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**).

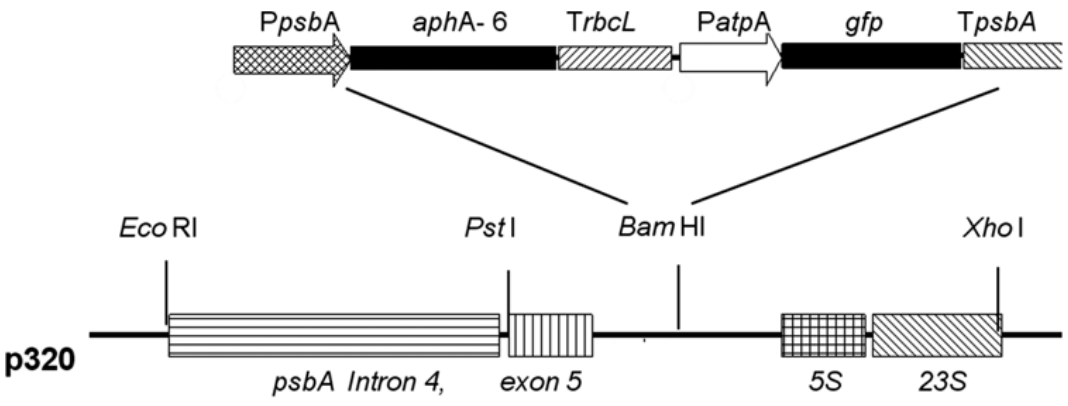

Fig. 1 p320-aphA6-GFP vector used for chloroplast transformation in *Chlamydomonas reinhardtii.* p320-aphA6-GFP targets the insertion of the expressions cassettes for *aphA-6* and *gfp* to the region between intron 4/exon 5 from *psbA* and the ribosomal genes 5S rRNA/5′ 23S RNA. P*psbA* promoter from *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 2×10^4 cell/mL is obtained. Grow the cells under photoperiod (16 h light/8 h dark) at 25 °C until a cell density of 2×10^6 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 1×10^8 cell/mL. A volume in the range of 5–10 mL is usually needed.
4. Place 250 μL of the 1×10^8 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 1×10^8 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.

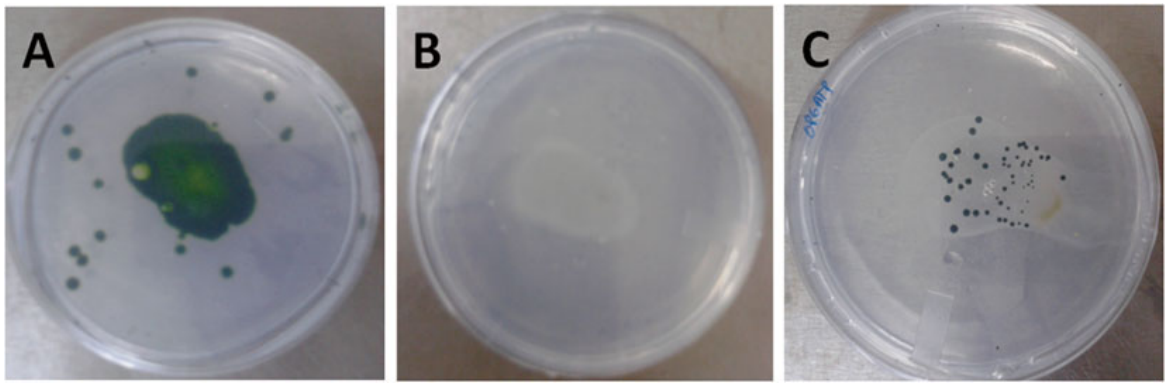

Fig. 2 *Chlamydomonas reinhardtii* cultures 3 weeks after bombardment with chloroplast transformation vector p320-aphA6-GFP. (**a**) Wild-type cells growing in medium without antibiotic; (**b**) Wild-type cells in the presence of kanamycin; (**c**) Transformed cellsforming colonies in the presence of 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 2×10^6 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.

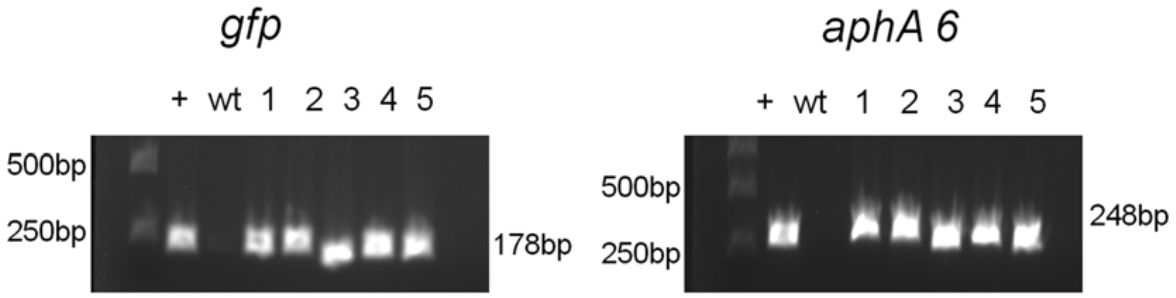

Fig. 3 Electrophoresis gel of PCR products from transformed colonies, using primers designed to amplify fragments of *gfp* and aphA-6 (*see* **Note 9**). Control (+) corresponds to transformation vector; lanes 1–5 correspond to PCR products of transformed colonies. wt corresponds to a wild-type untransformed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

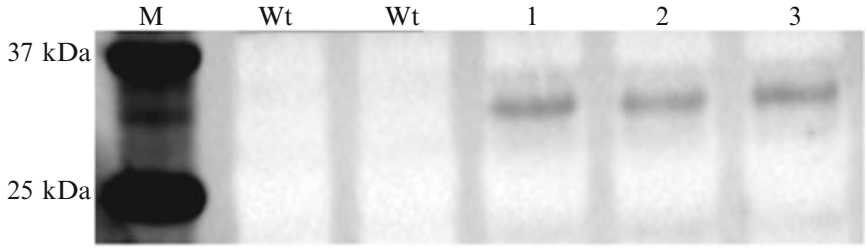

Fig. 4 Immunodetection of GFP in *Chlamydomonas reinhardtii* transformed lines. *M* molecular weight marker Precision Plus Protein WesternC Standards (Bio-Rad). Wt is total soluble protein of a wild-type strain, lanes 1–3 contain total soluble protein from three different transformed strains

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 1.5×10^7 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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