane from the sandwich and soak the membrane for 5 min in 2× SSC. Dry the membrane by holding it above paper towels, allowing it to drip, for 3 min. Place the membrane (DNA side facing down) on a clear plastic bag. Expose the wet membrane to UV light for 5 min at 302 nm in a transilluminator.

23. Use the membrane immediately in the prehybridization step (**steps 24** and **25**) or store the moist membrane (wrapped in plastic wrap and placed between two sheets of Whatman 3MM paper) at –20 °C.
24. Warm 50 mL of hybridization buffer in a shaking incubator at 42 °C for 30 min (*see* **Note 16**).
25. Place the membrane into a rectangular plastic container with sealed lid and add 50 mL of prewarmed hybridization buffer (*see* **Note 17**). Incubate the membrane for at least 3 h. Agitate the membrane gently during this prehybridization step.
26. Place 3 µL of the labeled probe into a 1.5-mL tube. Incubate the tube at 100 °C in a heating block for 10 min to denature the probe. Chill the probe quickly in an ice bath. Incubate on ice for 5 min. Proceed immediately to **step 27**.
27. Pour the hybridization buffer from the plastic container into a new vessel and add 2 µL of the denatured probe. Mix by inversion to form the hybridization solution. *Immediately* add the hybridization solution on top of the membrane in the plastic container. Allow the labeled probe to hybridize with the DNA by slowly shaking (80 rpm) overnight at 42 °C.
28. Next day, transfer the hybridization solution into a new vessel for storage (*see* **Note 18**).
29. Transfer the membrane to a new container. Wash the membrane two times for 20 min each at room temperature with 200 mL of low stringency wash buffer, followed by two times for 20 min each at 68 °C with 200 mL of prewarmed high stringency wash buffer (*see* **Note 19**).
30. Equilibrate the membrane for 2 min with 200 mL of washing buffer.
31. Incubate the membrane for 30 min to 3 h with 200 mL of blocking solution with slow shaking.
32. Incubate for 30 min with 40 mL of antibody solution.
33. Transfer the membrane to a new container. Wash off the excess antibody solution by incubating twice for 15 min each with 200 mL of washing buffer.
34. Equilibrate the membrane for 3 min with 40 mL of detection buffer.
35. Cut a rectangular, clear, plastic bag in half horizontally. Use the top portion, cutting one side and opening it to obtain a larger flat piece of plastic. Place the membrane (DNA side facing up) on one side of the plastic. Apply 3 mL 0.25 mM CDP-Star reagent, dropwise, over the surface of the blot until the entire surface is evenly soaked (*see* **Note 20**). As you are applying the substrate, immediately cover the dampened part of the

membrane with the second side of the plastic so the substrate is spread evenly over the membrane. Do not let air bubbles form between the membrane and the upper surface of the plastic. Incubate the membrane for 5 min at room temperature [12].

36. Squeeze excess liquid out of the plastic and seal the sides of the plastic close to the membrane using a bag sealer. Place the membrane, sealed in the plastic, in an autoradiography cassette.
37. In the dark room, place a sheet of X-ray film in the cassette over the plastic bag with the membrane and close the cassette.
38. After 2 min (*see* **Note 21**), remove the film from the cassette and transfer it into Developer and Replenisher solution. Move the film gently in the solution for 40 s.
39. Transfer the film into water and move it gently for 30 s.
40. Transfer the film to Fixer and Replenisher solution, again with gentle moving, for 1 min.
41. Rinse the film with running tap water and let it dry.
42. After exposure, wash the membrane in double distilled water for 1 min.
43. Incubate twice for 15 min each at 37 °C with 250 mL of stripping buffer.
44. Wash the membrane for 5 min with 250 mL of 2× SSC at room temperature.
45. Store the membrane wrapped in plastic wrap at –20 °C for up to 1 year.

### *3.2 Relative Quantitative Reverse Transcriptase PCR*

Thaw all frozen reagents before use. Briefly centrifuge them before starting the procedure. Keep all reagents on ice while setting up the reactions.

1. Extract RNA from transgenic and nontransgenic control plants using the total RNA extraction kit according to the manufacturer's instructions (*see* **Note 22**).
2. For each RNA sample, prepare two reactions of Reverse Transcription, i.e., one experimental reaction and one control without reverse transcriptase ("No Amplification Control" or NAC). The NAC will indicate whether there is any contaminating genomic DNA in the samples that is amplified by PCR. In addition, to test if one or more of the RT-PCR reagents is contaminated with the amplicon, include one "No Template Control" (NTC). The NTC includes all of the RT-PCR reagents except for the RNA template. Typically the RNA is simply substituted with nuclease-free water.
3. To remove DNA from the RNA samples, combine 1 μg of total RNA with components of the RNase-free DNase I kit, according to the manufacturer's instructions.

4. Inactivate the DNase I according to the manufacturer's instructions for the DNase kit. Typically, this is done by adding 1 μL of 25 mM EDTA to the reaction mixture and heating for 10 min at 65 °C (*see* **Note 23**).
5. Make cDNA using the DNA-free RNA samples and the First-strand cDNA synthesis kit, according to the manufacturer's instructions. High-temperature incubations can be performed in a thermal block cycler with a heated lid (*see* **Note 24**).
6. Measure the concentration of single stranded cDNA using a spectrophotometer. Equalize the concentration of all samples using PCR-grade water.
7. Choose one reference gene whose expression does not change in different tissues and experimental treatments. Design primers for amplification of reference and target genes using primer design software (*see* **Note 25**). To test the specificity of the primers, perform gradient PCR on a pooled cDNA sample with concentration of 25 ng/μL using the real-time PCR machine and SYBR Green real-time PCR master mix, or a conventional thermal cycler with conventional PCR reagents. The gradient should depend on the melting temperature of the specific primers (*see* **Note 26**). Typically, a gradient from 56 °C to 64 °C is used for primers with a melting temperature of 60 °C.
8. Prior to running the PCR, take note of the temperature of each column when a gradient step is set on a conventional thermal cycler. For a gradient of 56–64 °C, place five tubes containing the PCR reactions with sample cDNA in lanes that correspond to approximately 56 °C, 58 °C, 60 °C, 62 °C, and 64 °C annealing temperature. Place NTC in the 56 °C lane.
9. Start the PCR reaction using this cycling condition: 95 °C for 5 min; followed by 35 cycles of 95 °C for 10 s, a gradient step for 20 s, and 72 °C for 30 s; and finally, a single step at 72 °C for 7 min.
10. Run the amplicons on a 3 % agarose gel, along with a 50-bp molecular weight marker. A single band with the correct amplicon size indicates that the PCR primers are specific. Select the annealing temperature that produces the brightest single band without the presence of primer dimers. If primer dimers are present in all samples, adjust the annealing time and the primer concentration accordingly (*see* **Note 27**).
11. Make twofold serial dilutions (at least five dilutions) of the pooled cDNA by adding PCR-grade water.
12. Thaw one vial of SYBR Green real-time PCR master mix (*see* **Note 28**).

13. Prepare a PCR Master Mix of primers and SYBR Green mix for 20 μL reactions according to the manufacturer's instructions. Withhold 5 μL volume for the DNA (*see* **Note 29**).
14. Mix carefully by pipetting up and down. Do not vortex.
15. Pipet 15 μL PCR Master Mix into the wells of a 96-well plate. Add 5 μL of the serial dilutions of pooled cDNA. As negative controls, replace pooled cDNA with NAC, NTC, or PCR-grade water. Seal the plate with Sealing Foil.
16. Place the plate in the centrifuge and balance it with a suitable counterweight. Centrifuge at 1690 × *g* for 2 min.
17. Load the 96-well plate into the real-time PCR instrument. Start the PCR program using this protocol: pre-incubation program (95 °C for 5 min); amplification and quantification program repeated 45 times (denaturation at 95 °C for 10 s, annealing at the optimized temperature for 5–20 s, elongation at 72 °C for 5–30 s with a single fluorescence measurement) (*see* **Note 30**); melting curve program (95 °C for 5 s, 65 °C for 1 min, with a continuous fluorescence measurement ending at 97 °C as the temperature is increased with 0.5 °C increments) (*see* **Note 31**); and finally a cooling step to 40 °C.

    Ramp rates should be programmed as follows:

    Assign 4.4 °C/s ramp rate for the pre-incubation, denaturation, and elongation steps. Annealing ramp rate should be 2.2 °C/s if target temperature is greater than or equal to 50 °C, and 1.5 °C/s if target temperature is less than 50 °C. For the melting curve, input 4.4 °C/s for the 95 °C step, and 2.2 °C/s for the 65 °C step. For the cooling step, the ramp rate should be 1.5 °C/s.
18. Plot the crossing point (CP), or quantification cycle, (*y*-axis) versus the cDNA concentration input (*x*-axis). Determine the slope of the line (this is usually done automatically by the software of the real-time PCR instruments).
19. PCR efficiency is then calculated by the equation $E = 10^{(-1/m)}$, where $m$ is the slope of the line and $E$ is the efficiency [9]. PCR efficiency should be within 1.90–2.10 range. If the value is outside the acceptable range, try to optimize primer concentration and thermal protocol, use better quality reagents, redesign a new set of primers, or use better methods for RNA isolation to eliminate any source of PCR inhibitors.
20. Observe the peaks generated from the melting curve to assess the specificity of the amplified PCR product (*see* **Note 32**).
21. Repeat **steps 12–17** and **step 20** using equalized cDNA samples of transgenic and nontransgenic plants. Use three biological replicates with three technical replicates for each biological replicate. Include NAC, NTC as negative controls.

22. Calculate the relative expression ratio ($R$) of the gene of interest by the equation $R = (E_{\text{target}})^{\Delta\text{CPtarget}} / (E_{\text{ref}})^{\Delta\text{CPref}}$, where $E_{\text{target}}$ is the PCR efficiency of the gene of interest, ΔCPtarget is the CP of the nontransgenic plant for the target gene minus the CP of the transgenic plant, $E_{\text{ref}}$ is the PCR efficiency of the reference gene, and ΔCPref is the CP of the nontransgenic plant for the reference gene minus the CP of the transgenic plant [9] (*see* **Note 33**). The $R$ value tells us the fold change of expression of the gene of interest in the transgenic plant relative to the expression of the gene of interest in the nontransgenic plant.

### 3.3 Western Blot

1. Prepare the separating gel by mixing 4 mL of 4× Tris/SDS pH 8.8, 6.4 mL of acrylamide mixture, and 5.6 mL water in a 50-mL conical flask. Add 53.4 μL of ammonium persulfate and 10.6 μL of TEMED, and cast the gel within a gel cassette. Pour to a height of approximately 7 cm, leaving 3 cm for the stacking gel. Allow the separating gel to polymerize for 20 min.
2. Prepare the stacking gel by mixing 1 mL of 4× Tris/SDS pH 6.8, 0.533 mL of acrylamide mixture, and 2.467 mL of water in a 50-mL conical flask. Add 31.5 μL of ammonium persulfate and 6.3 μL of TEMED. Pour the stacking gel on top of the separating gel. Insert a gel comb immediately without introducing air bubbles. Allow the stacking gel to polymerize for 10 min.
3. Take 50 μg of total plant soluble protein for each sample. Add an equal volume of 2× sample buffer. Heat the samples for 2 min in a boiling water bath and then immediately put them on ice.
4. Fill the electrophoresis chamber with 1× SDS electrophoresis buffer. Load 30–50 μL of sample per well. Run the gel at 20 mA while the sample moves through the stacking gel, then at 30 mA while the sample moves through the separating gel (*see* **Note 34**).
5. Cut the Whatman 3MM chromatography paper into six pieces of 7.0 × 10 cm, and the nitrocellulose membrane into a 6 × 9 cm piece.
6. Following electrophoresis, remove the gel from the gel apparatus. Trim off the stacking gel and cut one corner to mark the orientation of the gel.
7. Assemble the sandwich according to Fig. 5 for a semidry transfer system.
8. Run the semidry transfer cell at 15 V for 30 min. After transfer, mark the positions of the molecular weight markers on the membrane with a pencil.

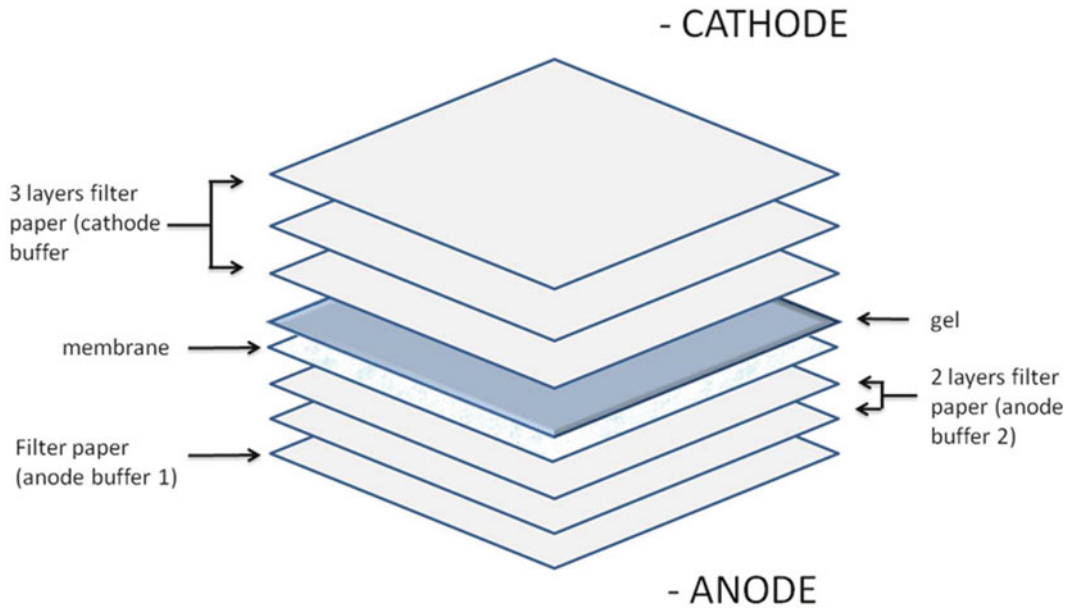


**Fig. 5** Setup for semidry transfer

9. Shake the membrane in 30 mL of blocking solution at room temperature for 60 min.
10. Rinse with 30 mL of TBST quickly, shake for 5 min, and pour off the TBST completely. Repeat.
11. Add 30 mL of TBST and primary antibody (1:2000 dilution). Shake slowly at room temperature for at least 2 h or overnight (*see* **Note 35**).
12. Wash the membrane with 25 mL of TBST for 5 min. Repeat 5 times.
13. Add 30 mL of TBST and 20 μL of conjugated peroxidase/antibody IgG. Shake slowly at room temperature for at least 2 h.
14. Wash with 25 mL of TBST for 5 min. Repeat 5 times.
15. Wash twice with 25 mL of 1× TBS for 5 min.
16. Add color development solution and incubate for 5 min at room temperature with shaking at 50 rpm. Do not exceed 10 min (*see* **Note 36**).
17. Pour off the color development solution. Wash the membrane with water 3 times. Take a picture.
18. Dry the membrane with paper towel, cover it with aluminum foil, and place it in a plastic bag. Store at −20 °C.

## 4 Notes

1. It is best to prepare this fresh each time.
2. Add up to 8 g of NaOH pellets while stirring the solution. The pH will reach about 6.0 and Maleic acid will become soluble. At this point, start adding 1 N NaOH drop by drop as it is easy for the pH to increase very quickly. Discard the solution if the pH goes above 7.5.
3. Prepare this solution 2 h before use.
4. Centrifuge the Anti-Digoxigenin-AP at 9000 × *g* for 5 min. Pipette 4 μL from the surface and add it to 40 mL blocking solution.
5. Wear a mask and gloves when working with 2-mercaptoethanol. It is hazardous in case of skin contact (irritant), of eye contact (irritant), of inhalation.
6. Dissolve 4CN immediately before use and protect solution from light.
7. Wear a mask and gloves when weighing acrylamide. Unpolymerized acrylamide is a potent neurotoxin and is absorbed through the skin.
8. We find that it is best to prepare this fresh each time.
9. Adjust the annealing temperature and extension time based on the Tm of the primers and expected amplification product.
10. If the labeling reaction was successful, your labeled experimental probe will migrate slower (i.e., appear to be larger) than your unlabeled control probe, due to the presence of DIG. The staining of the labeled DNA will be equal to or somewhat less than that of the unlabeled control DNA due to the presence of DIG [12].
11. A single cutter enzyme (cuts once within the transgene) in combination with a probe that hybridizes to the transgene will provide an estimate of transgene copy number (Fig. 1, *BamHI*). An enzyme that cuts twice in the transgene and liberates a specific DNA cassette in combination with a probe that hybridizes to the cassette will provide information about intactness of the cassette (*see* Fig. 1, *AscI*). An enzyme known not to cut within the transgene in combination with a probe that hybridizes to the transgene will provide an estimate of transgene locus number (*see* Fig. 1, *AvrII*).
12. For Southern hybridization, DNA must be pure, intact, and of high molecular weight. Impurities in the DNA may interfere with digestion and lead to overestimates of DNA concentration using spectrophotometry. Degraded and partially degraded DNA may produce false-negative and false-positive

results, respectively. Therefore, it is important to assess the integrity of the isolated DNA through electrophoresis prior to Southern analysis.

13. If digestion is complete there should be a homogeneous smear throughout the lane. If the DNA is not digested well, add more enzyme and continue digestion.
14. The high molecular weight Ficoll-400 stays at the bottom of the well, unlike sucrose or glycerol which diffuse quickly, thus yielding sharper DNA bands.
15. Acetic acid, a component of TAE, is a volatile acid. If we heat TAE in a microwave, some of acetic acid will escape, and hence changes the pH.
16. If using DIG Easy Hyb, the optimal hybridization temperature (Thyb) can be calculated as follows: $Tm = 49.82 + 0.41\ (\%\ G + C) - 600/l$ and $Thyb = Tm - (20\ °C \text{ to } 25\ °C)$, where Tm = melting point of the probe-target hybrid, (% G + C) = % of G and C residues in the probe sequence, Thyb = Optimal temperature for hybridization of probe to target in DIG Easy Hyb, $l$ = length of the hybrid in base pairs [12].
17. To avoid evaporation, cover the container with plastic wrap and place the lid tightly. A dry membrane will produce high background.
18. The hybridization solution with the DIG-labeled probe is stable at −20 °C for more than 12 months and can be reused 5 times. Immediately before reuse, denature the probe again by heating the hybridization solution at 68 °C for 10 min.
19. Place the high stringency wash buffer at 68 °C 1 h before use.
20. Since CDP-Star is light-sensitive, avoid exposure to light when applying it to the membrane.
21. Based on this result, adjust the exposure time to get more intense bands with acceptable background.
22. To get an accurate representation of gene expression, use intact and pure RNA prepared from fresh tissue. Test the purity of the total RNA using a spectrophotometer. If the RNA is pure, the value of $A_{260}/A_{280}$ should be 2.00 and the value of $A_{260}/A_{230}$ should be $\geq 2.0$. Test the RNA integrity by running 2 μg of total RNA on a 1 % agarose gel. For eukaryotic samples, intact total RNA run on a gel will have sharp 28S and 18S rRNA bands. The 28S rRNA band should be approximately twice as intense as the 18S rRNA band. Additional smaller distinct bands may also appear representing rRNA from plant organelles and small RNAs.
23. It is important not to exceed the 15 min incubation time or the room temperature incubation. Higher temperatures (≥28 °C) and longer incubation time could lead to $Mg^{2+}$-dependent

hydrolysis of the RNA. Additionally, it is vital that the EDTA be added to at least 2 mM prior to heat inactivation to minimize this problem.

24. Use the heated lid to minimize evaporation.
25. Design primers so that one half hybridizes to the 3′ end of one exon and the other half to the 5′ end of the adjacent exon, or alternatively, design primers to flank a region that contains at least one long intron. Primers should have a length of 20–30 bp, GC content of 40–60 %, and an amplicon length of 100–150 bp. The primer sequences should be specific to the template. They should have no complementarity of two or more bases at the 3′ ends of primer pairs to minimize primer-dimer formation, no mismatches between the 3′ end of primers and the template sequence, no runs of three or more Gs or Cs at the 3′ end, no T at the 3′-end (primers with a T at the 3′ end have a greater tolerance of mismatch), no complementary sequences within a primer sequence and between the primers in a pair.
26. Adjust the annealing temperature based on the Tm of the primers. For an initial experiment, set the annealing temperature 4 °C below the calculated primer Tm.
27. To remove primer dimers, decrease the annealing time (minimum is 5 s) and/or decrease the primer concentration by half. This will increase the stringency of primer binding and will ensure that only the desired gene is amplified.
28. Master mix should be kept away from light.
29. To prepare the PCR Mix for more than one reaction, multiply the volume of each component by the number of reactions to be run plus sufficient additional reactions (10 % of the number of reactions).
30. Calculate the hold time for the PCR elongation step by dividing the amplicon length over 25 (e.g., a 150 bp amplicon requires 6 s elongation time).
31. In the third step of the Melting Curve, the number of acquisitions per °C should be 5–10. The calculated Tm might differ by approximately 0.5 °C when using either the lowest (5) or highest (10) possible value of acquisitions/°C.
32. The experimental samples should yield a single sharp peak at the melting temperature of the amplicon, whereas the NAC, NTC, and water will not generate significant fluorescent signal. This result indicates that the products are specific, and that SYBR Green I fluorescence is a direct measure of accumulation of the product of interest. If there are more peaks, increase the concentration of the cDNA sample and/or perform re-optimization of the profile (e.g., melting and annealing temperature). New better primers may need to be designed.

33. $\Delta CP = CP_{control} - CP_{transgenic}$. If the control sample does not cross the threshold line, try to increase the concentration of the cDNA template by 2× or more.
34. Do not run the gel at a very high current, as this can overheat the gel and lead to band smiling (each band curves upward at the ends to look like a smile).
35. Overnight incubation of the membrane in antibody solution produces strong bands without significant increase in background.
36. Incubation of the membrane in color development solution for more than 10 min can produce high background.

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# Chapter 16

# Analysis of Recombinant Proteins in Transgenic Rice Seeds: Identity, Localization, Tolerance to Digestion, and Plant Stress Response

**Yuhya Wakasa and Fumio Takaiwa**

## Abstract

Rice seeds are an ideal production platform for high-value recombinant proteins in terms of economy, scalability, safety, and stability. Strategies for the expression of large amounts of recombinant proteins in rice seeds have been established in the past decade and transgenic rice seeds that accumulate recombinant products such as bioactive peptides and proteins, which promote the health and quality of life of humans, have been generated in many laboratories worldwide. One of the most important advantages is the potential for direct oral delivery of transgenic rice seeds without the need for recombinant protein purification (downstream processing), which has been attributed to the high expression levels of recombinant products. Transgenic rice will be beneficial as a delivery system for pharmaceuticals and nutraceuticals in the future. This chapter introduces the strategy for producing recombinant protein in the edible part (endosperm) of the rice grain and describes methods for the analysis of transgenic rice seeds in detail.

**Key words** *Oryza sativa* L., Protein body, Recombinant protein, Seed storage protein, Transgenic rice

## 1 Introduction

Rice seeds are an ideal production platform for molecular farming, as recombinant proteins can accumulate at high levels in the rice endosperm through established production systems [1, 2]. Furthermore, recombinant proteins that accumulate in rice seeds can be stored stably for several years at room temperature (a cold-chain infrastructure is not required for transport or storage) and delivered orally without purification. The functions of these proteins can be maintained even when the rice is cooked due to high thermal tolerance [3]. Orally administered rice seed-based pharmaceuticals are effectively delivered to the intestinal tract because they can withstand harsh, acidic conditions and digestive enzymes due to bioencapsulation within the cell wall and protein

Jacqueline MacDonald et al. (eds.), *Recombinant Proteins from Plants: Methods and Protocols*, Methods in Molecular Biology, vol. 1385, DOI 10.1007/978-1-4939-3289-4_16, 

bodies. In addition, the mechanisms used for the cultivation, harvesting, processing, and storage of rice have been well developed worldwide.

Rice endosperm cells have three organelles that are suitable for the accumulation of recombinant proteins: protein body I (PB-I), protein body II (PB-II), and the endoplasmic reticulum (ER) (Fig. 1). PB-I is an ER-derived protein body that contains prolamins; a group of seed storage proteins that include 10 kDa prolamin, 13 kDa cys-poor prolamin, 13 kDa cys-rich prolamin, and 16 kDa prolamin [4]. PB-II is a protein storage vacuole that contains 26 kDa globulin and glutelins [5]. Twenty-six kilodaltons globulin is encoded by a single gene, whereas glutelins constitute a multi-gene family that has been classified into four groups (subfamilies) including GluA (GluA1, GluA2, and GluA3), GluB (GluB1, GluB2, and GluB4), GluC, and GluD [6]. These storage proteins are exported to PB-II from the ER via two routes; through the Golgi apparatus via dense vesicles, or directly via precursor-accumulating vesicles.

To target recombinant proteins to PB-I, PB-II, or the ER, a suitable ER-targeting signal peptide must be attached to the N-terminus of the recombinant protein, in addition to a C-terminal retention signal such as Lys/His-Asp-Glu-Leu [(K/H)DEL], which is involved in the transport of proteins from the Golgi apparatus to the ER [7, 8] (Fig. 2a). ER retention signals can result in higher accumulation of recombinant proteins: a previous study reported that recombinant protein levels were two- to tenfold higher with the addition of an ER retention signal than without this signal [9].

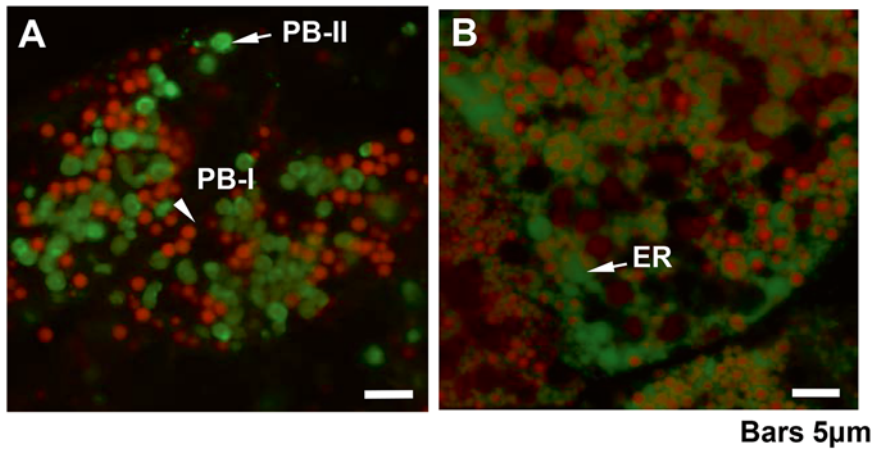


**Fig. 1** Target organelles for the high accumulation of recombinant proteins. Confocal microscopy observations of transgenic rice seeds expressing GFP-fused glutelin A2 (**a**) and a GFP-attached signal peptide and ER retention signal to its 5′ and 3′ ends (**b**). Seed sections stained with rhodamine B to visualize PB-I are examined using confocal microscopy. (**a**) *Arrowhead* (*red signal*) shows PB-I, *arrow* (*green signal*) shows PB-II. (**b**) *Arrow* (*green signal*) shows ER

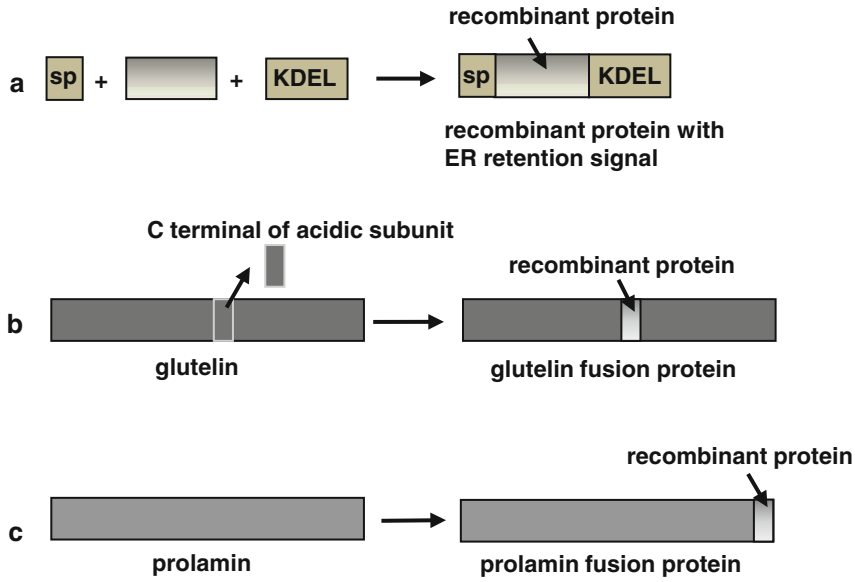


**Fig. 2** Structures of coding regions required for the high accumulation of recombinant proteins. (**a**) Recombinant proteins carrying a heterologous signal peptide and ER retention signal accumulate in PB-I or the ER (Table 1). Recombinant peptides fused to full-length glutelin (**b**) or prolamin (**c**) accumulate in PB-II and PB-I (Table 1). This figure was reprinted with permission from Wiley

Recombinant proteins containing signal peptides and ER retention signals are generally sorted to PB-I or the ER lumen in transgenic rice seed. However, the use of these sequences alone does not guarantee the accurate trafficking of recombinant proteins to the desired organelle. The translational fusion of a recombinant protein to a seed storage protein ensures that the recombinant protein is deposited into the desired protein bodies. A recombinant protein is typically fused with prolamin or glutelin in order to transport it into PB-I or PB-II, respectively (Fig. 2b, c) [10]. An effective strategy has already been established for the accumulation of a small recombinant peptide (<200 amino acids) as a part of glutelin. Glutelin is synthesized as preproglutelin on the rough ER and is then posttranslationally processed into mature acidic and basic subunits in PB-II. The target recombinant peptides can be inserted into the C-terminal region of the acidic subunit of glutelin as part of a glutelin fusion protein (Fig. 2b). The glutelin-fused recombinant peptide then typically accumulates in PB-II. Soybean seed storage proteins such as glycinin can also be used as carrier proteins, similar to rice seed storage proteins [11].

Promoters are also one of the most important factors that determine the level of accumulation of transgene products in rice seeds. Promoter strength and tissue specificity (seed-specific expression) are critical because they directly affect the accumulation of recombinant proteins. Several suitable promoters have been identified and used as seed-specific and high expression promoters

without detrimental effects on plant growth [12]. Some seed storage protein gene terminators have also been used successfully. Although a nopaline synthase (Nos) terminator is typically used in the field of plant molecular biology, it is not recommended for recombinant expression in rice seed tissue because its incomplete termination ability has been shown to reduce the accumulation of recombinant proteins [13].

Table 1 and Fig. 2 summarize suitable promoters, coding regions, and terminators for the high accumulation of recombinant proteins in transgenic rice seeds as well as the predicted deposit sites of target proteins. Several strategies are now available for the construction of plasmid vectors, including the conventional combination of restriction enzymes and ligases as well as the Gateway system (Invitrogen) and In-Fusion system (Clontech); therefore, vector plasmids can be easily constructed to express the desired gene.

Transgenic rice plants of both *japonica* and *indica* varieties are generally produced by *Agrobacterium*-mediated transformation. Useful information on rice transformation methods can be found in "Transgenic Plants: Methods and Protocols 2nd edition" (Chapters 5, 6, and 36), published by Springer [14], and in Chapter 2 of this volume.

**Table 1**
**Preferable gene structure to express recombinant proteins in rice seeds**

| | Gene name | RAP locus[a] |
|---|---|---|
| Promoter | *2.3 kb GluB1* promoter | Os02g0249800 |
| | *1.4 kb GluB4* promoter | Os02g0268300 |
| | *2.0 kb GluC* promoter | Os02g0453600 |
| | *1.0 kb Glb-1* promoter | Os05g0499100 |
| | *0.9 kb 10 kDa prolamin* promoter | Os03g0766200 |
| | *0.9 kb 16 kDa prolamin* promoter | Os06g0507200 |
| | Expression type | Predicted localization in seed cells |
| Coding region | Signal peptide + KDEL | ER or PB-I |
| | Glutelin fusion | PB-II |
| | Prolamin fusion | PB-I |
| | Gene name | RAP locus[a] |
| Terminator | *0.65 kb GluB1* terminator | Os02g0249800 |
| | *0.6 kb GluB4* terminator | Os02g0268300 |
| | *0.3 kb 10 kDa prolamin* terminator | Os03g0766200 |
| | *0.28 kb 16 kDa prolamin* terminator | Os06g0507200 |

[a]Please refer to http://rapdb.dna.affrc.go.jp/

In this chapter, we describe methods for the analysis of recombinant proteins produced in rice seed. These include protein extraction, confirming recombinant protein identity and subcellular localization, determining tolerance to digestive enzymes, and assessing unwanted ER stress responses.

## 2 Materials

### 2.1 Extraction of Total Protein from Mature Rice Seeds

1. Total protein extraction buffer: 50 mM Tris–HCl, pH 6.8, 8 M urea, 4 % SDS, 20 % glycerol, 5 % 2-mercaptoethanol, 0.01 % bromophenol blue.
2. Vortex mixer.
3. Microcentrifuge.
4. Grinder.

### 2.2 SDS-PAGE (for the Mini-slab Gel)

1. 1.5 M Tris–HCl, pH 8.8.
2. 0.5 M Tris–HCl, pH 6.8.
3. Distilled water.
4. 30 % acrylamide stock solution: 29 % (w/v) acrylamide, 1 % (w/v) methylene bis acrylamide.
5. 10 % ammonium peroxodisulphate (APS).
6. TEMED (N,N,N′,N′-tetramethylethylenediamine).
7. SDS-PAGE running buffer: 25 mM Tris hydroxyaminomethane, 192 mM glycine, 0.1 % (w/v) SDS.
8. Coomassie brilliant blue (CBB) R250 staining solution.
9. Destaining solution: 5 % (v/v) methanol, 7 % (v/v) acetic acid.
10. Electrophoretic apparatus for SDS-PAGE.
11. Size marker proteins (10–150 kDa).
12. SDS-PAGE loading buffer.
13. 10 % SDS.
14. 2-propanol.
15. Filter paper.
16. Kimwipes.
17. Shaker.

### 2.3 Immunoblot Analysis (for the Mini-slab Gel)

1. 10× Tris-buffered saline (TBS): 20 mM Tris–HCl, pH 7.5, 9 % NaCl.
2. Polyvinyl difluoride (PVDF) or nitrocellulose membrane (pore size 0.22–0.45 μm, 5.5 cm × 9 cm).
3. Filter paper (7.5 cm × 10.5 cm).

4. Blotting buffer: 25 mM Tris hydroxymethyl aminomethane, 192 mM glycine, 20 % methanol.
5. TTBS: 1× TBS, 0.05 % Tween 20.
6. Blocking buffer: 5 % skim milk powder in TTBS.
7. Primary antibody.
8. Horseradish peroxidase (HRP)-labeled secondary antibody (including a rabbit IgG antibody and HRP-linked antibodies).
9. ECL detection reagent.
10. X-ray film.
11. Devices for blotting.
12. Ice pack.
13. Shaker.
14. Methanol.
15. Darkroom.

### 2.4 Confocal Laser Scanning Microscopy

1. 10× phosphate buffered saline (PBS): 1.37 M NaCl, 81 mM $Na_2HPO_4$, 26.8 mM KCl, 14.7 mM $KH_2PO_4$.
2. Fixation solution: 3.7 % formaldehyde in 1× PBS.
3. Cell wall digestion solution: 1 % cellulase, 0.1 % pectolyase, in 1× PBS.
4. Permeabilization solution: 0.1 % Triton X-100 in 1× PBS.
5. Blocking solution: 1 % BSA, 0.01 % Triton X-100, in 1× PBS.
6. Primary antibody.
7. Secondary antibody with Alexa 488 conjugate.
8. 10 μg/mL rhodamine B (1× solution).
9. Slide glass.
10. Cover glass (e.g., 24×45 mm).
11. Razor blade.
12. Nail polish (inexpensive).
13. Confocal laser scanning microscope.
14. Low-melt agarose.
15. Gel tray.
16. Manicure pen.
17. Vibratome with accessories.

### 2.5 Digestibility Analysis of Recombinant Proteins

1. Grinder.
2. Microcentrifuge.
3. Pepsin dissolution buffer: 30 mM NaCl, pH 1.2 with HCl.

4. Pancreatin dissolution buffer: 50 mM $K_2HPO_4$–$KH_2PO_4$, pH 7.5.
5. Artificial gastric juice: 0.1 % pepsin in dissolution buffer.
6. Artificial intestinal juice: 1 % pancreatin in dissolution buffer.
7. Stop solution for the pepsin reaction: 160 mM $Na_2CO_3$.
8. Total protein extraction buffer (*see* **item 1** in Subheading 2.1).
9. Methanol.
10. Chloroform.
11. Distilled water.
12. Heat block or incubator.

### 2.6 Stepwise Extraction of Seed Storage Proteins

1. Globulin extraction buffer: 20 mM Tris–HCl, pH 6.8, 0.5 M NaCl.
2. Cysteine-rich prolamin extraction buffer: 60 % *n*-propanol.
3. Cysteine-poor prolamin extraction buffer: 60 % *n*-propanol, 5 % 2-mercaptoethanol.
4. Glutelin extraction buffer: 1 % lactic acid.
5. Total protein extraction buffer (*see* **item 1** in Subheading 2.1).
6. Litmus paper (pH 1–8).
7. 1 M NaOH.
8. Microcentrifuge.
9. Sonicator (the handheld type is easy to use).
10. Grinder.

### 2.7 Investigation of Undesirable Effects of Recombinant Proteins on Transgenic Rice Seeds

1. Liquid $N_2$.
2. Water-saturated phenol:chloroform:isoamyl alcohol (25:24:1).
3. RNA extraction buffer: 100 mM Tris–HCl, pH 9.0, 1 % SDS, 100 mM NaCl, 5 mM EDTA.
4. 99 % ethanol.
5. 3 M sodium acetate buffer, pH 5.2 with acetic acid.
6. 70 % ethanol.
7. DEPC-treated water (RNase-free water).
8. 8 M LiCl.
9. 2 M LiCl.
10. Vortex mixer.
11. Microcentrifuge.
12. Reagents for RT-PCR (reverse transcriptase, DNA polymerase, buffers, primers, dNTPs).
13. Thermal cycler or real-time PCR machine.
14. Grinder.

## 3 Method

### 3.1 Extraction of Total Protein from Matured Rice Seeds

When transgenic rice seeds are obtained, the first step is to prepare total seed protein for SDS-PAGE and immunoblot analyses. An adequate amount of seed protein can be extracted from one rice seed for SDS-PAGE or immunoblotting.

1. Grind mature seeds (*see* **Note 1**) individually into a fine powder.
2. Add 500 μL of total protein extraction buffer to the seed powder (approximately 20 mg) and vigorously vortex for 1 h at room temperature.
3. Centrifuge the mixture in a microcentrifuge at maximum speed (14,000 rpm; 10,000 × *g*) for 10 min at room temperature and collect the supernatant in a new tube by decantation. Typically, only 2–3 μL of supernatant is needed for SDS-PAGE and immunoblot analysis (Subheadings 3.2 and 3.3).

### 3.2 SDS-PAGE

To analyze total rice seed proteins, a small (e.g., 5.5 cm height × 9 cm width) 12 % acrylamide gel is suitable for the separation of these proteins. Thus, the SDS-PAGE method using a 12 % mini-slab gel is summarized in this section.

1. Assemble a mini-slab gel device, glass plate, comb, clip, and other necessary equipment (Fig. 3a).
2. Mark the level of the separation (running) gel on the glass plate (Fig. 3b).
3. Prepare a separation gel (Table 2) and mix gently without generating bubbles.
4. Immediately transfer the separating gel solution into the gap between the glass plates.
5. Add 2-propanol on top of the separation gel and keep the glass plate at room temperature for at least 30 min, until gel polymerization (*see* **Note 2**).
6. Completely remove 2-propanol from the gel plate by absorption with filter paper and prepare the stacking gel (Table 2).
7. After transferring the stacking gel to the top of the separation gel, put the comb in the stacking gel without generating bubbles. The stacking gel will completely polymerize within a few minutes.
8. Set the SDS-PAGE gel and running buffer in an electrophoresis gel tank.
9. After removing the comb, apply the samples (2–5 μL) and size marker proteins, mixed with loading buffer, to the wells of the SDS-PAGE gel.

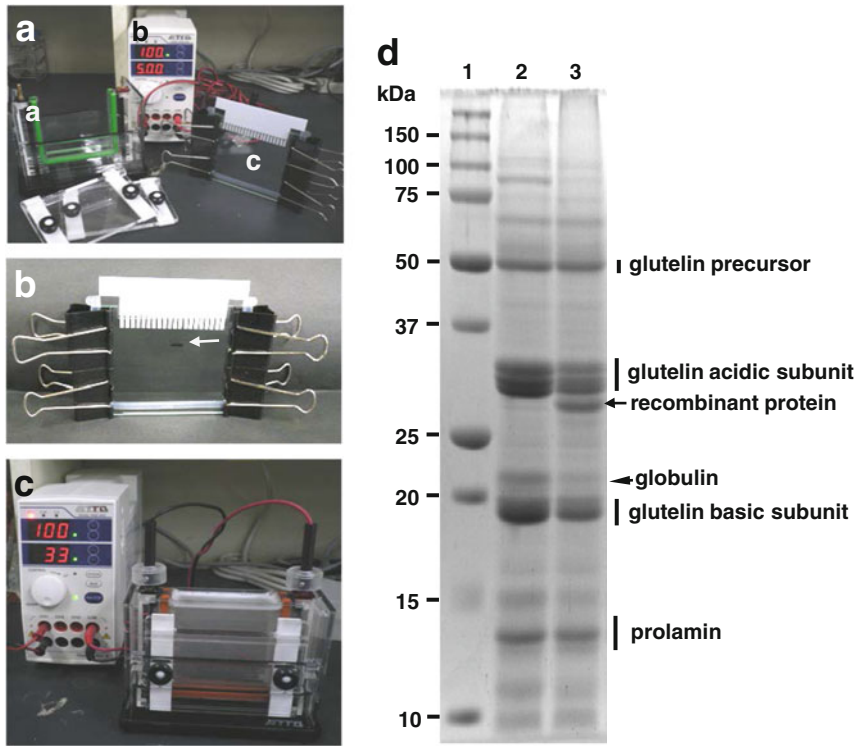


**Fig. 3** SDS-PAGE. **a**, Assembly for SDS-PAGE, the electrophoretic apparatus (a), power supply (b), glass plate (c). **b**, Determine the amounts of separation (running) gel on the gel plate (*white arrow*). **c**, Electrophoresis. Electrophoresis generally takes approximately 1 h (200 V) ~ 2 h (100 V) to complete. **d**, CBB-stained SDS-PAGE gel. Lane 1, size marker; Lane 2, total rice seed protein (cv. Kitaake); Lane 3, total rice seed protein (transgenic rice expressing one recombinant protein). The positions of the glutelin precursor, acidic or basic glutelin, globulin, prolamin, and recombinant proteins on the SDS-PAGE gel are indicated on the right side of the panel. If a clear recombinant protein signal is detected by CBB staining such as in lane 3, the accumulation of the recombinant protein is considered to be high. If no signal derived from the recombinant protein is detected at this step, proceed to immunoblot analysis

10. Perform electrophoresis according to standard methods (Fig. 3c). Electrophoresis is completed when the tracking dye moves to the lower edge of the PAGE gel.
11. After electrophoresis, stain the SDS-PAGE gel with CBB or by immunoblotting (*see* Subheading 3.3 for immunoblotting).
12. In the case of CBB staining, transfer the SDS-PAGE gel to a small container. Add CBB R250 solution to the container and gently shake for at least 15 min at room temperature (*see* **Note 3**).
13. Wash the stained SDS-PAGE gel with destaining solution for at least 2 h at room temperature by gently shaking (*see* **Note 4**).
14. Ensure the signal pattern of the seed protein samples (Fig. 3d).

**Table 2**
**Components of separation (12 %) and stacking gels for SDS-PAGE**

| | Final concentration | Mini-slab gel × 1 | Mini-slab gel × 2 | Mini-slab gel × 4 |
|---|---|---|---|---|
| Separation (Running) gel | | Total approx. 8 mL | Total approx. 16 mL | Total approx. 32 mL |
| Water | – | 2.8 mL | 5.6 mL | 11.2 mL |
| 30 % acrylamide | 12 % | 3.2 mL | 6.4 mL | 12.8 mL |
| 1.5 M Tris–HCl pH 8.8 | 375 mM | 2 mL | 4 mL | 8 mL |
| 10 % SDS | 0.1 % | 80 µL | 160 µL | 320 µL |
| 10 % APS | – | 30 µL | 60 µL | 120 µL |
| TEMED | – | 4 µL | 8 µL | 16 µL |
| Stacking gel | – | Total approx. 2.5 mL | Total approx. 5.0 mL | Total approx. 10.0 mL |
| Water | – | 1.44 mL | 2.88 mL | 5.76 mL |
| 30 % acrylamide | 4.8 % | 400 µL | 800 µL | 1.6 mL |
| 0.5 M Tris–HCl pH 6.8 | 125 mM | 625 µL | 1.25 mL | 2.5 mL |
| 10 % SDS | 0.1 % | 25 µL | 50 µL | 100 µL |
| 10 % APS | – | 30 µL | 60 µL | 120 µL |
| TEMED | – | 2 µL | 4 µL | 8 µL |

### *3.3 Immunoblot Analysis*

Described here is a typical immunoblotting method using immersion (wet)-type transfer, primary and secondary antibodies, an enhanced chemiluminescence (ECL) detection system, and X-ray films. These methods remove the need for a commercial kit and specific machines, thereby reducing costs.

In immunoblot analysis, a specific antibody is required as a primary antibody for the target protein. Prepare the primary antibody needed or fuse a tag (e.g., His-tag) to the recombinant protein beforehand.

1. Prepare the blotting apparatus (blotter, membrane, filter paper, ice pack, and blotting buffer) (Fig. 4a). If a PVDF membrane is used for blotting, immerse the PVDF membrane in 100 % methanol before use and then transfer to the blotting buffer.
2. Perform blotting according to standard conditions (e.g., 80 V for 1 h) (Fig. 4b, c). Use an ice pack to prevent excessive temperature increases in the blotting buffer due to electrification (Fig. 4c).

3. Transfer the membrane to a container filled with 10–20 mL of 1× TTBS containing 5 % skim milk powder. Shake gently for at least 1 h at room temperature (Fig. 4d).
4. Add the appropriate primary antibody (1000–10,000× dilution) and react by gently shaking for 3 h at room temperature or overnight at 4 °C.
5. Wash the membrane with 1× TTBS for 10 min at room temperature.
6. Repeat the wash step two times. Prepare 1× TTBS containing 0.5 % skim milk powder while washing the primary antibody.
7. After washing, add 1× TTBS containing 0.5 % skim milk powder to the container. Add appropriate amounts of the HRP-labeled secondary antibody (e.g., Rabbit IgG antibody or HRP-linked antibody) according to the manufacturer's protocol (5000–10,000× dilution).
8. React by shaking gently for 1–3 h at room temperature.
9. Wash the membrane with 1× TTBS for 10 min at room temperature three times.
10. Transfer the membrane to a new container and prepare the ECL detection reagent, X-ray film, and cassette.
11. Detect signals using the ECL detection reagent in a dark room according to the manufacturer's protocol (Fig. 4e) (*see* **Notes 5** and **6**).

### 3.4 Confocal Laser Scanning Microscopy

This experiment is conducted to determine the intracellular localization of recombinant proteins in rice seed cells using confocal microscopy and an antibody to the recombinant protein (confocal immunohistochemical microscopy). Seeds collected approximately 10–15 days after flowering (DAF) are suitable as samples for confocal microscopy. Sample preparation becomes difficult at more than 20 DAF due to seed firmness.

1. Harvest premature seeds (10–15 DAF) and carefully remove the hull (lemma and palea) from the seeds (Fig. 5a).
2. Cut seeds in half using a razor blade (Fig. 5a).
3. Suspend 4 g of low melting agarose in 100 mL water. Dissolve the agarose well in a microwave oven.
4. Pour liquefied agarose into a gel tray and cool the agarose 50 °C at room temperature (Fig. 5b).
5. Place the rice seeds, with the cut surface facing down, into the liquefied agarose with fine-tipped tweezers (Fig. 5c).
6. Leave the samples at room temperature for at least 15–20 min until gelling is complete.
7. While waiting for gelling, prepare the vibratome and its accessories.

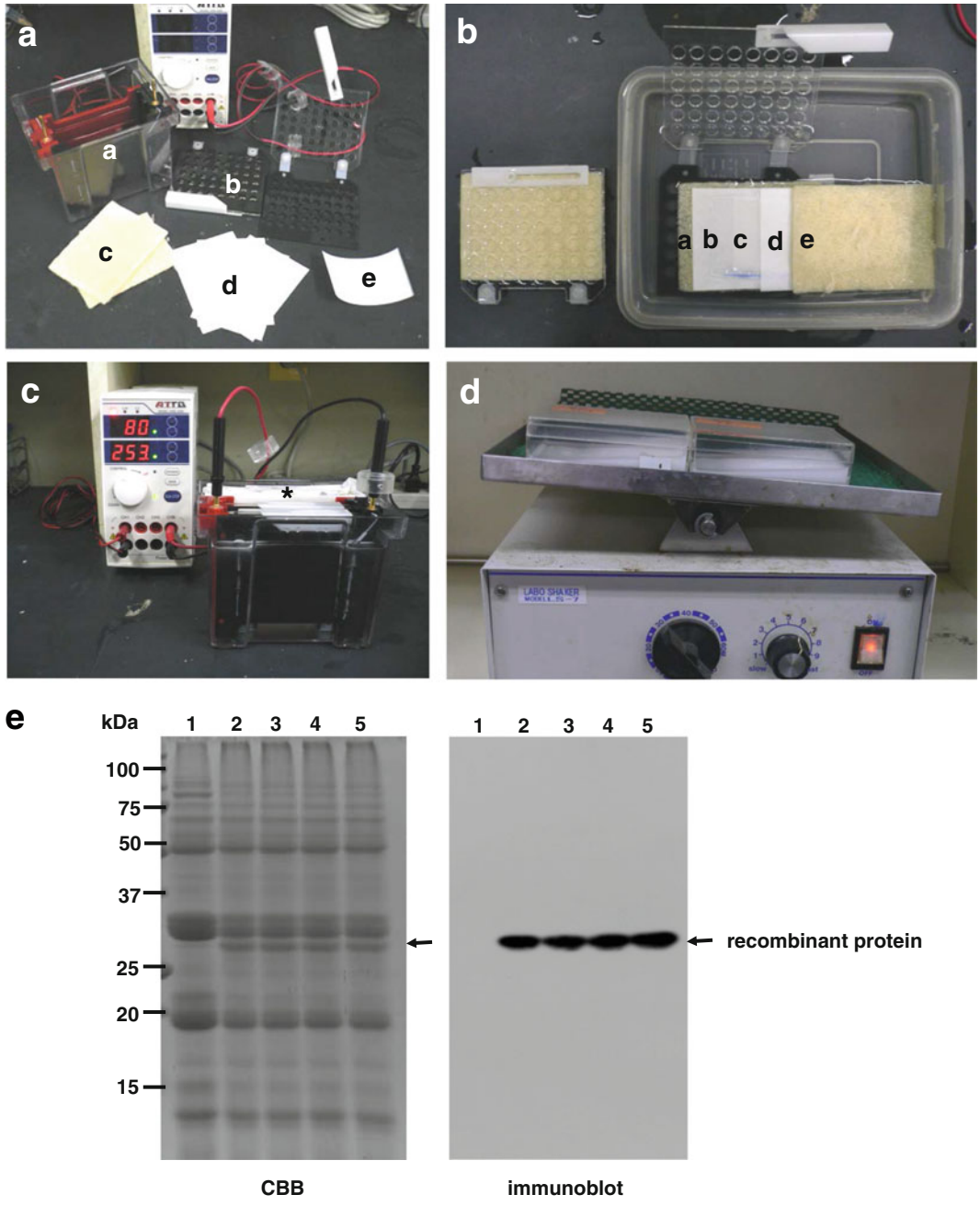


**Fig. 4** Immunoblotting. **a**, Blotting apparatus for wet (immersion) type transfer. a, buffer tank; b, Sandwich plate; c, urethane pads; d, filter paper; e, PVDF membrane. **b**, Assembly of the sandwich plate. (a) Urethane pad, (b) filter paper, (c) PAGE gel, (d) filter paper, (e) urethane pad. **c**, Blotting. An *asterisk* indicates an ice pack. **d**, Gentry shaking of membranes. **e**, CBB-stained SDS-PAGE gel (CBB) and X-ray film after signal detection (immunoblot). Lane 1, nontransgenic rice seed; lanes 2–5, transgenic rice seed expressing a recombinant protein. An *arrow* indicates the signal of a recombinant protein

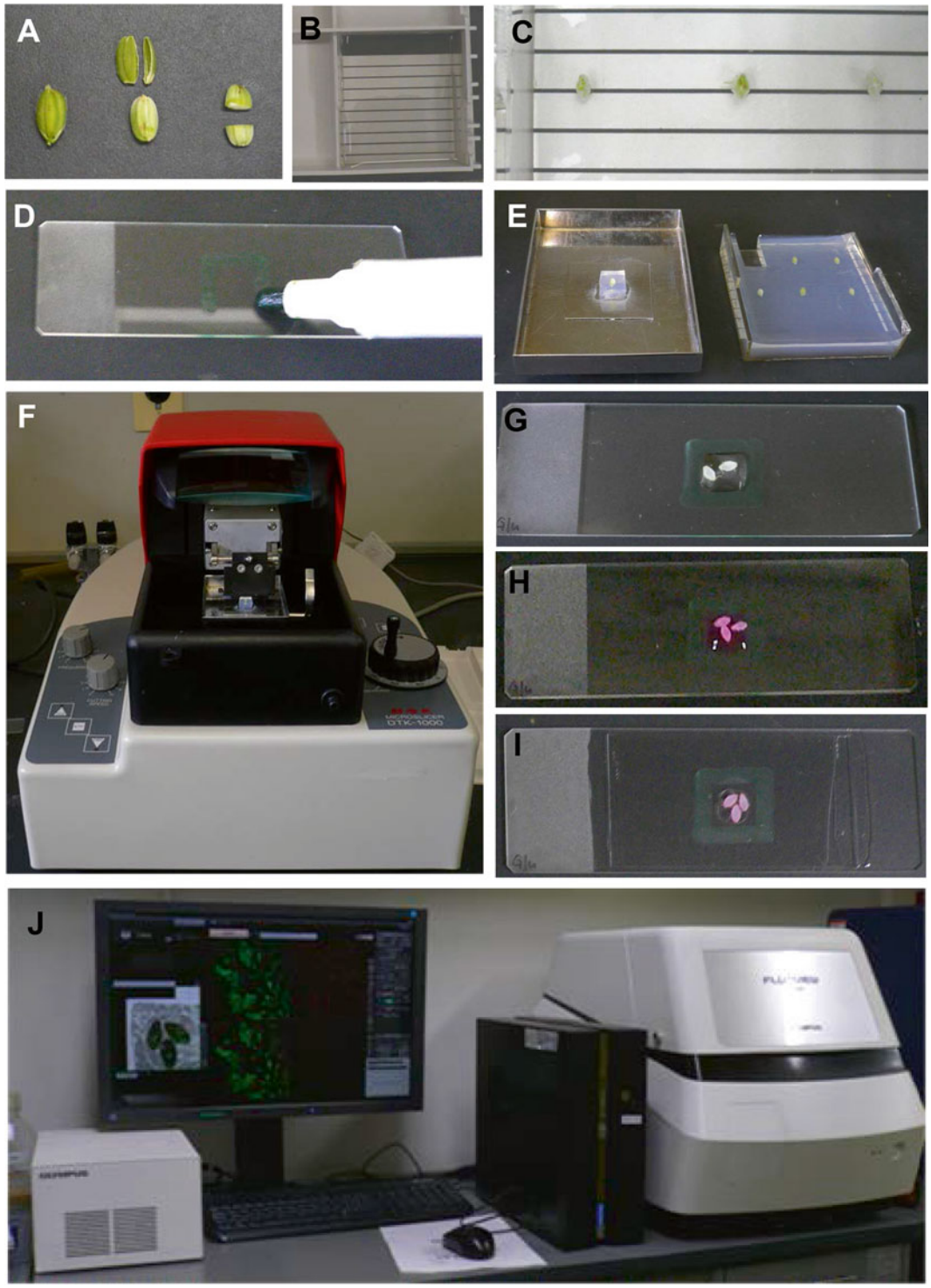


**Fig. 5** Confocal immunohistochemical microscopy analysis. (**a**) Premature seed (*left*), hulled premature rice (*middle*), and cut premature seed (*right*). (**b**) Agarose gel polymerization on the gel plate. (**c**) Agarose gel-embedded samples. (**d**) Making a square frame on a slide glass. (**e**) Agarose block on a steel dish. (**f**) Setting of the steel dish on a vibratome. (**g**) Sample sections on slide glass. (**h**) Rhodamine staining of samples. (**i**) Preparation for observations using confocal microscopy. (**j**) Confocal laser scanning microscopy

8. Draw an approximately 1-cm square frame on a slide glass with a manicure pen (Fig. 5d).
9. Use a razor blade to cut blocks of agarose containing the samples.
10. After excising the agarose containing the seed samples, adhere the gel block onto the steel dish of the vibratome (Fig. 5e).
11. Set the steel dish on the vibratome (Fig. 5f).
12. Use the vibratome to cut approximately 100–200 μm-thick slices from the agarose gel block. Place the sections (one to six sections) in distilled water within the square frame of the slide glass (Fig. 5g).
13. Remove the distilled water, apply 3.7 % formaldehyde in 1× PBS to the sections, and incubate at room temperature for 1 h (fixation).
14. Wash three times with 1× PBS at intervals of 10 min at room temperature.
15. Apply cell wall digestion solution to the sections and incubate for 10 min at room temperature.
16. Wash three times with 1× PBS at intervals of 10 min at room temperature.
17. Apply permeabilization solution to the sections and incubate for 15 min at room temperature.
18. Wash three times with 1× PBS at intervals of 10 min at room temperature.
19. Apply blocking solution to the sections and incubate for 1 h at room temperature.
20. Wash three times with 1× PBS at intervals of 10 min at room temperature.
21. Dilute the primary antibody (100–1000× dilution) in blocking solution and apply the primary antibody solution to the sections. Incubate for 3 h at room temperature or overnight at 4 °C (*see* **Note 7**).
22. Wash three times with 1× PBS at intervals of 10 min.
23. Dilute the secondary antibody (100–500× dilution) in blocking solution and apply the secondary antibody solution to the sections. Incubate for 3 h at room temperature.
24. Use rhodamine B to stain PB-I. Apply 1× rhodamine B solution to samples and incubate for 15 min at room temperature (Fig. 5h).
25. Wash three times with 1× PBS at intervals of 10 min at room temperature.
26. Mount the cover glass on sections and seal the cover glass by using nail polish (Fig. 5i). Large cover glasses (e.g., 24 × 45 mm) may be more easy to use than normal cover glasses (18 × 18 mm).

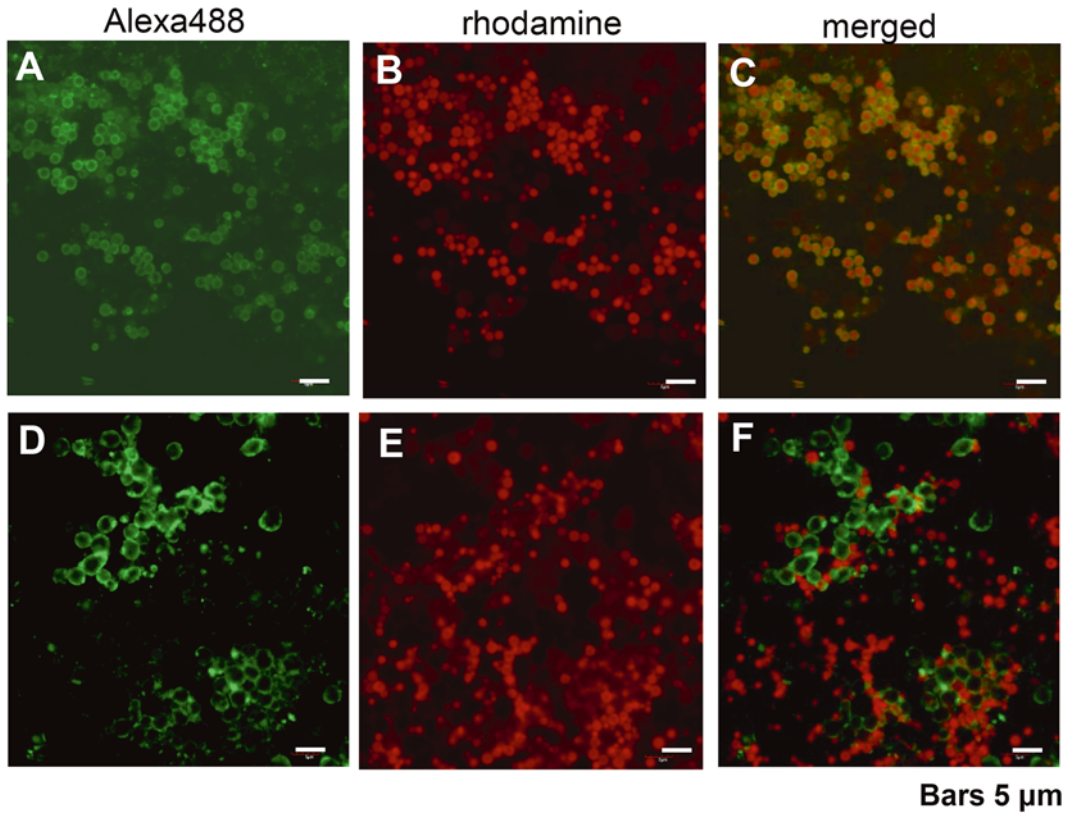


**Fig. 6** Indirect immunohistochemical analysis using a confocal laser scanning microscope. *Left panels* show the localization of recombinant proteins (*green*, Alexa 488), *middle panels* show the localization of PB-I (*red*, rhodamine), and *right panels* show merged images. (**a–c**) This recombinant protein accumulated in PB-I. (**d–f**) This recombinant protein accumulated in PB-II (*see* **Note 17**)

27. View the preparation with a confocal laser scanning microscope (e.g., FV10i, Olympus) according to the manufacturer's protocol (Figs. 5j and 6). Magnification of 60–300× is usually suitable for rice endosperm cells. Recommended excitation wavelengths are around 499 nm (Alexa 488, emission 520 nm) and 558 nm (rhodamine B, emission 575 nm).

### *3.5 Stepwise Extraction of Seed Storage Proteins*

Unexpected subcellular localization of recombinant proteins has been reported in transgenic rice seeds. For example, a protein expressed as a glutelin fusion was sequestered to PB-I, but was not detected in PB-II [15, 16]. This was attributed to free cysteine residues in the recombinant protein, which formed intermolecular disulfide bonds with cysteine-rich prolamins that accumulate mainly in PB-I, such as 10 kDa prolamin, 13 kDa cys-rich prolamin, and 16 kDa prolamin. However, the accumulation of recombinant proteins in PB-I has several advantages. Human pharmaceutical proteins deposited in PB-I are more effectively

delivered to the small intestine without being degraded than those targeted to PB-II or to the ER.

Interactions between cysteine-rich prolamins and recombinant proteins expressed as glutelin fusion proteins can be confirmed experimentally. This section describes a stepwise seed protein extraction method, consisting of three separate stepwise extractions, to determine colocalization of recombinant glutelin fusions with endogenous seed storage proteins. An outline of this experiment is shown in Table 3 and Fig. 7a.

1. Grind individual mature transgenic rice seeds into fine powder. Three samples are needed: one for each of the three extractions.
2. Add 500 μL of globulin extraction buffer to each sample and sonicate for 2 min on ice.
3. Centrifuge in a microcentrifuge at maximum speed (20,000 × *g*) for 10 min. Remove the supernatant (*see* **Note 8**) and wash the pellet three times by resuspending in 500 μL of globulin extraction buffer and centrifuging at maximum speed for 10 min. For one of the samples, proceed to **step 8** (Extraction 1). For the other two samples, continue with **step 4**.
4. Resuspend the pellet in 500 μL of cys-poor prolamin extraction buffer and sonicate for 2 min on ice.
5. Centrifuge at maximum speed (14,000–20,000 × *g*) for 10 min. Remove the supernatant (*see* **Note 9**). Wash the pellet three times by resuspending in 500 μL of the cys-poor prolamin extraction buffer and centrifuging at maximum speed for 10 min. For one of the samples, proceed to **step 8** (Extraction 2). For the remaining sample, continue with **step 6** (Extraction 3).

**Table 3**
**Extraction order of the stepwise extraction**

| | Buffer composition | Extraction 1 | Extraction 2 | Extraction 3 |
|---|---|---|---|---|
| Globulin extraction buffer | 0.5 M NaCl, 10 mM Tris–HCl (pH 6.8) | + | + | + |
| Cys-poor extraction buffer | 60 % *n*-propanol | – | + | + |
| Cys-rich extraction buffer | 60 % *n*-propanol, 5 % 2-mercaptoethanol | – | – | + |
| Glutelin extraction buffer | 1 % lactic acid | + | + | + |
| Extraction steps in Subheading 3.5 | | **1–3, 8–12** | **1–5, 8–12** | Perform all steps |

+, with pre-extraction; –, without pre-extraction

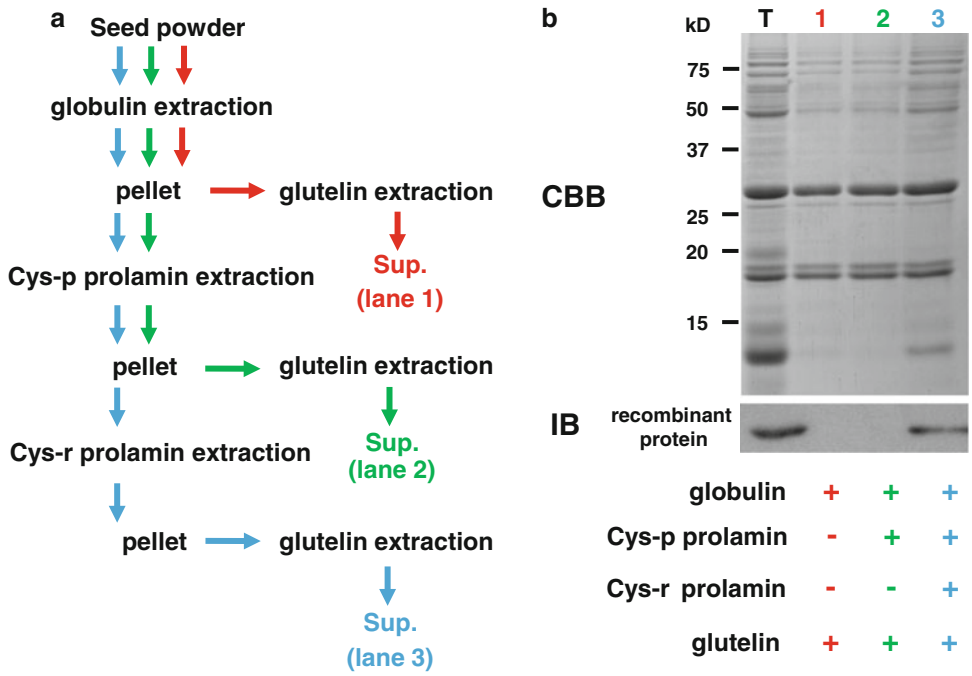


**Fig. 7** Stepwise extraction of seed storage proteins. (**a**) Flow chart of this experiment. (**b**) SDS-PAGE (CBB) and immunoblot (IB) analyses are shown. Proteins were extracted with 1 % lactic acid following a pre-extraction with different solvents. Total seed proteins; +, with pre-extraction; –, without pre-extraction; Cys-p, cysteine-poor; Cys-r, cysteine-rich. This figure was reprinted with permission from Wiley. *Red*, *green*, and *blue arrows* indicate extraction 1, extraction 2, and extraction 3 in Table 3, respectively

6. Resuspend the pellet in the cys-rich prolamin extraction buffer and sonicate for 2 min on ice.
7. Centrifuge at the maximum speed (14,000–20,000 × *g*) for 10 min. Remove the supernatant (*see* **Note 9**). Wash the pellet three times by resuspending in 500 μL of the cys-rich prolamin extraction buffer and centrifuging at maximum speed for 10 min.
8. Resuspend the pellet in the glutelin extraction buffer and sonicate for 2 min on ice.
9. Centrifuge at the maximum speed (14,000–20,000 × *g*) for 10 min. Collect the supernatant.
10. Adjust the pH of samples to pH 7.0 with 1 M NaOH (*see* **Note 10**).
11. Centrifuge at the maximum speed (14,000–20,000 × *g*) for 10 min. Remove the supernatant and dry the pellet in air or by using a vacuum pump.

12. Resuspend the pellet in 500 μL of total protein extraction buffer and subject the samples to SDS-PAGE and immunoblotting according to Subheadings 3.2 and 3.3 (Fig. 7). If the recombinant protein is observed in the sample from Extraction 3, this indicates that the protein interacts with cysteine-rich prolamins and accumulates in PB-I (*see* **Note 11**).

### 3.6 Digestibility Analysis of Recombinant Protein

This experiment determines whether recombinant proteins in rice seed cells exhibit higher resistance to digestive enzymes than bare proteins. When recombinant proteins for oral intake are expressed in transgenic rice seeds, this experiment should be conducted to validate their resistance to gastric and intestinal juices. Control proteins are needed for a comparison of resistance to digestive enzymes. Although purified recombinant proteins derived from *E. coli* are often available, the use of proteins extracted from rice seeds is preferable, since identical glycosylation patterns are assumed. This section explains the methodologies used in the digestion experiment with an artificial gastric juice (pepsin; Subheading 3.6.1) or artificial intestinal juice (pancreatin; Subheading 3.6.2) as well as the preparation of control proteins from rice seeds (Subheading 3.6.3).

#### 3.6.1 Pepsin Digestion

1. Set a heat block or incubator to 37 °C.
2. Grind five to ten dry seeds.
3. Prepare seven microcentrifuge tubes per sample. Six tubes are needed for the pepsin time course treatment (for, e.g., 0, 1, 5, 10, 20, and 30 min), and one is for a negative control.
4. Transfer 5 mg of seed powder into each microcentrifuge tube.
5. Add 150 μL of artificial gastric juice (pepsin solution) to each tube and immediately mix well. Incubate the tubes at 37 °C for 0–30 min, removing each tube at a different time point. For the negative control, add the same volume of pepsin dissolution buffer without pepsin and incubate at 37 °C for 30 min.
6. After this incubation, immediately add 100 μL of the stop solution and mix well.
7. Add 750 μL of the total protein extraction buffer and mix well (*see* **Note 12**).
8. Centrifuge in a microcentrifuge at maximum speed for 10 min and collect the supernatant.
9. Subject 8 μL of the supernatant to SDS-PAGE and immunoblotting according to Subheadings 3.2 and 3.3 (Fig. 8).

#### 3.6.2 Pancreatin Digestion

1. Follow **steps 1–4** of Subheading 3.6.1 (pepsin digestion).
2. Add 200 μL of artificial intestinal juice (pancreatin solution) into each microcentrifuge tube and immediately mix well.

Incubate the tubes at 37 °C for 0–120 min, removing each tube at a different time point. As a negative control, add the same volume of pancreatin dissolution buffer without pancreatin and incubate at 37 °C for 120 min.

3. After this incubation, add 800 μL of total protein extraction buffer and mix well (*see* **Note 13**).
4. Boil the samples for 5 min to inactivate the pancreatin enzyme.
5. Mix the samples again. Centrifuge at maximum speed for 10 min and collect the supernatant.
6. Subject 8 μL of samples to SDS-PAGE and immunoblotting according to Subheadings 3.2 and 3.3. Results of the pancreatin digestion would be similar to the pepsin digestion (Fig. 8).

### *3.6.3 Preparation of Control Proteins*

1. Extract proteins from transgenic rice as described in Subheading 3.1. Approximately 400–450 μL of total protein solution can be obtained from one seed.
2. Transfer 200 μL of the total protein sample to a new microcentrifuge tube.
3. Add 600 μL of methanol and mix well.
4. Add 200 μL of chloroform and vortex vigorously for 1 min.
5. Add 600 μL of distilled water and mix well.

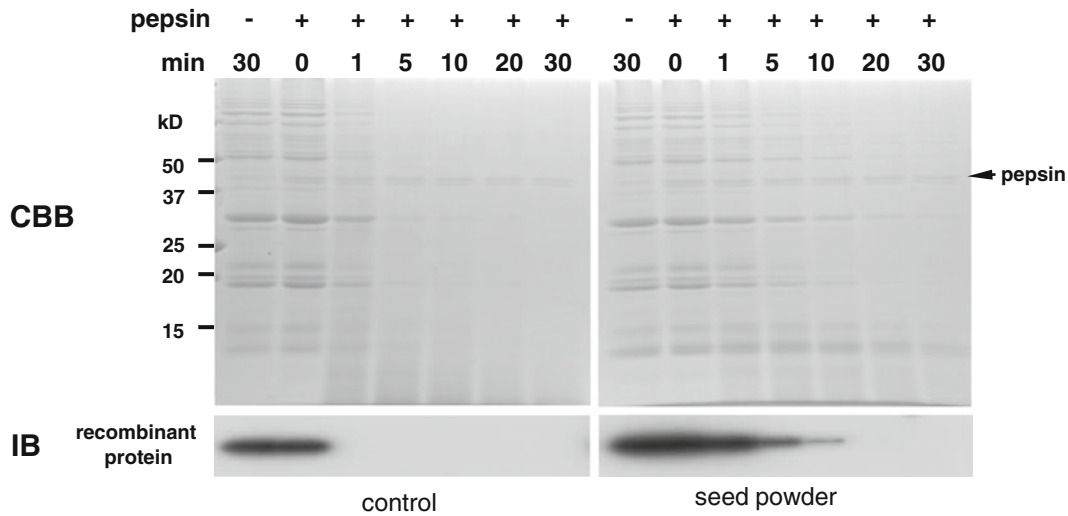


**Fig. 8** Digestibility analysis of recombinant protein. The digestibility of recombinant proteins by 0.1 % pepsin at 37 °C for up to 30 min (0, 0.5, 1, 5, 10, 20, and 30 min). The *left panel* shows recombinant proteins after extraction from transgenic rice seeds (control). The *right panel* shows transgenic seed powder (seed powder). SDS-PAGE (CBB) and immunoblot (IB) analyses are shown. +, with 0.1 % pepsin; –, without 0.1 % pepsin. An *arrowhead* indicates pepsin

6. Centrifuge the sample in a microcentrifuge at maximum speed (14,000–20,000 × *g*) for 1 min.
7. Remove the aqueous phase carefully, ensuring not to touch the middle layer (protein layer).
8. Add 600 μL of methanol and mix well.
9. Centrifuge the sample at maximum speed for 1 min.
10. After air-drying the pellet, grind the pellet into a fine powder.
11. Use the powder for control samples for comparison to rice seed powder in the pepsin and pancreatin digests (Fig. 8) (*see* **Note 14**).

### *3.7 Investigation of Undesirable Effects of Recombinant Proteins on Transgenic Rice Seeds*

When recombinant proteins or peptides are accumulated as secretory proteins in rice seeds for oral intake, grain qualities such as yield, appearance, and flavor are important. Some recombinant proteins perturb homeostasis in rice seed cells, resulting in ER stress responses. ER stress responses maintain ER homeostasis by balancing the folding capacity and folding demands imposed on the ER [17]. Transgenic rice seeds under ER stress conditions have shrunken and floury phenotypes that lead to reductions in grain quality and yield [18]. Low grain quality due to ER stress conditions during seed development in transgenic rice is an issue that needs to be addressed. A clearer understanding of the molecular mechanisms underlying ER stress is needed in the near future. Here, we describe a method to estimate relative ER stress levels in transgenic rice seeds using RT-PCR of ER stress marker genes (Table 4). The expression of these genes is closely associated with ER stress levels in rice [19].

**Table 4**
**Primer sets for amplification of ER stress responsive genes in rice**

| Gene name | Accession no. | Forward primer (5′–3′) | Reverse primer (5′–3′) |
|---|---|---|---|
| *OsBiP2* | Os03g0710500 | TACTCCTCTGTTCAGGGTG | GCTTGATGTCGAAGATGGTG |
| *OsBiP3* | Os05g0367800 | GGCGTCAAGAACGCCGTGGTGGG | AAATAAATTCTAGCTAGCCGGCGG |
| *OsBiP4* | Os05g0428600 | CAAGGAGGAGTACGAGGAGAAG | CACACTTTCGATCGAATCCAAAC |
| *OsBiP5* | Os08g0197700 | AACATCAAGAACACGCTCGGCG | GCAGCTAGCACCTAGCAGCGAC |
| *SAR1B*-like | Os06g0225000 | TTCTACATGCTCTCACAAG | ACCACGTAAACTACTGCATC |

### 3.7.1 Total RNA Extraction from Rice Seed Tissue

1. Freeze premature seed samples using liquid $N_2$ (1–3 seeds per tube).
2. Grind the frozen samples into a fine powder and add 400 μL of water-saturated phenol:chloroform:isoamyl alcohol and 400 μL of RNA extraction buffer immediately to the powder.
3. Vortex for at least 15 min at room temperature
4. Centrifuge in a microcentrifuge at maximum speed (14,000–20,000 × *g*)
5. Collect the supernatant in a new tube. Add 400 μL of water-saturated phenol:chloroform:isoamyl alcohol.
6. Mix well and centrifuge at the maximum speed.
7. Collect the supernatant in a new tube.
8. Add 40 mL of 3 M sodium acetate buffer and 1 mL of 99 % ethanol and mix well.
9. Incubate for at least 10 min on ice.
10. Centrifuge samples at maximum speed for 10 min.
11. Discard the supernatant and dry the pellet in air or by using a vacuum pump.
12. Dissolve the pellet in 60 μL of DEPC-treated water (or RNase-free water).
13. Add 20 μL of 8 M LiCl to the sample and mix well.
14. Incubate samples for at least 1 h or overnight at 4 °C.
15. Centrifuge samples at maximum speed for 10 min.
16. Discard the supernatant and rinse the pellet with 2 mM LiCl.
17. Rinse the pellet well with 70 % ethanol (*see* **Note 15**)
18. Dry the pellet in air or by using a vacuum pump and dissolve in 30–100 μL of DEPC-treated water (or RNase-free water) (*see* **Note 16**)

### 3.7.2 RT-PCR

Perform RT-PCR or quantitative real-time RT-PCR using equal amounts of RNA template according to the manufacturer's protocols (Fig. 9). The RT reaction can be primed with a mixture of oligo-dT and random hexamers. Some candidate maker genes and their primer sets for the amplification of these genes are shown in Table 4. Thirty cycles of PCR reaction will give preferable results. No amplification signal should be detected in normal seed RNA, but a clear amplification signal is detected in RNA from an ER-stressed seed. To normalize for differences in starting RNA concentrations, use a reference RNA such as *17S ribosomal* RNA (Forward primer 5′-tccatcttggcatctctcag-3′ and reverse primer 5′-gtacccgcatcaggcatctg-3′). ER stress levels are estimated based on the accumulation of mRNA from genes in Table 4 and matured seed phenotypes [18].

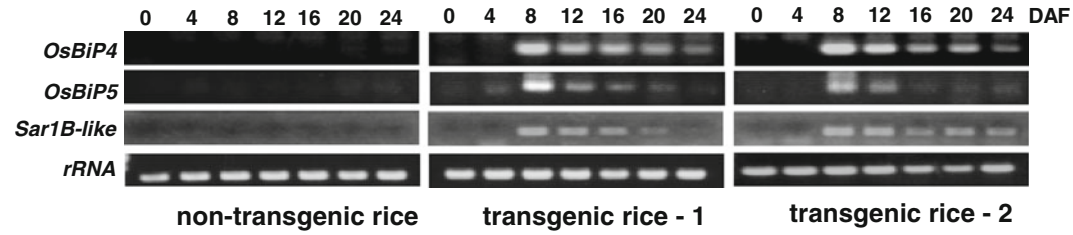


**Fig. 9** RT-PCR analysis of ER stress marker genes in nontransgenic rice or transgenic rice seeds. Total RNA was extracted from 0, 4, 8, 12, 16, 20, and 24 DAF seed tissues. The expression of *OsBiP4*, *OsBiP5*, and *Sar1B-like* genes is shown as examples. *17S ribosomal RNA* (*rRNA*) was used as a control. Transgenic rice 1 and transgenic rice 2 exhibit ER stress in response to recombinant protein accumulation, as revealed by the expression of the stress marker genes

## 4 Notes

1. If the aim is to extract total protein from premature seeds, the seed sample should be frozen by liquid $N_2$ before grinding.
2. Once the separation gel is polymerized, a clear boundary line can be observed between the gel and 2-propanol.
3. CBB-G250 is also available for gel staining. If you use CBB-G250, washing buffer should be replaced with distilled water.
4. Placing Kimwipes in the destaining solution helps decolorize the stained gel by adsorbing the released dye.
5. If a high background and/or nonspecific signals are detected, use high stringency washing buffer (0.2 % SDS, 0.5 % Triton X-100 in 1× TBS) instead of TTBS.
6. After ECL signal detection, the membrane can be stained with CBB to ensure blotting efficiency. Transfer the membrane to CBB-R250 and shake gently for 1–2 min. Wash the membrane with distilled water and dry at room temperature.
7. When the sample is reacted for a long time with the primary antibody on glass slides, the reaction should be performed in an airtight plastic container (such as Tupperware) together with wet Kimwipes to prevent the primary antibody solution from drying.
8. To ensure that globulin dissolved adequately, add an equal volume of total protein extraction buffer to the supernatant and perform SDS-PAGE. You can see a visible band of globulin together with water and saline soluble proteins (Fig. 10, lane 1).
9. To ensure that cys-poor or cys-rich prolamins dissolved adequately, add an equal volume of distilled water to the supernatant and incubate for at least 1 h on ice. After centrifuging at the maximum speed (14,000–20,000×*g*), dissolve the pellet in total protein extraction buffer. Subject the sample to SDS-PAGE. You can see visible bands of cys-poor or cys-rich

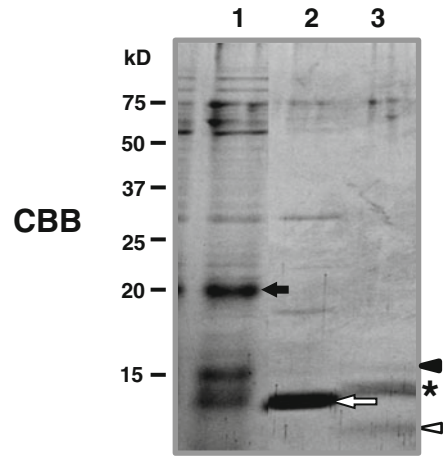


**Fig. 10** SDS-PAGE of globulin, cys-poor prolamin, and cys-rich prolamin fractions. Lane 1, globulin fraction; Lane 2, cys-poor prolamin fraction; Lane 3, cys-rich prolamin fraction. Bands corresponding to globulin (*black arrow*), 13 kDa cys-poor prolamins (*white arrow*), 16 kDa cys-rich prolamin (*black arrowhead*), 13 kDa cys-rich prolamins (*asterisk*), and 10 kDa cys-rich prolamins (*white arrowhead*) are indicated

prolamin (Fig. 10, lanes 2 and 3, respectively). Because cys-rich prolamins accumulate at lower levels than the other seed storage proteins, the entire cys-rich prolamin fraction sample should be subjected to SDS-PAGE.

10. Use litmus paper (wide range) to measure approximate pH. Glutelins are precipitated as insoluble matter as pH levels approach 7.0. Glutelins can redissolve if the pH increases above 8.0, for example, if too much NaOH is added. In this case, the pH can be lowered to around 7.0 by adding more glutelin extraction buffer.
11. Endogenous glutelins can typically be extracted after the removal of globulins from the seed powder (extraction 1), or the removal of cysteine-poor prolamins after globulin has been removed (extraction 2). However, recombinant proteins are sometimes extracted by lactic acid only after cysteine-rich prolamins have been removed by a 2-propanol and 2-mercaptoethanol treatment following th extraction of globulin (extraction 3). In this case, the necessity for the removal of cysteine-rich prolamins before the extraction of recombinant proteins suggests that the free-cys of recombinant proteins aggregates with cysteine-rich prolamins via intermolecular disulfide bonds.
12. Bromophenol blue in the total extraction buffer is used as a pH indicator. The color of the solution, after adding the extraction buffer, should be blue. An orange or yellow color indicates that the solution is too acidic. In such a case, slowly add more of the stop solution (which is basic) until the color changes to blue.

13. The addition of total protein extraction buffer to samples is not sufficient to inactivate pancreatin. Samples should be immediately boiled after adding the total protein extraction buffer or stored at −30 °C until the boiling step.
14. Since only a small amount of seed protein is lost during these steps, the amount of protein collected should be adequate. Because seeds weigh approximately 17–20 mg per seed, control proteins equivalent to approximately 6.8–8 mg of seed powder can be obtained from 200 μL of the total protein extract. Control proteins are typically digested less than 1–2 min after pepsin or pancreatin treatment.
15. $Li^+$ remaining in the samples may inhibit the activity of reverse transcriptase in subsequent steps (RT-PCR).
16. A DNase I treatment should be performed before RT-PCR. On the other hand, an RT-PCR kit in which a separate DNase I treatment is unnecessary is also commercially available (e.g., ReverTra Ace qPCR RT Master Mix with gDNA Remover).
17. Signals of recombinant proteins that accumulate in PB-I completely merge with rhodamine signals. When recombinant proteins accumulate in PB-II or the ER, a double immunostaining experiment using two antibodies [e.g., an anti-recombinant protein antibody (rabbit) and anti-marker protein (mouse)] is required to confirm the correct localization of recombinant proteins. OsTiP3 and calnexin are generally used as PB-II and ER marker proteins, respectively. On the other hand, not only PB-I but also PB-II can be stained with treatment of rhodamine (for more than 1 h) but PB-II may be stained pale red (Fig. 11) [20].

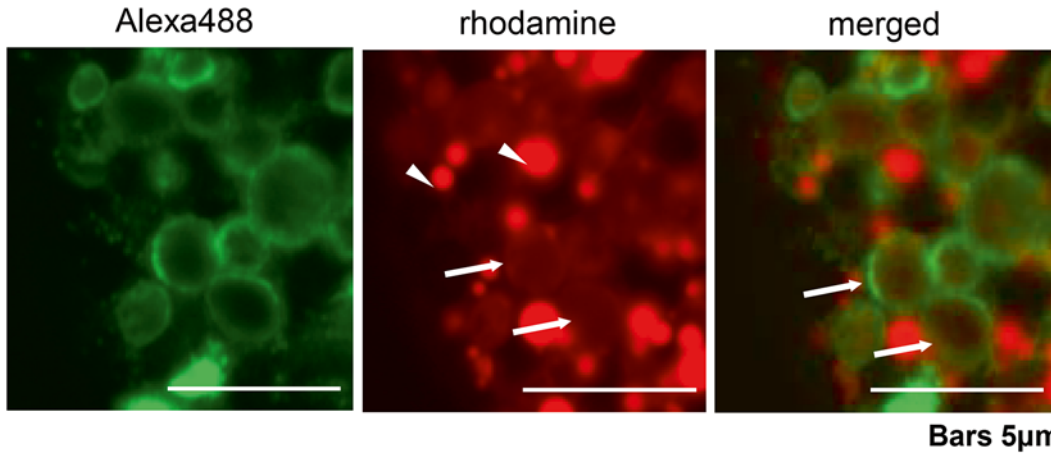


**Fig. 11** Over-staining (for more than 1 h) of rhodamine B in transgenic rice seeds. The *left panel* shows the localization of recombinant proteins (*green*, Alexa 488), the *middle panel* shows the localization of PB-I (*arrowhead*) and PB-II (*arrow*), and the *right panel* shows merged images

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# Chapter 17

# Real-Time PCR-Based Quantitation Method for the Genetically Modified Soybean Line GTS 40-3-2

**Kazumi Kitta, Reona Takabatake, and Junichi Mano**

## Abstract

This chapter describes a real-time PCR-based method for quantitation of the relative amount of genetically modified (GM) soybean line GTS 40-3-2 [Roundup Ready® soybean (RRS)] contained in a batch. The method targets a taxon-specific soybean gene (lectin gene, *Le*1) and the specific DNA construct junction region between the *Petunia hybrida* chloroplast transit peptide sequence and the *Agrobacterium* 5-enolpyruvylshikimate-3-phosphate synthase gene (*epsps*) sequence present in GTS 40-3-2. The method employs plasmid pMulSL2 as a reference material in order to quantify the relative amount of GTS 40-3-2 in soybean samples using a conversion factor ($C_f$) equal to the ratio of the RRS-specific DNA to the taxon-specific DNA in representative genuine GTS 40-3-2 seeds.

**Key words** Real-time PCR, Genetically modified soybeans, GTS 40-3-2, TaqMan® chemistry, Conversion factor

## 1 Introduction

The production of genetically modified (GM) crops has been increasing since the start of full-scale commercialization of GM crops in 1996 [1]. Numerous safety assessments of GM crops and their derived foods and feeds have been mandatorily conducted by authorities in countries around the world, and commercially available GM crops are considered to be as safe as their conventional (non-GM) counterparts. Nonetheless, although GM cultivation area has been steadily increasing, the commercial use of GM crops has not always been embraced by the general consumer. To expand consumers' choices, many countries have introduced legislation requiring labels to be applied to agricultural products that contain more than a certain threshold level of approved GM events. In addition, in order to affirm the appropriateness of labeling, a variety of detection methods for GM crops have been developed.

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At the present time, polymerase chain reaction (PCR)-based detection methods are the standard for routine analysis of the GM content of food samples. For the quantification of GM content, a real-time PCR-based analysis of bulk sample homogenates is widely used and GM content is evaluated based on the ratio of GM- to taxon-specific DNA copy numbers. In some countries, such as Japan, the quantified GM value based on the copy number ratio is converted into a weight percentage (weight %) using an empirically defined conversion factor ($C_f$).

In this chapter, we describe a real-time PCR-based quantitation method for GTS 40-3-2, in which a $C_f$ is employed and GM values are expressed as a weight % [2–5]. The method amplifies a 121-bp fragment of GTS 40-3-2 construct-specific sequence and a 118-bp fragment of the taxon-specific soybean lectin gene (*Le*1) in separate real-time PCR reactions. PCR products are measured based on TaqMan® chemistry [6], over each PCR cycle by means of specific probes labeled with two fluorescent dyes: carboxyfluorescein (FAM) as a reporter dye and carboxytetramethylrhodamine (TAMRA) as a quencher. The method uses plasmid pMulSL2 as the reference material, which includes the construct-specific sequence of GTS 40-3-2 and the soybean taxon-specific sequence (*Le*1) (Fig. 1). The use of plasmid as the reference material enables us to consistently draw a stable calibration curve. Separate standard curves with each primer/probe system are generated in the same analytical amplification run. The standard curves are created using five concentrations of pMulSL2: 20, 125, 1500, 20,000, and 250,000 copies per reaction. The copy number for the unknown sample DNA is obtained by interpolation from the standard curves. To determine the proportion of GTS 40-3-2 in the unknown sample, the copy number of the GTS 40-3-2 construct-specific sequence is divided by the copy number of the *Le*1 gene in the unknown sample, divided by the $C_f$ of GTS 40-3-2, and then multiplied by 100 %.

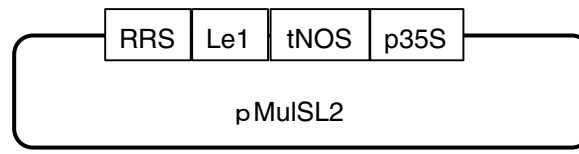


**Fig. 1** Schematic diagram of the pMulSL2. *RRS* construct-specific sequence of GTS 40-3-2, *Le1* soybean taxon-specific sequence (*Le*1), *tNOS* target sequence of nopaline synthase terminator, *p35S* target sequence of cauliflower mosaic virus 35S promoter

## 2 Materials

### 2.1 Sample Preparation

1. Water: autoclaved ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C).
2. A rotor mill, such as Rotor-Speed Mill P14 with the 0.5-mm sieve ring (Fritsch, Idar-Oberstein, Germany) (*see* **Note 1**).
3. A laboratory centrifuge (capable of 3000 × *g*) equipped with a swing-out rotor.
4. A refrigerated microcentrifuge set at 4 °C.
5. A water bath set at 65 °C.
6. A UV spectrophotometer.
7. DNeasy® Plant Maxi kit (Qiagen, Hilden, Germany) (*see* **Note 1**).
8. Tris-EDTA (TE) buffer: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA.
9. Isopropanol.
10. 70 % ethanol.

### 2.2 PCR

1. Real-time PCR instrument: Although this method was originally validated with ABI PRISM® 7700 (Applied Biosystems, currently Life Technologies, Carlsbad, CA, USA), the method described in this chapter is adjusted to ABI PRISM® 7900 (Life Technologies) (*see* **Note 1**).
2. A laboratory centrifuge equipped with a swing-out rotor for a PCR plate.
3. MicroAmp® Optical 96-Well Reaction Plate (Life Technologies) (*see* **Note 1**).
4. MicroAmp® Optical Adhesive Film (Life Technologies) (*see* **Note 1**).
5. TaqMan® Universal PCR Master Mix (Life Technologies) (*see* **Note 1**).
6. Reference material (Plasmid): The reference material used to develop and validate the method is the plasmid pMulSL2 which is included in the GM Soybean (RRS) Detection Set (Fasmac, Atsugi, Japan; or Nippon Gene, Tokyo, Japan). The GM Soybean (RRS) Detection Set includes pMulSL2 adjusted at 20, 125, 1500, 20,000, and 250,000 copies per 2.5 μL, and 5 ng/μL of ColE1/TE solution as no template control (NTC) in separate tubes.
7. Primer/Probe Mix: The sequences of the primers and probes for the soybean line GTS 40-3-2 construct-specific and the taxon-specific genes are listed in Table 1. Primer/Probe Mix contains 1.25 μmol/L each of the 5′ and 3′ primers and 0.5 μmol/L TaqMan® probe.

**Table 1**
**Primers and TaqMan® probe for GTS 40-3-2 quantitation**

| Name | Oligonucleotide DNA sequence | Final concentration in PCR | Length (bp) |
|---|---|---|---|
| Taxon-specific gene target sequence (Le1n02) | | | |
| Le1n02-5′ | 5′-GCC CTC TAC TCC ACC CCC A-3′ | 500 nmol/L | 118 |
| Le1n02-3′ | 5′-GCC CAT CTG CAA GCC TTT TT-3′ | 500 nmol/L | |
| Le1-Taq | 5′-FAM-AGC TTC GCC GCT TCC TTC AAC TTC AC-TAMRA-3′ | 200 nmol/L | |
| GMO target sequence (RRS) | | | |
| RRS 01-5′ | 5′-CCT TTA GGA TTT CAG CAT CAG TGG-3′ | 500 nmol/L | 121 |
| RRS 01-3′ | 5′-GAC TTG TCG CCG GGA ATG-3′ | 500 nmol/L | |
| RRS-Taq | 5′-FAM-CGC AAC CGC CCG CAA ATC C-TAMRA-3′ | 200 nmol/L | |

*FAM* 6-carboxyfluorescein, *TAMRA* 6-carboxytetramethylrhodamine

## 3 Methods

Since PCR amplifies template DNA even in minute amounts, contamination with unintended DNA should be avoided. Contamination with deoxyribonuclease (DNase) secreted from the human body (such as from skin, and in saliva) should also be avoided in order to prevent degradation of samples. Disposable gloves should be properly worn. Autoclaved micro-tubes and micro-pipet tips should be used, or filtered micro-pipet tips are preferable.

### *3.1 Sample Grinding and DNA Extraction*

1. Grind dry soybean seed samples with a rotor mill (*see* **Notes 2–4**).
2. Put the ground sample into a plastic bag or similar container, and shake well so that the entire batch is evenly mixed.
3. Extract DNA from 1.0 g of ground sample using the DNeasy Plant Maxi Kit, according to the manufacturer's manual, with some modifications. Briefly, add 10 mL of Buffer AP1 (preheated to 65 °C) and 20 μL of RNase A stock solution (100 mg/mL) to 1 g of ground sample and vortex vigorously. Incubate the mixture for 1 h at 65 °C. Mix 2–3 times during incubation by inverting the tube. Spin lysate at 3000 × *g* for 10 min at room temperature. Pipet 7 mL of supernatant to a new 15-mL tube. Add 2.5 mL of Buffer P3 to the lysate, mix, and incubate for 15 min on ice. Spin lysate at 3000 × *g* for 35 min at room temperature. Pipet 8 mL of supernatant to the QIA shredder maxi spin column (lilac) placed in a 50-mL collection tube and spin

at 3000 × *g* for 5 min at room temperature. Transfer 7.5 mL of flow-through, without disturbing the pellet in the collection tube, to a new 50-mL tube. Vortex the lysate and transfer 6.8 mL of the lysate to a new 50-mL tube. Add 10.2 mL of Buffer AW1 directly to the lysate and mix immediately by vortexing. Apply sample to the DNeasy maxi spin column (colorless spin column) including any precipitate that may have formed. Centrifuge at 3000 × *g* for 15 min. Discard flow-through and reuse collection tube. Add 12 mL Buffer AW2 to the maxi spin column and centrifuge at 3000 × *g* for 15 min to dry the membrane. Discard flow-through. Transfer the DNeasy maxi spin column to a new 50-mL tube. Pipet 1 mL of water (preheated to 65 °C) directly onto the DNeasy maxi spin column membrane and leave for 5 min at room temperature. Centrifuge for 10 min at 3000 × *g* to elute.

4. Measure the volume of the eluent. Add an equal volume of isopropanol to the eluent, and incubate the mixture for 5 min at room temperature after inverting the tube 10 times.
5. Centrifuge at 12,000 × *g* for 15 min at 4 °C. Discard supernatant, and rinse the precipitant with 500 μL of 70 % ethanol (*see* **Note 5**).
6. Centrifuge at 12,000 × *g* for 3 min at 4 °C, discard supernatant, and dry the pellet.
7. Dissolve the pellet completely with 50 μL TE buffer and let it sit in a refrigerator overnight. After the overnight refrigeration, visually inspect it to make sure there is no insoluble matter (*see* **Note 6**).
8. Determine the DNA concentration of the solution by measuring the UV absorbance at 260 nm with a UV spectrophotometer [7] (*see* **Note 7**). Adjust the DNA concentration of the solution with TE buffer to 20 ng/μL and store below –20 °C.

### 3.2 Real-Time PCR

The relative standard curve method is used for quantitation of copy numbers in extracted DNA from unknown test samples. Separate calibration curves with each primer/probe system are generated in the same analytical amplification run. The calibration curves are created using five concentrations including 20, 125, 1500, 20,000, and 250,000 copies of pMulSL2 plasmid DNA; each in triplicate (i.e., three technical replicates per calibration point). Triplicate reactions using an appropriate dilution of the DNA extracted from the unknown samples are also measured in the same analytical amplification run.

Frozen reagents should be thawed at room temperature and then immediately transferred to ice. Reagents which have been taken out of the refrigerator should also be kept on ice. Before use, the reagents should be vortexed and spun down.

1. For GTS 40-3-2 quantification, prepare two Master Mixes: one for the taxon-specific gene (*Le*1) and one that is GTS 40-3-2 specific. Prepare each Master Mix by blending TaqMan® Universal PCR Master Mix and Primer/Probe Mix in a 5:4 ratio (*see* **Note 8**). Vortex, and spin down.
2. Pipet 78.75 μL of each Master Mix into a separate 500 μL tube (*see* **Note 9**). Add 8.75 μL of template DNA to the tubes in which Master Mix has been dispensed (*see* **Note 10**). Vortex, and spin down.
3. Determine the layout of the reaction solution on the 96-well reaction plate. Dispense 25 μL of the reaction solution prepared above into each of 3 wells. Components of the amplification reaction mixture are as shown in Table 2. Centrifuge at 1000 × *g* for 1 min.
4. Place the PCR plate in the real-time PCR instrument, select the 9600 emulation mode, and set the temperature-time program as indicated in Table 3. Start the reaction and data capture according to the manufacturer's manual.

**Table 2**
**Amplification reaction mixture in the final volume/concentration per reaction vial**

| | |
|---|---|
| Total volume | 25 μL |
| Template DNA (50 ng soybean genomic DNA) | 2.5 μL |
| Reaction buffer (TaqMan® Universal PCR Master Mix) | 12.5 μL |
| Primers (5′ and 3′) | 500 nmol/L each |
| Probe | 200 nmol/L |

**Table 3**
**Reaction conditions**

| | | Time (s) | Temperature (°C) |
|---|---|---|---|
| Pre-PCR: decontamination | | 120 | 50 |
| Pre-PCR: activation of DNA polymerase and denaturation of template DNA | | 600 | 95 |
| PCR (45 cycles) | | | |
| Step 1 | Denaturation | 30 | 95 |
| Step 2 | Annealing and elongation | 60 | 59 |

### 3.3 Data Analysis

1. To obtain stable standard curves and to eliminate variation caused by different methods for determining threshold lines ($T$), $T$ is drawn manually. First, multiple candidates of $Tn$ are set at $2n^{-1}$-fold ($n$: natural number) of the standard deviation of the mean baseline emission calculated between cycles 3 and 15. The $n$ is increased one by one until one of the amplification plots does not cross over $Tn$. The optimal $T$ ($To$) is determined by comparing the amplification rates ($An$) and $|\Delta An, n_{+1}|$ calculated from the slope of the standard curves generated at multiple candidates $Tn$. Values for $An$ and $|\Delta An, n_{+1}|$ are calculated using the following formulas 1 and 2:

$$A_n = 10^{(-1/\text{slope})} \quad (1)$$

$$|\Delta A_{n,n+1}| = |(\Delta A_{n+1} - A_n) / A_n| \times 100 \quad (2)$$

$To$ is determined as satisfying one of the following two conditions:

*Condition 1*—The section of the linear phase is defined as total sections of $|\Delta An, n_{+1}|$, which are <1 % continuously in 2 or more sections. Adopt the average of $n$ values for the section of linear phase to determine the $To$. If the average is not an integral number, round up the decimal fraction of the average. If the section of the linear phase splits into two groups, adopt the section in which the average of $n$ values is larger. In this case, the correlation value of the standard curve must be >0.990, and $An$ value < 2.1. The $To$ should not cross the amplification plots of NTC.

*Condition 2*—If "Condition 1" could not be fulfilled, the allowable $|\Delta An, n_{+1}|$ value should be changed to 2 %. If it is not enough, the allowable $|\Delta An, n_{+1}|$ value shall be increased by 1 %, up to 5 %. If the $To$ cannot be adopted, even if the allowable $|\Delta An, n_{+1}|$ value is changed to 5 %, the tests should be abandoned.

2. The $C_t$ (cycle of threshold) values determined for the calibration points in *Le*1 or the GTS 40-3-2 construct-specific target are plotted against the logarithm of the copy number of plasmid DNA of pMulSL2. The copy numbers for the unknown sample DNA are obtained by interpolating to the standard curves. This can be done automatically using the software from the real-time PCR instrument, according to the manufacturer's instructions.
The copy number of each sample is obtained as the mean value of triplicates compared with the optimal standard curve. The ratio of recombinant-DNA (rDNA) to taxon-specific sequence *Le*1 in a fully transgenic sample is calculated by formula 3 and defined as a conversion factor ($C_f$).

$$C_f = \frac{\begin{array}{c}\text{Copy numbers of r DNA se quence}\\ \text{in the DNA extracted from GM seeds}\end{array}}{\begin{array}{c}\text{Copy numbers of taxon specific sequence}\\ \text{in the DNA extracted from GM seeds}\end{array}} \quad (3)$$

GM content in the test sample is then calculated by formula 4 (*see* **Note 11**).

$$\text{GM content} = \frac{\begin{array}{c}\text{Copy numbers of r DNA sequence in}\\ \text{the DNA extracted from unkown sample} \times 100\,\%\end{array}}{\begin{array}{c}\text{Copy numbers of taxon specific sequence}\\ \text{in the DNA extracted from unkown sample} \times C_f\end{array}} \quad (4)$$

## 4 Notes

1. This is an example of suitable products available commercially. Equivalent products may be used if they can be shown to produce the same results.
2. Contamination can easily occur when handling powders. Therefore, milling and other operations must be done in separate rooms. The mill should always be washed, and the rooms kept clean and tidy.
3. Check that the sample is sufficiently dry. If it is moist, air-dry it prior to milling.
4. When it is necessary to define the conversion factor (*see* Subheading 3.3, **step 3**), include a sample of fully transgenic GTS 40-3-2 soybean seed.
5. After adding 500 μL of 70 % ethanol, tap the bottom of the tube so that pellet comes off.
6. If there is insoluble matter in the solution, centrifuge at $12{,}000 \times g$ for 3 min at 4 °C, and use the supernatant for subsequent steps.
7. The quality of DNA solutions is evaluated in terms of the absorption ratios at 260/280 nm and 260/230 nm. DNA solutions should have absorption ratios at 260/280 nm and 260/230 nm of 1.7–2.0 and >1.7, respectively.
8. Before blending, determine the multiple of each reagent in order to prepare sufficient Master Mix for all samples, making sure to include extra to cover losses which may arise during pipetting.

   [Mixture amounts]

   TaqMan® Universal PCR Master Mix: $12.5 \times (X + \alpha)$ μL

Primer/Probe Mix: $10\times(X+\alpha)\,\mu L$

$X$: Reagent multiple (3 wells are used per sample, so this is 3 times the number of template DNA types)

$\alpha$: Compensation for pipetting losses

9. This dispensed amount of 78.75 μL is suggested for three replicates of one sample. The amount of Master Mix necessary for 1 well is 22.5 μL, and thus theoretically the amount can be anything at or above 67.5 μL.
10. The added amount of 8.75 μL is suggested for three replicates, and corresponds to the amount of Master Mix, 78.75 μL. The ratio of template to Master Mix should be maintained at 1:9.
11. For the Japanese standard methods, the $C_f$ for GTS 40-3-2 construct-specific quantitation was defined as 1.04 with an ABI PRISM 7900 96-well in a ring-trial for method validation [4]. The defined $C_f$ value has been confirmed as a fixed value with the validated GST 40-3-2 construct-specific quantitation method.

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# Chapter 18

# A Standardized Lepidopteran Bioassay to Investigate the Bioactivity of Insecticidal Proteins Produced in Transgenic Crops

**Gerson Graser and Frederick S. Walters**

## Abstract

Insecticidal bioassays are the only reliable method to investigate the biological activity of an insecticidal protein and therefore provide an essential toolkit for the characterization and potency determination of these proteins. Here we present a standardized method for a lepidopteran larval bioassay, which is optimized to specifically estimate activity of insecticidal proteins produced in transgenic plants. The treatment can be either applied to the surface of the artificial diet, or blended into the diet.

**Key words** Insecticidal bioassays, Larval bioassay, Insecticidal proteins, Diet surface assay, Diet incorporation assay, European corn borer (ECB)

## 1 Introduction

Insect larval bioassays are widely used to assess and characterize the biological activity of transgenic crops that express insecticidal proteins. As the method employs living organisms, a key step to producing high quality data is to design the bioassays in a standardized way that minimizes the risk of introduced experimental error. Here we describe a robust and sensitive method that allows for a consistent execution of lepidopteran larval bioassays in which the treatment can be applied to the surface of the artificial diet or incorporated by blending into the diet. Choice of whether to use the surface application or the diet incorporation method is in the hands of the researcher, depending on considerations such as the amount of available insecticidal protein (diet incorporation assay requires more), concerns about potential temperature sensitivity of the insecticidal protein (diet incorporation has more potential for concern), or the likelihood of the insect larvae burrowing below the surface and being able to reduce treatment exposure (only of concern for the surface application assay). The described method

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can be used to evaluate the insecticidal activity of insecticidal molecules (e.g., small molecules and proteins) against freshly hatched first instar lepidopteran insects including *Spodoptera frugiperda* (J.E. Smith) (fall army worm) and *Ostrinia nubilalis* (Hübner) (European corn borer) but should also be applicable to other species with a similar biology. The percent mortality in response to the treatment doses is determined over time to assess the test molecule's insecticidal activity. In dose response experiments, an estimate of the $LC_{50}$ (Lethal Concentration that kills 50 % of the tested insect population) of the insecticidal protein can be determined and quantitatively compared to that of proteins from different sources. Due to the unique nature of the insect bioassays, it is worth mentioning that the inherent variability from individuals, both within and across populations, can result in significant differences in their responses even when the same bioassay is conducted repeatedly over time [1–4] and is therefore largely out of the control of the scientist. For the direct comparison of the potencies of different insecticidal proteins, it is therefore essential to compare them side-by-side by using the same insect population, the same ingredients (e.g., base diet, buffers etc.), and the same experimental conditions to produce meaningful data.

## 2 Materials

All solutions are prepared using ultrapure deionized water and analytical grade reagents. All solutions are prepared at room temperature unless otherwise noted.

### 2.1 Insects

1. Freshly hatched (1st instar) lepidopteran larvae (*see* **Note 1**).

### 2.2 Bioassay Materials

1. 24-well plates such as Costar No. 3527 (Fisher Scientific, Inc., Pittsburgh, PA, USA).
2. Sealing tape, clear polyolefin (Fisher Scientific, Inc., Pittsburgh, PA, USA).
3. Fine tip paintbrush (*see* **Note 2**).
4. 18 × 24 in. white paper.
5. Blender.
6. General Lepidopteran Insect diet (Bio-Serv, Frenchtown, NJ, USA).
7. Antibiotics (optional) (available from Sigma-Aldrich Corp., St. Louis, MO, USA):

   Aureomycin.

   Cefotaxime.

   Nystatin.

   Streptomycin.

8. Repeater pipette tips (i.e., Eppendorf Combitips 10 mL, 22 26 650-1 Fisher Scientific, Inc., Pittsburgh, PA, USA).
9. Mortar and pestle, or Grindomix Knife Mill Model GM 200 (Retsch Inc. Newtown, PA, USA).
10. Homogenizer.
11. Water bath.
12. Cheesecloth.
13. Agar.
14. Diurnal incubator.

### 2.3 Plant Extraction Buffers

1. Borate extraction buffer: Weigh 38.1 g sodium tetraborate decahydrate, 2 g polyvinylpyrrolidone (PVP), and 0.5 g sodium azide into a 2-L glass beaker or flask. Add purified water to a volume of 900 mL (*see* **Note 3**). Add 12 mL concentrated HCl (35–38 %), 5 mL Tween®20, mix and bring to a final volume of 1 L with purified water. The solution pH will be approximately 7.5. Filter and store at 2 °C to 8 °C for up to 1 year.
2. Phosphate buffered saline plus Tween®20 buffer (PBST): Add 1 pack of phosphate buffered saline with Tween®20 (P-3563, Sigma-Aldrich Corp., St. Louis, MO, USA) to a 2-L glass beaker or flask. Add purified water to a final volume of 1 L. The solution pH will be approximately 7.4. Filter and store at 2–8 °C for up to 1 year.

## 3 Methods

### 3.1 Preparation of Plant Extracts

Prepare crude plant extracts from frozen or lyophilized leaf material (*see* **Note 4**). Younger leaves are often easier to sample and process (e.g., approximately V6 stage maize leaves or younger). When working with frozen samples, it is important to maintain them in the frozen state at all times during handling or preliminary grinding and processing using either liquid nitrogen or dry ice to ensure sample integrity.

1. If using frozen leaf samples, grind the samples into a homogeneous powder. Various methods of grinding are suitable (e.g., mortar and pestle, Grindomix Knife Mill).
2. Weigh the desired amount of frozen ground or lyophilized plant leaf powder into appropriate containers (e.g., 20-mL Greiner tubes; 14-mL polypropylene Falcon tubes) (*see* **Note 5**).
3. Add a suitable extraction buffer, such as Borate buffer or PBST, and mix the sample by gentle rotation or repeated inversion in preparation for the homogenization step (*see* **Note 6**).

4. Homogenize the wetted sample using an available homogenizer device (e.g., Omni Prep™, Turrax®, glass/glass or Teflon/glass homogenizer), and keeping the extract chilled, filter the extract across several layers of cheesecloth to remove large solids (alternatively, a large pore size centrifugal filtration device can be used).
5. Centrifuge the recovered filtrate at 3000 × *g* or greater for 10 min at 4 °C. The recovered supernatant is ready for dilution and surface application or use in the diet incorporation procedure.

### 3.2 Insect Diet Preparation for Surface Treatment Bioassay

1. Set a water bath at 52.5 °C ± 2.5 °C (*see* **Note 7**).
2. Calculate the total volume of diet needed (*see* **Note 8**). For the preparation of 500 mL General Lepidopteran diet, add 437.5 mL purified water to 9.5 g agar (final concentration 1.9 %) in an appropriately sized microwaveable beaker or flask and bring to a full boil in the microwave, allowing the agar to dissolve completely. When the agar is removed from the microwave, carefully swirl the solution to assess if the agar is completely dissolved. Pour immediately into an appropriately sized blender (a 1 L size blender is appropriate for making 500 mL diet) and add 72 g wheat germ based dry mix (final concentration 14.4 %).
3. Secure the top of the blender and gradually increase blender speed, then mix for approximately 1 min or until the mixture is smooth in consistency (*see* **Note 9**).
4. The prepared diet is ready for immediate use. It can also be solidified and stored at 5 °C ± 3 °C for a maximum of 3 days, or cut into smaller sections, placed in aluminum foil, and sealed in a bag for storage at –20 °C ± 8 °C for up to 2 months (*see* **Note 10**).
5. You may use a repeater pipette to aliquot diet into 24-well plates. The pipette tip may need to be cut in order to allow the diet to pass through the pipette more easily. Pipette approximately 800 μL per well (*see* **Note 11**).
6. Allow the diet to cool and harden before applying treatments to the surface (*see* **Note 12**).
7. Diet can be placed into 24-well plates the day before the bioassay is set up and stored covered at 5 °C ± 3 °C. If this option is used, allow excess condensation from storage to evaporate before using the plates for surface treatment.

### 3.3 Insect Diet Preparation for Diet Incorporation Bioassay

1. To prepare insect diet for the diet incorporation assay, follow **steps 1** through **4** of Subheading 3.2, with the following modification: Instead of using 437.5 mL of water, use 219 mL of water if antibiotics are included (*see* **Note 9**) or 263 mL of water if antibiotics are not included, to facilitate the subsequent pipetting and mixing steps.

2. For diet incorporation assays it is essential to bring the diet to the water bath temperature (52.5 °C ± 2.5 °C) prior to addition of insecticidal protein treatments, as excess heat could inactivate the protein. It is also possible to substitute a low-melt agar and use an even lower water bath temperature for proteins which are especially sensitive to increased temperatures (*see* **Note 13**).

### 3.4 Plate Labeling

1. Prepare labels for each 24-well plate with the date the bioassay is initiated (e.g., date the insects are added to the plate) and treatment information such as the name and concentration of the insecticidal protein (*see* **Note 14**) (Fig. 1a).

### 3.5 Preparation of Treatments and Controls for Surface Treatment Bioassay

1. Treatments and controls need to be determined prior to every experiment. For example, a treatment may be a transgenic plant crude extract of a known concentration of the insecticidal protein (*see* Subheading 3.1). An appropriate negative control would be the buffer/solvent used to prepare the treatment (excluding the test protein). When conducting experiments with plant-derived protein, it is suggested that a negative plant extract be processed in the same way as the positive plant extract

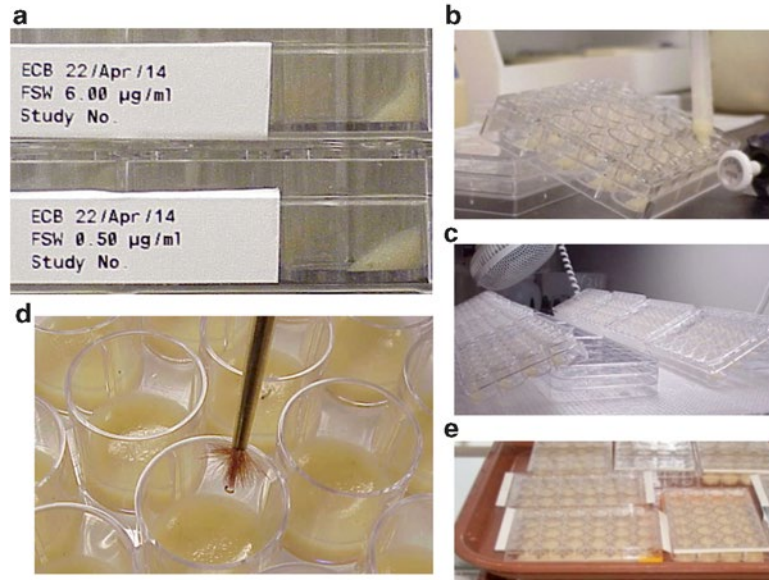


**Fig. 1** Preparation of artificial diet plates for bioassay. (**a**) An example of label information for prepared bioassay plates (e.g., used for European corn borer, ECB) (**b**) Dispensing of diet into a 24-well plate which is positioned at an angle for the diet incorporation bioassay. (**c**) Diet incorporation plates completing the drying step. (**d**) Addition of neonate larva to a surface treatment bioassay well. (**e**) Surface treatment bioassay plates arranged on a tray in incubator after the addition of insects and sealing of plates

and be included in the insecticidal bioassay as an additional negative control. A negative matrix or buffer/water control always needs to be added to each experiment to assess the control (no effect) mortality rate. For a dose–response experiment, typically five doses plus a zero dose control will provide a minimum dataset. Better analyses usually result from increasing the number of doses to eight or more (use of a greater number of doses will increase the likelihood that the response will be well characterized and suitable for statistical analysis), but it is also important to have the doses span the response range (e.g., not having too many at the low or high response level).

2. Prior to the initiation of an insecticidal bioassay, it is also necessary to determine the number of replicates which are appropriate for each experiment and which are in alignment with the statistical design of the study. Typically, three replicates of 10–24 insects each (for a given treatment or control) will afford a minimal dataset for subsequent statistical analysis or comparisons, but it is recommended to evaluate specific requirements in advance (*see* **Note 15**).
3. Calculate the concentration of the treatment application which is suitable to deliver the desired treatment dose per single well of the 24-well plate (the surface area is approximately 1.9 $cm^2$ for Costar 3527 plates). The suggested application amount is approximately 50 μL of solution to each well. An example calculation for determining the concentration of the treatment being applied to the diet is shown below.

   Example calculation:

   *Desired application dose*: 150 ng/$cm^2$ (variable, depends on the experiment)

   *Surface area of one well*: 1.9 $cm^2$ (constant for Costar 3527 plates)

   *Application volume*: 50 μL (suggested)

$$285\,\text{ng protein} = 150\,\text{ng}/\,\text{cm}^2 \times 1.9\,\text{cm}^2$$

$$5.7\,\text{ng}/\;\;\text{L} = 285\,\text{ng protein} \div 50\;\;\text{L}$$

Therefore, the concentration of the treatment being applied to the diet should be 5.7 ng/μL (*see* **Note 16**).

### 3.6 Preparation of Treatments and Controls for Diet Incorporation Bioassay

1. Follow the **steps 1** and **2** of Subheading 3.5.
2. For a diet incorporation assay, prepare each insecticidal protein treatment in an appropriate solvent/buffer at a concentration twice as high as the intended final concentration to be used in the bioassay (as it will be mixed with an equal volume of insect diet prepared at twice the regular strength) (*see* **Note 17**)

Example calculation (*see* **Note 17**)

*Desired dose used in bioassay*: 5 μg insecticidal protein/mL

*Concentration of insecticidal protein treatment stock*: 10 μg insecticidal protein/mL

*Insect diet at 2× strength*: follow instructions as described under Subheading 3.3, **steps 1** and **2**.

*Volume added per well*: 200 μL (suggested)

### 3.7 Bioassay Setup for Surface Treatment Bioassay

1. Use freshly prepared plates containing diet, or remove stored plates from 5 °C ± 3 °C and equilibrate to room temperature (also allow excess condensation from storage to evaporate before using the plates for surface treatment).
2. Pipette 50 μL of each treatment directly onto the surface of the diet contained in each well of the 24-well plates. This volume can vary up to 100 μL depending on the assay needs, but volumes below 50 μL may not cover the surface uniformly, introducing error. Use a repeater pipette with an appropriate tip for the volume needed to be delivered. Repeat this process for each treatment dose to be used in the surface treatment bioassay.
3. Allow plates to air dry with circular agitation using an orbital shaker to aid in evenly spreading treatments onto the diet surface. The drying process may take up to 1.5 h or the time can be shortened by using a rotating fan. The surface of the diet will usually appear duller when completely dry (*see* **Note 18**).
4. Tap the freshly hatched lepidopteran larvae from the original container onto an 18 × 24 in. white paper to make the insects available for transfer (*see* **Note 19**).
5. Using a clean fine tip paintbrush, place a single freshly hatched lepidopteran larva in each well containing treated diet (Fig. 1d) (*see* **Note 20**). We recommend using only a single larva per well as the experimental error could rise if any surviving insects preyed upon other live/dead larvae and therefore avoided feeding on the treatment surface. Cover the wells tightly (e.g., by using a pressure-sensitive transparent sealing tape with a roller to assure sealing) (*see* **Note 21**).
6. Place assay plates in a controlled incubator (e.g., 22 °C ± 5 °C) with a constant light/day cycle (e.g., 14 h light/10 h dark). Place plates in random order within the incubator (Fig. 1e), repositioning each successive day to minimize any potential systematic error associated with the environmental condition.

### 3.8 Bioassay Setup for Diet Incorporation Bioassay

1. Mix each treatment with an equal volume of temperature-equilibrated insect diet prepared at twice the regular strength and pipette immediately into a 24-well plate. It is also suggested to add the diet incorporation mix while the plate is at a fixed angle on the bench, so that the diet mix can be added to a

smaller surface/edge (Fig. 1b). Repeat this process for each treatment dose to be used in the bioassay.

2. Allow plates to air dry while in the slanted position (Fig. 1c). The drying process will generally be shorter than with the surface treatment method as the volume per well is much less (0.2 mL suggested).
3. Follow **steps 4–6** of Subheading 3.7.

### 3.9 Scoring the Bioassay

1. Mortality readings for each plate are taken over the time course of the assay and need to be recorded. Readings usually begin at 72–96 h (or as needed by the experimental design) and continue daily as needed for the experiment or until the percent mortality of any negative control treatments is greater than 20 %.
2. You should observe the insect through the transparent sealing tape. Tapping the well plate edge may aid in determining larval status, as the larvae may then move afterwards and be scored more easily. If you need to touch the insect to confirm its status, carefully use a probe and insert it through the sealing tape (*see* **Note 22**).
3. Insects that do not respond to touch (by a gentle probe technique) are considered dead (*see* **Note 23**). A dissecting microscope or other magnifying device may aid in the assessment of the insects. Wells missing insects are not used to calculate the % mortality. The percent mortality is calculated as follows (*see* **Note 24**):

$$\%\,\text{Mortality} = \#\,\text{of dead insects} \div \text{Total}\,\#\,\text{of insects in plate} \times 100\,\%$$

Example:

$$30.4\,\%\,\text{Mortality} = 7\,\text{dead insects} \div 23\,\text{insects total (one empty well)} \times 100\,\%$$

### 3.10 Data Evaluation and Statistical Analysis

If an $LC_{50}$ or an $LC_{90}$ is to be determined from a dose response experiment, calculate using Probit analysis [5] software programs, such as EPA Probit Analysis v. 1.5 or Proc Probit procedure in SAS (SAS Institute Inc., Cary, NC, USA) or any similar programs and data analysis methods.

## 4 Notes

1. Only those individuals which are moving and appear healthy should be selected.
2. Clean the brush with bleach, then with soap, rinse well with water, and allow to dry. Alternatively, 70 % EtOH can be used.

3. Mix all ingredients in the order listed for ease of preparation. PVP will dissolve slowly.
4. Crude plant extracts are recommended as opposed to highly purified plant protein preparations to keep the form of the insecticidal protein as close as possible to that which exists in planta. A purification process would introduce steps which have the potential for actually altering the integrity or activity of the insecticidal protein. The use of crude plant extracts also allows the scientist to set up an assay to quantify the concentration of the insecticidal plant protein (e.g., ELISA assay) in a timeframe which facilitates design of the bioassay doses in the same day.
5. Be sure to keep samples cold after weighing and preparation for subsequent extraction.
6. The exact ratio of plant material to extraction buffer, and the extraction buffer chosen, may need to be optimized based on insecticidal protein recovery (e.g., as determined by ELISA assay, or estimated by Western blot). A starting point might be a ratio of 0.1 g per 3 mL of the selected buffer. It may also be beneficial to add protease inhibitors such as Complete protease inhibitor cocktail tablets (Roche Applied Science) with the extraction buffer, but any adverse effects resulting from this on the lepidopteran larvae must first be tested at the final expected concentration of protease inhibitor mix which will be present in the diluted crude extract.
7. Use of gloves, safety glasses, and lab coat is recommended, where appropriate, while performing this procedure. Care should be taken when handling/blending boiling agar during the diet preparation.
8. It is advisable to prepare more diet than is needed for all of the plates (e.g., make 600 mL if 400 mL is needed) as this will be important to minimize transfer of surface bubbles/froth when pipetting diet into plate wells.
9. Optional: If microbial contamination has been observed as a recurrent problem in the laboratory, or if the bioassay endpoint is particularly prolonged, e.g., longer than 7 days, it may be advantageous to add antibiotics such as nystatin, cefotaxime, aureomycin, and streptomycin to the General Lepidopteran Diet. Add 1 mL of each 25 mg/mL antibiotic per 9 mL of prepared diet once the diet has cooled to 52.5 °C ± 2.5 °C. For each optional antibiotic stock solution, add 9.5 mL of purified water to 250 mg antibiotic. Mix until completely dissolved, and bring the volume up to 10 mL with purified water. The nystatin will usually be present as a suspension at this concentration, which will simply need to be mixed well prior to pipetting and use. These antibiotic solutions should be prepared

fresh on the day of use. Keep on ice or store at 5 °C ± 3 °C until use. Discard the leftover antibiotics or diet containing antibiotics appropriately after each use.

10. If the diet is stored prior to dispensation into the 24-well plate, it will need to be heated again using a microwave to liquefy it prior to pipetting.
11. Be careful not to splash diet on the sides of the wells when pipetting as these surfaces cannot be covered adequately with the subsequent surface-applied treatment. If diet splashes onto the sides of the well in discrete areas, allow diet to harden and carefully remove the unwanted diet splashes using a spatula without disturbing the actual diet surface. If diet splashes are too extensive, those plates should be discarded.
12. Care must be taken to ensure you do not over-dry the diet. When the diet is over-dried, it may crack or pull away from the wall of the well, rendering it unusable for surface application.
13. When working with low-melt agar, it can also help to pre-heat the blender by temporarily filling it with hot water before the melted agar is prepared. Then, remove the water immediately prior to adding the melted agar and blending of the diet ingredients.
14. Add label information on the plate, not the lid, to avoid confusion; also, be sure to use the correct units of concentration which pertain to the assay type, e.g., mass of insecticidal protein per unit volume for the diet incorporation bioassay, mass of insecticidal protein per unit area for the surface treatment bioassay.
15. A knowledge of the expected variation and the magnitude of the difference to be detected are essential to best inform the experimental design. The expected variation and the necessary replication will depend on the particular insect, test molecule, and magnitude of the difference to be detected.
16. When calculating the volume of treatment needed for each dose, plan for a higher volume than needed for 24 wells per plate (e.g., 0.05 mL per plate well × 40 = 2 mL to prepare). This will allow for an overage to facilitate sample application and accommodate any volumetric error during the pipetting process.
17. When calculating the volume of treatment needed for each dose, plan for a higher volume than needed for 24 wells per plate (e.g., 0.2 mL final diet incorporation mixture per plate well × 40 = 8 mL to prepare; 4 mL insecticidal protein treatment + 4 mL 2× strength insect diet). This will allow for an overage to facilitate sample application and accommodate any volumetric error during the pipetting process. All diets containing the insecticidal protein should be used fresh.

18. Closely monitor the drying process after the treatment is applied to the diet surface. Do not over-dry the diet. Over-drying the diet surface will cause fractures and will make the wells/plate unusable as the insect larvae could feed below the surface without exposure to the treatment. If wells within the same plate are drying unevenly, rotate plate orientation in front of the fan.
19. During this infesting process, take precautions to contain the insects, such as a bent paper edge or border of water to minimize the chance of escape.
20. It is sometimes helpful to slightly moisten the paintbrush with purified water during transfer. The larva can be added to the well by tapping the edge of the paintbrush shaft against the well; alternatively, the larvae can be carefully placed on a side of the well, avoiding any contact of the brush with the diet surface.
21. It may be beneficial to use a small pin to poke four to five holes in the surface of each sealed well to reduce subsequent condensation build-up on the interior surface of the wells or sealing tape. The holes must be small enough to prevent escape of the neonate larvae.
22. Take care not to puncture a hole large enough for the insect to escape. Depending on the size of the probe, the same puncture site may need to be used during sequential reads. Alternatively, the puncture hole may be sealed with a small peel-off circle of label tape.
23. It is important to use this same endpoint metric when comparing across datasets. For example, if insects that did not molt to the next instar were considered dead during the scoring, the datasets could generate disparate results.
24. If one is only comparing a limited number of treatment samples, a corrected mortality can be generated for the comparisons using formulas such as Abbott's correction [6]. If the analyses will involve comparison across doses and a subsequent estimation of an $LC_{50}$ parameter, we recommend describing the data in terms of uncorrected mortality, as negative control mortality is routinely very low, and also to avoid confusion as the electronic analysis may actually require the separate entry of the number of control organisms which have responded.

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# Part V

## Antibodies for Pathogen Resistance

# Chapter 19

# Antibody-Mediated Pathogen Resistance in Plants

**Dieter Peschen, Stefan Schillberg, and Rainer Fischer**

## Abstract

The methods described in this chapter were developed in order to produce transgenic plants expressing pathogen-specific single-chain variable fragment (scFv) antibodies fused to antifungal peptides (AFPs), conferring resistance against fungal pathogens. We describe the selection from a phage display library of avian scFv antibodies that recognize cell surface proteins on fungi from the genus *Fusarium*, and the construction of scFv–AFP fusion protein constructs followed by their transient expression in tobacco (*Nicotiana* spp.) plants and stable expression in *Arabidopsis thaliana* plants. Using these techniques, the antibody fusion with the most promising in vitro activity can be used to generate transgenic plants that are resistant to pathogens such as *Fusarium oxysporum* f. sp. *matthiolae*.

**Key words** Transgenic plants, Antibody, Antibody fragments, Single-chain variable fragment, scFv, Pathogen, Fungal antigens, Antifungal peptide, *Arabidopsis thaliana*, *Fusarium* spp.

## 1 Introduction

Many different strategies have been explored to produce disease-resistant transgenic plants, involving the augmentation of natural defense responses or the import of specific disease resistance genes from other species or varieties [1–3]. The transfer of genes from resistant to susceptible plant species is one strategy that can be used to accelerate conventional breeding for disease resistance [4, 5]. Such genes often enhance general defense pathways such as the hypersensitive response [6] and systemic acquired resistance [7] or lead to the widespread expression of protective defense peptides, pathogenesis-related proteins, or avirulence factors that protect against specific pathogens [8]. In addition to the exploitation of natural plant genes, genes from non-plant sources can be expressed in plants to prevent or delay pathogen infections. Two major strategies have been developed: pathogen-derived resistance, in which a pathogen gene is expressed in the protected plant [9], and pathogen-targeted resistance, in which a foreign gene is used to interfere with

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pathogen infection or reproduction. Antibody-mediated resistance falls into this latter category [10, 11].

Antibody-mediated resistance is achieved by the expression of antibodies or antibody fragments that bind to and inactivate pathogens in planta. A greater understanding of plant diseases and the identification of many proteins necessary for infection, pathogen replication and spreading, have allowed the development of antibodies that bind to and inactivate key targets that play an essential role during pathogenesis. Antibody-mediated resistance has been used for the control of viral [12–20], bacterial [21], and fungal infections [22–24]. In the latter case, the potency of antifungal antibodies has been enhanced by fusing them to known antifungal peptides (AFPs) [22, 23] or enzymes [24].

Building on these efforts, the first steps towards the generation of plants with antibody-mediated pathogen resistance comprise antigen preparation, antibody generation and cloning, efficient antibody expression, and targeting to appropriate cellular compartments. In this chapter, we look at how antibodies against plant pathogens are generated, and how expression cassettes are designed and introduced into plants. We use our previously published work on *Fusarium* resistance in *Arabidopsis thaliana* [22] to demonstrate these methods. In this approach we used avian immune libraries prepared from chickens [22], although murine or human libraries can also be used [25], or libraries prepared from transgenic mice expressing human immunoglobulins [26], as well as nanobody libraries prepared from camelid species that produce unique antibodies comprising a heavy chain but no light chain [27].

## 2 Materials

### 2.1 Fungal Cultures and the Preparation of Fungal Antigens

1. Petri dishes and inoculation loop.
2. Potato dextrose agar (PDA).
3. Potato dextrose broth (PDB).
4. Czapek Dox liquid medium, 35 g/L.
5. CM sporulation medium: 0.75 % (w/v) carboxymethylcellulose, 0.05 % (w/v) yeast extract, 0.025 (w/v) $MgSO_4 \cdot 7H_2O$, 0.05 % (w/v) $NH_4NO_3$, 0.05 % (w/v) $KH_2PO_4$.
6. Incubator with orbital shaker.
7. Rotating/undulating turntable.
8. Miracloth.
9. Vacuum pump and tubing.
10. Liquid nitrogen, mortar and pestle.
11. Mid-size centrifuge and rotors/adapters suitable for tubes and bottles ranging from 10 mL to 250 mL.

12. Light microscope.
13. Cell counting chamber (hemocytometer).
14. Phosphate-buffered saline (PBS): 0.14 M NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO_4$, 1.5 mM $KH_2PO_4$ (pH 7.4 with HCl).
15. Sterile distilled water.
16. Glycerol.
17. Stock solution of 100 mM phenylmethylsulfonylfluoride (PMSF).
18. CWP suspension buffer: 10 mM Tris–Cl, pH 7.8, 1 mM PMSF.
19. CWP wash buffer: 1 M NaCl, 1 mM PMSF.
20. CWCBP extraction buffer: 50 mM Tris–Cl, pH 7.8, 2 % (w/v) SDS, 100 mM Na-EDTA, 40 mM β-mercaptoethanol, 1 mM PMSF.

### 2.2 Preparation of the Phage Display Library

1. Stock of young, healthy brawn chickens, 20–24 weeks old.
2. MM Gerbu 100 adjuvant (Gaiberg).
3. PBS, as listed in Subheading 2.1, **item 14**.
4. Bovine serum albumin (BSA).
5. Fc-specific polyclonal rabbit anti-chicken antibody used to determine antibody titer.
6. RNA extraction kit.
7. mRNA purification kit.
8. First-strand cDNA synthesis kit.
9. Standard PCR reagents and equipment: 10 mM dNTP mix, 25 mM $MgCl_2$, dimethyl sulfoxide (DMSO), *Taq* DNA polymerase, 10× reaction buffer (supplied by the manufacturer of the enzyme), PCR-grade distilled water, suitable tubes or plates, and compatible thermocycler.
10. Primers suitable for amplifying chicken $V_H$ and $V_L$ cDNA and adding restriction site linkers compatible with the phage display vector [22]. For vector pHENHi (*see* below) first-strand cDNA is synthesized using primer 5′-CGG TGG GGG ACA TCT GAG TGG G-3′ for the $V_H$ sequence and primer 5′-AGG GGT GGA GGA CCT GCA CCT C-3′ for the $V_L$ sequence. The $V_H$ sequence is then amplified using forward primer 5′-TCT AGG CAT CCC TTG GCC CAG CCG GCC ATG G*CT GCC GTG ACG TTG GAC GAG TCC*-3′ and reverse primer 5′-CTA GTG CAT GCT GGA GGT GAC C*TC GGT CCC GTG GCC CCA TGC GTC*-3′. Similarly the $V_L$ sequence is then amplified using forward primer 5′-TCC ACA CAT GCT CCA GGC GCG CC*T GCG CTG ACT CAG CCG TCC TCG GTG*-3′ and reverse primer 5′-TGA CCT TCG AGG

ATG CGC GGC CGC GTC GAC *GGG CTG GCC TAG GAC GGT CAG*-3′. In each case, the underlined segment represents the overhang region, the central segment introduces restriction enzyme sites (*see* Subheading 3.2.3, **step 4**), and the italic segment represents the binding region of the antibody.

11. Phage display library vector (we have described the procedure for pHENHi, but any phage display vector can be used if the cloning procedure is adapted accordingly).
12. Materials for gel extraction and ethanol precipitation of DNA.
13. Materials for DNA digestion with restriction enzymes, ligation, and the transfection of *Escherichia coli* with phagemid DNA.
14. *E. coli* strain, e.g., XL1-blue, stored either as a glycerol stock or as recently streaked plates.
15. Apparatus for agarose gel electrophoresis.
16. M13-K07 helper phage.
17. 2TY medium: 1.6 % (w/v) tryptone, 1.0 % (w/v) yeast extract, 0.5 % (w/v) NaCl (pH 7.4 with NaOH).
18. Stock solution of 50 mg/mL ampicillin.
19. Stock solution of 10 % glucose.
20. Incubator with orbital shaker.
21. Rotating/undulating turntable.
22. Centrifuge and rotors/adapters as listed in Subheading 2.1, **item 11**.
23. Stock solution of 10 mg/mL kanamycin.
24. Polyethylene glycol (PEG) 6000.
25. Stock solution of 2.5 M NaCl.
26. Sterile distilled water.
27. 2TYGA agar plates: 1.6 % (w/v) tryptone, 1.0 % (w/v) yeast extract, 0.5 % (w/v) NaCl, 1 % (w/v) glucose, 100 μg/mL ampicillin, 1.5 % (w/v) agar (pH 7.4 with NaOH).
28. Immunotubes.
29. Dried, skimmed milk.
30. Poly(oxyethylene)sorbitan monolaurate (Tween-20).
31. 100 mM triethylamine.
32. Luria-Bertani (LB) medium: 1.0 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 1.0 % (w/v) NaCl (pH 7.4 with NaOH).
33. Glycerol.
34. 1 M Tris–Cl, pH 7.4.
35. Gauge-3 syringe needle.
36. Miracloth.

37. Dialysis tubing.
38. Sodium azide.
39. Plasmid preparation kit or standard reagents for plasmid isolation.
40. Materials required for the transfer of single-chain variable fragment (scFv) cassettes into a plant expression vector.

### 2.3 Transient Expression (Tobacco)

1. Tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) seeds, available from seed suppliers.
2. Greenhouse or growth room.
3. Phytochamber with climate and photoperiod control.
4. *Agrobacterium tumefaciens* strain GV3101 [pMP90RK, $Gm^R$, $Km^R$, $Rif^R$].
5. Stock solution of 50 mg/mL carbenicillin.
6. Stock solution of 100 mg/mL rifamycin.
7. Stock solution of 10 mg/mL kanamycin, or another selection reagent appropriate for the marker carried on the plant expression vector.
8. YEB medium: 0.5 % (w/v) nutrient broth (62.5 % peptone, 37.5 % beef extract by weight), 0.1 % (w/v) yeast extract, 0.5 % (w/v) peptone, 0.5 % (w/v) sucrose, 2 mM $MgSO_4$ (pH 7.4 with NaOH). *See* **Note 1**.
9. Incubator with orbital shaker.
10. Centrifuge and rotors/adapters as listed in Subheading 2.1.
11. Ultrapure water.
12. Glycerol.
13. Electroporation system and compatible cuvettes.
14. SOC medium: 1.0 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl, 0.2 % (w/v) KCl, 0.2 % (w/v) $MgCl_2$, 0.25 % (w/v) $MgSO_4$, 0.02 % (w/v) glucose (pH 7.4 with NaOH). *See* **Note 1**.
15. YEB agar plates: 0.5 % (w/v) nutrient broth, 0.1 % (w/v) yeast extract, 0.5 % (w/v) peptone, 0.5 % (w/v) sucrose, 2 mM $MgSO_4$, 1.5 % (w/v) agar (pH 7.4 with NaOH). *See* **Note 1**.
16. Standard PCR reagents and equipment.
17. Potting soil ED73 Einheitserde (Patzer, Sinntal-Jossa).
18. Whatman 3MM paper.
19. YEB-I medium: 0.5 % (w/v) nutrient broth, 0.1 % (w/v) yeast extract, 0.5 % (w/v) peptone, 0.5 % (w/v) sucrose, 0.2 % (w/v) MES, 2 mM $MgSO_4$, 20 μM acetosyringone (pH 5.6 with KOH). *See* **Note 1**.

20. MMA medium: 0.43 % (w/v) Murashige & Skoog (MS) basic salts, 0.2 % (w/v) MES, 2.0 % (w/v) sucrose (pH 5.6 with KOH).
21. Large preserve jars (1 L capacity).
22. Vacuum chamber, pump and tubing.

### 2.4 Stable Transformation (Arabidopsis)

1. *A. thaliana* cv. Columbia seeds, available from seed suppliers.
2. **Items 2–18** from Subheading 2.3.
3. 70 % (v/v) ethanol.
4. Absolute ethanol.
5. Dipping medium: 0.43 % (w/v) Murashige & Skoog (MS) basic salts, 5.0 % (w/v) sucrose, 0.04 % (v/v) Silwet L-77 (Lehle Seeds, USA) supplemented with 50 μg/mL kanamycin (or antibiotic appropriate for the plant expression vector).
6. MSSA plates: 0.43 % (w/v) Murashige & Skoog (MS) basic salts (pH 5.7), 0.9 % (w/v) agar. Kanamycin (or antibiotic appropriate for the plant expression vector) is added to a final concentration of 50 μg/mL after autoclaving and cooling to at least 50 °C.

## 3 Methods

### 3.1 Choosing Appropriate Target Material

The initial objective is to isolate important pathogen antigens which can be used to generate antibodies. It is important to realize that not all antigens will be effective targets, and it is useful to develop a panel of antibodies and test them in vitro on the pathogen before progressing to transgenic plants. In the case of fungal pathogens, obvious targets include surface structures of the infecting spores, mycelia, and cell walls.

#### 3.1.1 Isolation of Fungal Spores

The following protocol is adapted specifically for *Fusarium* species. The media and incubation conditions may need to be modified for use with other fungi.

1. Grow fungus on PDA plates by seeding infected agar pieces onto the plates and incubating them for 5–7 days at 28 °C.
2. Using an inoculation loop, transfer two small infected agar pieces into 300 mL of CM sporulation medium [22] and shake for 3–10 days at 28 °C, 175 rpm.
3. On day three, the spore density should be ~$10^7$ spores/mL (*see* **Note 2**). Filter the liquid through two layers of sterile Miracloth to remove the mycelia, then centrifuge at 4000 × *g* for 5 min at room temperature.

4. Set aside 50,000 spores to use for the isolation of cell wall proteins in Subheading 3.1.2.
5. To obtain germinated spores, add $10^5$ spores to 1 L of PDB and incubate for 16 h at 28 °C prior to isolation (*see* **Note 3**). Use the germinated spores in Subheading 3.2.
6. Wash the remaining spores twice with 100 mL sterile PBS. Centrifuge at 4000 × *g* after each wash. These spores can be stored for at least 6 months in 1 mL PBS containing 25 % (v/v) glycerol at −80 °C.

### 3.1.2 Isolation of Cell Wall Proteins

1. From **step 4** in Subheading 3.1.1, transfer spores to 2 × 250 mL Czapek Dox liquid medium (*see* **Note 4**). Use a final concentration of $10^5$ spores/L, and shake for 3 days at 28 °C, 225 rpm [28].
2. On the fourth day, vacuum filter the suspension through three layers of Miracloth and wash with 500 mL distilled water to remove spores and media residue.
3. Transfer approximately 1 g of the fresh mycelia to liquid nitrogen, grind and re-dissolve in 10 mL ice-cold CWP suspension buffer to isolate the cell walls containing noncovalently bound proteins, and proceed to **step 4** below. Repeat this step with another 1 g of mycelia to isolate the cell walls containing covalently bound proteins, and proceed to Subheading 3.1.3.
4. To isolate noncovalently bound proteins, incubate for 30 min at 4 °C on a rotating/undulating turntable running at 50 rpm, then wash three times in 10 mL ice-cold CWP wash buffer and three times in 10 mL water containing 1 mM PMSF to remove the cytoplasm and NaCl. Each wash should consist of 10 min on the turntable at 4 °C, 50 rpm, and should be interspersed with a centrifugation step (2000 × *g*, 5 min, 4 °C) to pellet the cell wall debris.
5. Resuspend the cell wall debris in 10 mL PBS.
6. Store cell wall debris in 1 mL PBS containing 25 % (v/v) glycerol at −20 °C. Use this preparation in Subheading 3.2.

### 3.1.3 Isolation of Covalently Bound Cell Wall Proteins

1. Take a preparation of cell wall proteins in 10 mL CWP suspension buffer from **step 3** in Subheading 3.1.2 and centrifuge at 2000 × *g* for 5 min at room temperature to pellet the cell debris.
2. Resuspend in 10 mL CWCBP extraction buffer and boil for 5 min.
3. Centrifuge at 2000 × *g* for 5 min at 4 °C and wash six times in 10 mL water supplemented with 1 mM PMSF to remove SDS, β-mercaptoethanol, and EDTA. Each wash should consist of 10 min on the turntable at 4 °C, 50 rpm, and should be interspersed with a centrifugation step (2000 × *g*, 5 min, 4 °C) to pellet the cell wall debris.

4. Resuspend the cell wall pellet in 10 mL PBS.
5. Store cell wall debris in 1 mL PBS containing 25 % (v/v) glycerol at –20 °C. Use this preparation in Subheading 3.2.

#### 3.1.4 Isolation of Plate Culture Washing Proteins

1. Grow fungal cultures on PDA plates by seeding infected agar pieces onto the plates and incubating for 5–7 days at 28 °C. The agar color changes to red when the plates are full of mycelia.
2. Wash agar plates with 5 mL sterile PBS and collect the liquid.
3. Remove mycelia by centrifugation at 8500 × *g* for 15 min at 4 °C.
4. Aliquot the supernatant containing the plate culture washing proteins and store at –20 °C for up to 6 months. Use this preparation in Subheading 3.2.

### 3.2 Generating Antibodies Against Target Antigens

Phage display [29] is a rapid and versatile method for the generation of high-affinity antibodies against target antigens, and this is the method we have used to prepare scFv antibodies against the fungal antigens described above. We generated three immunized avian libraries by injecting chickens with, respectively, the cell wall proteins, plate wash proteins, and germinated spores prepared in Subheading 3.1. The following sections cover the immunization of chickens (*see* **Note 5**), RNA extraction from spleen cells, cloning the antibody genes, and the preparation and screening of a phage display library.

#### 3.2.1 Immunization of Chickens

1. Use one young and healthy brawn chicken for each antigen preparation. The chickens should be approximately 20–24 weeks old and reared in accordance with local animal welfare regulations.
2. Prepare a mixture comprising 120 μL of MM Gerbu 100 adjuvant and 580 μL of the antigen preparation from Subheading 3.1 (germinated spores, noncovalently bound cell wall proteins, covalently bound cell wall proteins, or plate wash proteins). The cell wall proteins should be prepared at a concentration of approximately 200 μg/mL, and the germinated spores should have a density of $1 \times 10^3$ spores/mL.
3. Inject each chicken in the pectoral muscle with 300 μL of the mixture. Three injections should be performed at 14-day intervals.
4. After the last immunization, collect eggs for 6 consecutive days. Isolate polyclonal antibodies from the yolk to determine the titer (*see* **Note 6**). When the titer is satisfactory (*see* **Note 7**), chickens should be sacrificed for spleen removal 7 days after the final immunization.

### 3.2.2 Spleen Preparation

1. To remove the spleen from a chicken, sacrifice the animal humanely, cut the pelt at the abdominal wall, identify and remove the spleen.
2. Surface sterilize the spleen with 70 % (v/v) ethanol, rinse briefly in sterile distilled water, and then cut off a section (about 25 %) allowing it to fall directly into the buffer for RNA extraction, which should be sitting on ice (*see* **Note 8**).
3. Homogenize the spleen in the extraction buffer by drawing backwards and forwards through a gauge-3 syringe needle.

### 3.2.3 Amplification of $V_H$ and $V_L$ Domains and Creation of Phage Display Libraries

1. Extract total RNA from spleen cells using the RNA extraction kit according to the manufacturer's instructions (*see* **Note 8**).
2. Isolate mRNA from the total RNA using the mRNA purification kit according to the manufacturer's instructions (*see* **Note 9**).
3. Synthesize cDNA from the isolated mRNA using primers specific for the $V_H$ and $V_L$ domains of the chicken immunoglobulin genes (*see* Subheading 2.2, **item 10**). We use the SUPERSCRIPT™ Preamplification Kit (Gibco BRL, Karlsruhe) for first-strand cDNA synthesis.
4. Amplify the resulting cDNAs using primers that introduce adapters at the 5′ and 3′ ends of the amplification product so that the cDNAs can be rendered compatible with the phage display vector. We use vector pHENHi (Fig. 1) and therefore introduce adapters for SfiI and BstEII at the termini of the $V_H$-domain cDNAs and adapters for AscI and NotI at the termini of the $V_L$-domain cDNAs using the primers listed in Subheading 2.2, **item 10**. The PCR conditions are discussed in **Note 10**.
5. Load the PCR reaction products onto separate lanes of an agarose gel and fractionate to remove contaminants and artifacts from the desired products, which are approximately 400 bp in size. Purify the desired product bands by gel extraction and ethanol precipitation. Resuspend separately in 20 μL sterile water.
6. Transfer 2 μL of the $V_H$ fragment library to a fresh tube and add the necessary components to carry out the appropriate restriction digest. Perform the same process, separately, on the $V_L$ fragment library. Purify the restricted cDNA populations by agarose gel electrophoresis, extraction, and ethanol precipitation as described above. Resuspend, separately, in 20 μL sterile water.
7. Transfer the restricted cDNA populations to a sterile tube and add the necessary components to ligate the cDNAs into the phage display vector. One ligation should be carried out for the $V_H$ fragments and one for the $V_L$ fragments.
8. To complete the library, repeat the restriction and ligation steps but this time use the complete $V_H$ library as the destination vector for the $V_L$ fragments and the complete $V_L$ library as the

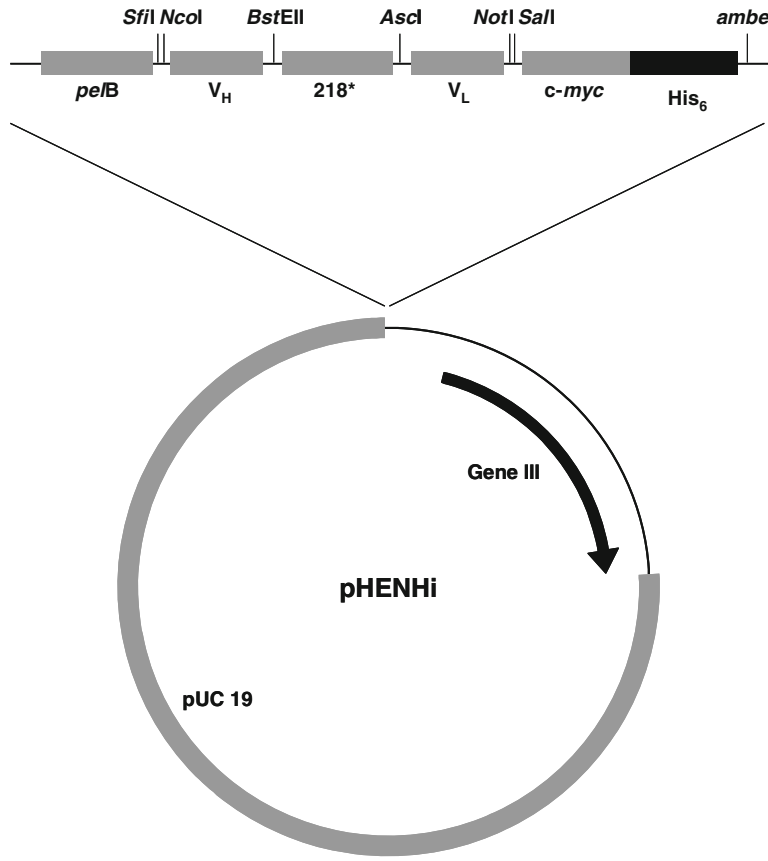


**Fig. 1** Map of the phagemid pHENHi. ***pelB*** signal sequence for transport into the periplasm, ***VH*** variable domain of the heavy chain, ***218**** 218 Linker with tyrosine changed to proline, ***VL*** variable domain of the light chain, ***c-myc*** myc epitope for scFv detection, ***His6*** hexahistidine sequence for scFv detection and purification, ***amber*** TAG stop codon, ***Gene III*** envelope protein gene III of the phage M13, ***pUC19*** pUC19 vector backbone, from which pHENHi was derived

destination vector for the $V_H$ fragments. The result should be two libraries containing $V_H$ and $V_L$ fragments.

9. When the ligation reactions are complete, transform *E. coli* with the ligation products, and plate out on appropriate selective medium for the vector.
10. Select ten colonies to check for library complexity (*see* **Note 11**).
11. Store the libraries (transformed *E. coli*) as glycerol stocks and plates as required.

*3.2.4 Propagation of Phage Library*

1. Seed the phage library into 250 mL 2TY medium to start the induction of phage replication.
2. Pool the phage and propagate in 50 mL 2TY medium supplemented with 100 µg/mL ampicillin and 1 % (w/v) glucose at 37 °C, shaking at 225 rpm, until an $E_{600nm}$ of 0.5 is achieved.

3. Decant into 15-mL tubes and add 50 μL M13-K07 helper phage (approximately $10^{11}$ phages/mL) to each 10 mL culture.
4. Incubate in a water bath at 37 °C (no shaking) for 30 min.
5. Centrifuge the infected cells at 4000 × *g* for 10 min, 4 °C, remove supernatant, and resuspend pellet in 200 mL 2TY medium supplemented with 100 μg/mL ampicillin and 25 μg/mL kanamycin.
6. Incubate overnight at 30 °C, shaking at 225 rpm.
7. To isolate the phages, centrifuge the overnight culture at 4000 × *g* for 30 min at 4 °C, remove the supernatant, and add to the culture bottle 40 mL (one fifth volume) of 20 % (w/v) PEG 6000 in 2.5 M NaCl.
8. Allow phages to precipitate for 1 h at 4 °C, then centrifuge at 6500 × *g* for 30 min at 4 °C.
9. Resuspend the pellet in 40 mL sterile distilled water and add 8 mL (one fifth volume) of 20 % (w/v) PEG 6000 in 2.5 M NaCl.
10. Allow phages to precipitate for 20 min at 4 °C, then centrifuge at 4000 × *g* for 10 min at 4 °C.
11. Resuspend the pellet in 2.5 mL sterile water and centrifuge at 4000 × *g* for 10 min at 4 °C to remove cell debris.
12. Store the supernatant, which contains the phages, at 4 °C. They will remain stable for at least 6 months.

### 3.2.5 Titration of the Phage Library

1. Seed a culture of untransformed *E. coli* cells from a glycerol stock or freshly streaked plate into 10 mL 2TY liquid medium and incubate at 37 °C with shaking until an $E_{600nm}$ of 0.5 is achieved.
2. Meanwhile, prepare tenfold dilutions from $10^{-4}$ to $10^{-13}$ of the supernatant from Subheading 3.2.4 **step 12** to measure the titer of the phages.
3. Add 100 μL of each dilution to 900 μL *E. coli* culture and incubate for 30 min at 37 °C in a water bath (no shaking).
4. Plate 100 μL of each infected *E. coli* culture onto 2TYGA agar plates and incubate overnight at 37 °C.
5. Count the number of colony forming units to determine the library titer (*see* **Note 12**). Each colony represents one phage. The number of colonies is therefore equal to the total number of phages added to the bacterial cells.

### 3.2.6 Panning to Select Antigen-Specific Phages

1. Seed a culture of untransformed *E. coli* cells from a glycerol stock or freshly streaked plate into 10 mL 2TY liquid medium and incubate at 37 °C until an $E_{600nm}$ of 0.5 is achieved.

Make one culture for each antigen preparation. These cultures will be used in **step 12**.

2. Meanwhile, take sterile Immunotubes and to each tube add 3.5 mL of fungal antigen preparation as described in Subheading 3.1. The concentration of protein should be approximately 250 μg/mL (for the protein extracts), and for the germinated spore preparation the spore density should be $1 \times 10^5$ spores/mL.
3. Incubate the Immunotubes for 2 h at 37 °C.
4. Discard the liquid. Wash the Immunotubes three times with PBS.
5. Block nonspecific binding sites with 4 mL PBS containing 2 % (w/v) dried skimmed milk for 2 h at 37 °C.
6. Wash the Immunotubes three times with PBS.
7. Add to each Immunotube 3.5 mL PBS containing 2 % (w/v) dried skimmed milk and $10^{12}$ to $10^{13}$ cfu of the phage preparation obtained in Subheading 3.2.4. Incubate on a rotating/undulating table at 50 rpm for 2 h at room temperature.
8. Wash the Immunotubes 20 times with PBS containing 0.05 % (v/v) Tween-20, and then 20 times with PBS, to thoroughly remove non-bound phages.
9. Elute the bound phages by adding 1 mL 100 mM triethylamine to each tube and incubating for 10 min at room temperature on a rotating/undulating table at 50 rpm.
10. Decant and save the eluate, and neutralize remaining phages by adding 200 μL of 1 M Tris–Cl (pH 7.4).
11. Pool the two eluates for each tube and store at 4 °C overnight.
12. Recover the bacterial cultures from **step 1** above and add 9 mL bacterial cells ($E_{600\text{nm}} = 0.5$) to each 1.2 mL of phage eluate ($1 \times 10^{13}$ cfu), and then incubate for 30 min in a water bath at 37 °C (no shaking).
13. Centrifuge the cells at $4000 \times g$ for 10 min at 4 °C, resuspend in 2 mL LB medium, and plate serial dilutions of $10^{-4}$ to $10^{-9}$ cfu per plate onto 2TYGA. Incubate overnight at 37 °C.
14. Next morning, scrape individual colonies from the plates into LB medium, add an equal amount of glycerol, and store at −80 °C.
15. Purify the phages by following **steps 1–12** of Subheading 3.2.4.
16. Return to **step 1** of the current section (Subheading 3.2.6) for the next round of panning. Use the purified phages from **step 15** in **step 7**.
17. After three rounds of purification, pure isolates of each antigen-specific phage are available. Cultures can then be used to

isolate specific scFv cassettes for insertion into plant expression vectors. DNA should be prepared from a single colony using a standard plasmid preparation method, and the scFv cassette can be transferred using restriction–ligation and enzymes appropriate for the destination vector.

### 3.3 Plant Transformation

Before plant transformation is possible, the scFv cassettes from antigen-specific phages must be inserted into a plant expression vector. Although many different vectors are available, the choice should be appropriate for the intended host species and compatible cloning sites would be advantageous. For expression in *A. thaliana* we use vector pTRAkc, in which the transgene is under the control of the *Cauliflower mosaic virus* 35S RNA promoter (CaMV 35S). Cassette DNA is isolated from the phage preparation, digested with restriction enzymes NcoI (5′) and NotI (3′), and inserted into the compatible NcoI and NotI sites in the destination vector. The vector is also suitable for the expression of AFP genes and AFP–scFv fusion constructs (Fig. 2).

In this section, we describe how to introduce the scFv and AFP–scFv coding sequences, now housed in a plant expression cassette, into *A. thaliana* and tobacco plants. We describe transient expression in tobacco, which can be used to confirm antibody expression and function within 2–3 days, and stable transformation

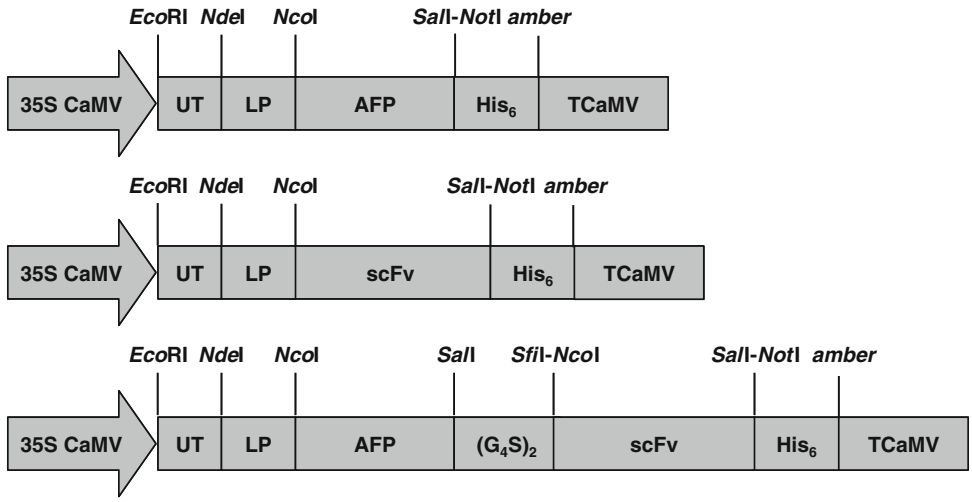


**Fig. 2** Map of the plant expression cassette from plasmid pTRAkc. ***35S CaMV*** cauliflower mosaic virus 35S RNA promoter, ***UT*** untranslated region, ***LP*** leader peptide, ***AFP*** antifungal peptide coding region, ***His6*** hexahistidine tag for recombinant protein detection and purification, ***TCaMV*** cauliflower mosaic virus 35S RNA polyadenylation site/terminator, ***scFv*** coding sequence for single-chain fragment variable polypeptide derived from phage display library, ***(G4S)2*** ten-amino-acid linker between the AFP and scFv cassettes comprising a tandem repeat of four glycine residues and one serine residue; ***SalI-NotI*** and ***SfiI-NcoI*** each represent two adjacent restriction sites

of *A. thaliana*, which is used to generate stable, disease-resistant plant lines for disease challenge testing. Ultimately the aim may be to stably transform other crop plants, but a detailed description of multiple transformation methods is beyond the scope of this chapter. The interested reader can refer to our earlier chapter on antibody expression in plants, which discusses in detail stable transformation methods for tobacco, rice, and wheat [30].

*3.3.1 Preparation of Recombinant A. tumefaciens Cells*

1. Seed a 10 mL culture of *A. tumefaciens* GV3101 in YEB medium containing 50 μg/mL rifamycin and 50 μg/mL carbenicillin, and incubate in an orbital shaker overnight at 28 °C, 250 rpm.
2. Transfer 5 mL of the overnight culture to 100 mL fresh YEB medium supplemented with antibiotics as described above, and incubate for 36–48 h in an orbital shaker at 28 °C, 250 rpm, until an $E_{600\text{nm}}$ of 1.5–2.0 is achieved.
3. Transfer the cells onto ice for 30 min and harvest by centrifugation at 4000 × *g* for 15 min at 4 °C. Wash the pellet three times with 100 mL ice-cold ultrapure water and once with 100 mL 10 % (v/v) glycerol, each wash interspersed with a 10-min centrifugation step at 4000 × *g*, 4 °C.
4. Resuspend the pellet in 2 mL ice-cold 10 % (v/v) glycerol and store 70-μL aliquots at −80 °C until required.
5. Allow an aliquot of the prepared competent cells to thaw on ice and add 400 ng of recombinant plasmid DNA containing the scFv transgene.
6. Transfer the mixture to a chilled cuvette with a 20-mm electrode separation and insert the cuvette into the electroporation system.
7. Carry out electroporation at 2.5 kV, 25 μF, and 200 Ω for 1 ms.
8. Resuspend cells in 500 μL SOC medium and allow them to recover for 1 h at 28 °C with shaking.
9. Transfer an aliquot of the transformed cells onto YEB agar plates supplemented with 50 μg/mL rifamycin, 50 μg/mL carbenicillin, and 25 μg/mL kanamycin (or another selection reagent appropriate for the marker carried on the plant expression vector) to select recombinants.
10. Incubate plates for 2–3 days at 28 °C.
11. Individual colonies should be picked, cultured, and tested by PCR for the presence of the transgene.

*3.3.2 Transient Expression in Tobacco by Agroinfiltration [31]*

1. Tobacco plants should be maintained in a glasshouse or growth room at 23 °C, 65 % humidity, with a 16-h photoperiod (10,000 lux). We cultivate tobacco seeds in ED73 Einheitserde soil.

2. Transfer 1 mL of *A. tumefaciens* culture from Subheading 3.3.1 to 200 mL fresh YEB medium supplemented as above with three antibiotics and incubate overnight in an orbital shaker at 28 °C, 250 rpm.
3. The next morning, centrifuge the cells at 4000 × *g* for 15 min at room temperature and resuspend the pellet in 200 mL YEB-I medium. Incubate overnight on an orbital shaker at 28 °C, 250 rpm.
4. The next morning, centrifuge the cells at 4000 × *g* for 15 min at room temperature and resuspend the pellet in 50 mL MMA medium. Adjust the $E_{600nm}$ to 1.5–2.0 by adding further medium as appropriate and then incubate for 2 h at room temperature.
5. Transfer 50–150 mL of the *A. tumefaciens* suspension to a large preserve jar and add two to three tobacco leaves, 6–15 cm in width. Place a lid on the preserve jar but prevent complete closure by affixing a small roll of paper under the lid, and place the jar in a vacuum chamber. Start the vacuum pump and apply a vacuum of 60–90 mbar for 20 min.
6. Switch off the vacuum pump, recover the leaves, and rinse them with water. Transfer the leaves to an incubation bowl lined with two to three sheets of wet Whatman 3MM paper. Seal the bowl with transparent film and incubate in a phytochamber at 23 °C, with a 16-h photoperiod (10,000 lux) for 2–3 days before harvesting the leaves for protein recovery.

### 3.3.3 Stable Transformation of A. thaliana by Floral Dip [32]

1. *A. thaliana* plants should be maintained in a glasshouse or growth room at 23 °C, 65 % humidity, with a 16-h photoperiod (10,000 lux). We cultivate *A. thaliana* seeds in ED73 Einheitserde soil.
2. Transfer 1 mL of *A. tumefaciens* culture from Subheading 3.3.1 to 100 mL fresh YEB medium supplemented as described above with three antibiotics, and incubate for 16 h in an orbital shaker at 28 °C, 250 rpm.
3. Centrifuge cultures at 4000 × *g* for 15 min at room temperature and resuspend the pellet in 50 mL dipping medium.
4. Transfer 30 mL of the suspension into a Petri dish and dip *A. thaliana* flowers, still attached to the plants, into the suspension for 3 s each.
5. Maintain the dipped plants in the glasshouse as described above and at the appropriate time recover the dry seeds.
6. Collect about 10,000 seeds (500 mg) in a 2-mL tube and surface sterilize in 1 mL 70 % (v/v) ethanol for 15 min on a rotating/undulating platform. Remove the supernatant and replace with 1 mL absolute ethanol. Rotate the tube for 1 min and wash the seeds again with absolute ethanol. This step should remove any residual water.

7. Tap seeds onto dry, sterile Whatman 3MM paper and transfer approximately 2000 seeds (100 mg) onto four MSSA plates. Incubate the plates for 2 days at 4 °C in the dark to synchronize germination, and then germinate in a phytochamber at 25 °C with a 16-h photoperiod (10,000 lux) for 14 days.
8. Transfer strong green plants into the ED73 Einheitserde soil.

### 3.4 Disease Resistance Test

1. Propagate kanamycin-resistant F1 *A. thaliana* plants and recover F2 seeds.
2. Germinate F2 seeds and grow plants under the conditions described above until the rosette leaves are approximately 5 cm long (4–5 weeks). Simultaneously propagate wild-type plants to use as controls.
3. Spray plants with a preparation of fungal spores ($1 \times 10^5$ spores/mL in PDB) from Subheading 3.1.1 **step 6** before the beginning of the photoperiod.
4. Incubate in a phytochamber at 23 °C, 65 % humidity, and a 12-h photoperiod (10,000 lux).
5. Document the condition of the plants 10, 15, and 20 days after infection and take comparative photographs of wild-type and transgenic plants from the infected and noninfected groups.

## 4 Notes

1. Magnesium compounds ($MgCl_2$ and $MgSO_4$) and sugars such as glucose should be added to the media after autoclaving from a filter-sterilized stock solution and made up to the correct final concentration, adding sterile distilled water if necessary.
2. Fungal density can be established using a cell counting chamber. If the density is too high or too low, the spores can be diluted in CM medium or concentrated by centrifugation and then resuspending in CM medium.
3. Germination should be confirmed under a light microscope. The incubation time can be extended if necessary.
4. This culture medium contains sucrose as its sole carbon source and nitrate as its sole nitrogen source. Fungi grow well in this medium, but only a few species of bacteria.
5. Mice can also be used for the preparation of scFv libraries but the methods are not covered here.
6. Polyclonal chicken antibodies (IgY) are isolated by separating the yolk from the egg white, washing the yolk with ice-cold water, and determining the volume in a glass measuring cylinder. Four volumes of ice-cold PBS are then added and the

mixture stirred for 10 min before precipitating the yolk proteins with PEG-6000. Precipitation is achieved by stepwise increases in PEG concentration, initially 3.5 %, then 8.5 %, then 12 %. Each precipitation is carried out by stirring PEG into the solution at 150 rpm on ice for 20 min and then centrifuging for 20 min at 12,000 × *g* and 4 °C, and filtering the supernatant through three layers of Miracloth into a clean vessel. After the final spin, the pellet contains the IgY. This is resuspended in ¼ yolk volume of cold PBS and mixed with an equal volume of cold 50 % ethanol to precipitate the rest of the PEG. The IgY is then pelleted for 25 min at 15,000 × *g* at 4 °C and resuspended in ¼ yolk volume PBS before dialyzing overnight against 2 L PBS, using dialysis tubing, to remove ethanol residue. The antibody concentration is determined by spectrophotometry assuming that an extinction of 1 at 280 nm corresponds to 1.4 mg IgY/mL. Sodium azide should be added to a final volume of 0.02 % (w/v) before aliquoting and storing at −20 °C.

7. The antigen concentration for coating ELISA plates is 100 μg/mL. The IgY concentration is adjusted to 1 mg/mL and then diluted to between $1 \times 10^{-3}$ and $1 \times 10^{-9}$ per mL and used in the ELISA, followed by detection with antibodies and a substrate reaction. If the detection limit is less than $1 \times 10^{-5}$, the chicken must be immunized again.
8. For total RNA extraction we use the RNeasy Midi Isolation Kit (Qiagen, Hilden) and therefore homogenize spleen tissue directly in the RLT buffer supplied by the manufacturer. To confirm the presence of total RNA, 1 μL of the final product should be mixed with an equal amount of glycerol running buffer and separated by agarose gel electrophoresis to determine quantity and quality.
9. For mRNA purification, we use the Oligotex mRNA Mini Isolation Kit (Qiagen, Hilden) according to the manufacturer's instructions. Generally, 1 μL of the final product from the total RNA isolation is sufficient input at this stage.
10. Each PCR comprises 3 μL cDNA from the first-strand synthesis reaction, 1 μL of each primer listed in Subheading 2.2, **item 10** (10 pmol/μL), 0.5 μL of a 10 mM dNTP mix, 3 μL 25 mM $MgCl_2$, 2.5 μL DMSO, 5 μL 10× reaction buffer (supplied by the manufacturer of the enzyme), 0.4 μL *Taq* DNA polymerase (4 U/μL) made up to 50 μL with distilled water. The reaction mix is then overlain with mineral oil, if required, and placed in a thermocycler set to carry out an initial denaturation at 95 °C for 5 min, followed by 33 cycles of denaturation (94 °C, 1 min), annealing (2 min), and elongation (72 °C, 2 min). After 33 cycles, there should be a final elongation step at 72 °C for 5 min. At the end of the run, 5 μL of reaction product should be checked by agarose gel electrophoresis.

11. Pick ten clones from each scFv library and perform a colony PCR. Separate the PCR products by 1 % agarose gel electrophoresis. The products should be approximately 1.1 kb in size because they comprise the $V_H$ and $V_L$ domains as well as the linker introduced by the vector. Sequence the PCR products to check for library complexity. The complexity should decline with successive rounds of panning (*see* Subheading 3.2.6) and eventually reveal multiple clones representing specific binders.
12. Initially, the titer should be approximately $10^7$–$10^{10}$ colonies per 100 μL of *E. coli* culture, but after the first round of panning this should fall significantly to $10^4$–$10^7$ colonies. In subsequent panning rounds, the titer should increase again by 10- to 100-fold per round.

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ERRATUM TO

# Chapter 1
# Chloroplast-Based Expression of Recombinant Proteins by Gateway® Cloning Technology

Johanna Gottschamel and Andreas Lössl

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# INDEX



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## E



## F



## G



## H



## I



## K



## L



## M



## N



## O



## P

## R



## S



## T

## V



## W



## Y