Chapter 17

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A Simple Technology for Generating Marker-free Chloroplast Transformants of the Green Alga *Chlamydomonas reinhardtii*

Marco Larrea-Alvarez, Rosanna Young and Saul Purton

Abstract

The availability of routine methods for the genetic engineering of the chloroplast genome of *Chlamydomonas reinhardtii* is allowing researchers to explore the use of this microalga as a phototrophic cell platform for synthesis of high value recombinant proteins and metabolites. However, the established method for delivering transforming DNA into the algal chloroplast involves microparticle bombardment using an expensive 'gene gun'. Furthermore, selection of transformant lines most commonly involves the use of a bacterial antibiotic resistance gene. In this chapter, we describe a simple and cheap delivery method in which cell/DNA suspensions are agitated with glass beads: a method that is more commonly used for nuclear transformation of *Chlamydomonas*. We also describe the use of plasmid expression vectors that target transgenes to a neutral site within the chloroplast genome between *psbH* and *trnE2*, and employ *psbH* as the selectable marker – thereby avoiding issues of unwanted antibiotic resistance genes in the resulting transgenic lines. Finally, we highlight a feature in our latest vectors in which the presence of a novel tRNA gene on the plasmid results in recognition within the chloroplast of UGA stop codons in transgenes as tryptophan codons. This feature simplifies the cloning of transgenes that are normally toxic to *E. coli*; serves as a biocontainment strategy restricting the functional escape of transgenes from the algal

chloroplast to environmental microorganisms, and offers a simple system of temperature-regulated translation of transgenes.

Key Words Algae, Biocontainment, *Chlamydomonas reinhardtii*, Chloroplast, Glass beads, Homologous recombination, Transformation.

1 Introduction

The green freshwater alga *Chlamydomonas reinhardtii* occupies a special niche in the history of chloroplast biotechnology. Early studies using this organism confirmed that chloroplasts contain their own genetic system, and forward-genetic screens for photosynthetic mutants resulted in the isolation of numerous strains carrying mutations and deletions in essential photosynthetic genes located on the chloroplast genome [1]. It was one such deletion mutant that was used for the first demonstration of stable chloroplast transformation: a strain with a deletion affecting the ATP synthase gene, *atpB* was rescued to phototrophic growth by microparticle bombardment using a plasmid carrying the wild-type *atpB* [2]. Since that time, ever more advanced tools for genetic engineering of the *Chlamydomonas* chloroplast have been developed including a range of selectable markers, reporter genes, expression vectors and recipient strains, together with techniques for controlling transgene expression [3–8].

The low cost of cultivation and fast growth rate of *Chlamydomonas*, combined with the ease of chloroplast engineering, is now fuelling interest in the use of this alga as

an industrial platform for the production of high-value recombinant products such as therapeutic proteins [9,10], and also as a test-bed for chloroplast metabolic engineering studies aimed at producing designer metabolites such as novel bioactive compounds [11,12]. As part of our own research efforts in these areas, we have sought to simplify and accelerate the process of generating transgenic lines. Whilst the delivery of foreign DNA into the chloroplast compartment typically involves the use of a 'gene gun' in which a lawn of algal cells is bombarded with DNA-coated gold microparticles, we employ a simpler and cheaper technique that requires just a basic laboratory vortex [6]. The transformation of eukaryotic cells by agitation of a cell suspension in the presence of glass beads and naked DNA was first developed as a nuclear transformation method for yeast, and subsequently for *Chlamydomonas* [13]. Kindle et al then showed that the method could also be applied to chloroplast transformation of *Chlamydomonas* [14], but the low transformation rates achieved when compared with those obtained by microparticle bombardment appear to have dissuaded researchers from adopting this method. However, the recovery of only a handful of colonies is not really an issue when generating chloroplast transformant lines since all should be genetically identical: the integration of foreign DNA into the chloroplast genome occurs exclusively via homologous recombination mediated by left and right flanking sequences [3]. As a consequence, the outcome of a transformation experiment is the precise targeting of the transgenic DNA to the same locus in each of the recovered transformant lines.

The success of the glass bead transformation method relies on the prior removal (or significant weakening) of the proteinaceous cell wall of *Chlamydomonas*. This can be achieved either by using a cell wall-deficient mutant such as *cw15* (as illustrated in Fig.

1) or by pre-treatment of walled cells with the lytic enzyme, gametolysin – an extracellular metalloprotease produced during the mating of *Chlamydomonas* gametes that mediates cell wall removal [1].

In this chapter we cover the methodology of transformation using either walled strains pre-treated with gametolysin or *cw15* strains. Furthermore, we provide details of how the selection for transformant colonies on solid media can employ either the standard '*aadA* cassette' to generate colonies resistant to spectinomycin [15], or can use an alternative strategy in which selection involves the phototrophic rescue of a strain (TN72) that carries a deletion in the essential photosystem II gene *psbH* (Fig. 2). In this latter approach, integration of the gene-of-interest (GOI) is accompanied by the restoration of the *psbH* lesion using a copy of the native gene carried on the transforming plasmid, and therefore avoids the use of any bacterial antibiotic resistance gene. This is attractive from the point of view of biosafety since the only foreign DNA in the resulting transgenic lines is the GOI. Furthermore, our latest transformation vectors (pWUCA2 and pWUCA4) for use with TN72 also carry an additional useful feature. A modified copy of the native chloroplast gene, *trnW* encoding a tryptophan tRNA has been added to the vectors such that expression of the gene gives rise to a novel tRNA (tRNA-W^{UCA}) with a modified anticodon able to recognise the UGA stop codon as a codon for tryptophan [16]. In the case of pWUCA4, this gene has been further modified to encode a temperature-sensitive version of tRNA-W^{UCA} [8]. The inclusion of the tRNA gene in the vectors allows the replacement of one or more tryptophan codons with TGA when designing synthetic transgenes. This offers three benefits: Firstly, the presence of internal stop codons in transgene coding sequences avoids issues of product toxicity in *E. coli* when

cloning into the chloroplast expression vector since full translation of the transgene does not occur in the bacterium, only in the chloroplast [16]; secondly, the stop codons ensure functional biocontainment of transgenes in the algal chloroplast since any DNA escape and horizontal transfer into a bacterial genome is unlikely to give rise to a translatable gene [16], and thirdly, the temperature sensitive version of the tRNA can be used to regulate synthesis of the products of the transgenes simply by switching the temperature of the algal culture between the permissive and non-permissive temperatures [8].

2 Materials

2.1 Chlamydomonas Strains and transformation plasmids

1. Wild-type and mutant strains can be obtained from the Chlamydomonas Resource Center (www.chlamy.org) based at the University of Minnesota. Strains detailed in this chapter are CC-620 (wild-type, mating-type +), CC-621 (wild-type, mt–), CC-400 (*cw15*, mt+), and CC-5168 (cell-wall deficient strain TN72: *cw15*, *psbH*::*aadA*, mt+ [ref. 6]). (*see* **Note 1**).
2. Chloroplast expression vectors pSRSapI [6], pWUCA2 [16] and pWUCA4 [8], together with the pUC-atpX-AAD plasmid carrying the *aadA* cassette selectable marker [15] are also available from the Chlamydomonas Resource Center.

2.2 Growth of Chlamydomonas

1. Use AnalaR grade chemicals (*e.g.* from Sigma or BDH) and purified water (distilled or reverse-osmosis quality). Two media are most commonly used for culturing of *Chlamydomonas*: TAP or "Tris-Acetate-Phosphate" medium and

HSM or "High-Salt-Minimum" medium (*see* **Note 2**). Recipes for both media can be found at www.chlamycollection.org/methods/media-recipes or in ref. 1. 'HSM-N' is medium lacking a source of nitrogen, and is made by simply omitting the NH_4Cl from the Beijerink salts during preparation of HSM. Liquid media are autoclaved in bottles, or in glass Erlenmeyer flasks (with the medium filling the flask to no more than 50% volume) firmly stoppered with a cotton wool bung that is covered with foil. Sterile media can be stored at room temperature.

2. For solid media, a high-grade agar such as Bacto™ Agar (Becton Dickinson) is required. This is added to liquid media at 2 % (w/v) prior to autoclaving. Following autoclaving and cooling to a temperature below ~50 °C, the medium is poured into 90 mm Petri dishes (Sterilin) to a depth of ~5 mm, and allowed to set. TAP and HSM plates are stored at room temperature and inspected for any bacterial or fungal contamination prior to use.
3. Haemocytometer: Depth 0.1 mm, 1/400 mm^2 (Hawksley) and Tincture of Iodine solution: 20 mM iodine in 95 % (v/v) ethanol. Store at room temperature.

2.3 Glass Bead Transformation

1. Acid washed glass beads, 425-600 μm (Sigma). Aliquot 0.3 g of glass beads into 5 ml test tubes, cap and autoclave (*see* **Note 3**).
2. Gametolysin stock as prepared in section 3.1. Stored at -80 °C.
3. HSM soft agar: 0.5 % (w/v), store at room temperature. Melt agar in a microwave and equilibrate in a water bath for 1 hour at 42 °C before use.
4. HSM plates (for selection using *psbH*) or TAP+Spc^{100} plates (for selection using *aadA*).

2.4 Genomic Extraction and PCR Analysis

1. Chelex 100 resin (BioRad). 5 % (w/v) in sterile water. Store at room temperature.
2. Q5 High-Fidelity DNA Polymerase (New England Biolabs), with supplied 5xQ5 reaction buffer.
3. Mix of four dNTPs at 25 mM (Sigma).
4. Oligonucleotide primers (*see* **Note 4**).
5. 1% (w/v) agarose gel prepared in Tris-Acetate-EDTA buffer.
6. DNA size markers (New England Biolabs).

3 Methods

The introduction of exogenous DNA into the single chloroplast of the *Chlamydomonas* cell relies on the brief agitation of an aqueous suspension of cells and DNA with glass beads. It is believed that the abrasive action of the beads creates transient holes within the cell membrane allowing entry of DNA into the cell. Where the two membranes of the chloroplast are appressed against the cell membrane, the opportunity exists for entry of the DNA directly into the organelle [13]. Since the cell wall represents an additional barrier to DNA delivery, then transformation is best achieved if the cell wall is first removed using gametolysin. Alternatively, a cell wall-deficient strain carrying the *cw15* mutation can be used.

3.1 Preparation of Chlamydomonas Gametolysin

1. Re-streak the two wild-type strains CC-620 and CC-621 on separate TAP plates several times over a period of about 1 week to ensure that the algae are healthy

and actively growing. Incubate the plates at 25 ^{o}C in the light (~20-50 µE/m^2/s PAR).

2. Set up liquid cultures of both strains by inoculating 25 ml of HSM medium in 50 ml flasks with a large loopful of cells, and grow with shaking (~100 rpm) for 2 days at 25 ^{o}C in the light (~50 µE/m^2/s).
3. Transfer each culture to 300 ml HSM in 1000 ml flasks and grow until the cultures reach early stationary phase (~5 x 10^6 cells/ml). Cell concentration is determined by adding 10 µl of tincture of iodine to a 1 ml aliquot of the culture to kill the motile cells, placing an aliquot onto the counting grid of a standard haemocytometer, overlaying the cover slip and counting total cells within the 5 x 5 gridded area using a light microscope with x40 objective lens (total magnification x 400). Repeat the count for the second gridded area and take the average. Cell number per ml is calculated by multiplying the count by 10^4.
4. Pellet the cells using 250 ml centrifuge bottles spun at 4000 x g for 5 minutes. Pour off supernatant and re-suspend each pellet in 5 ml of HSM-N (*see* **Note 5**).
5. Transfer each strain to 1.2 litres of HSM-N in 3 litre flasks.
6. Grow overnight in the light at 25 ^{o}C with shaking, as before. Pellet the cells as above and concentrate 10-fold by re-suspending each pellet in 120 ml of HSM-N.
7. Mix the two cell suspensions in an empty sterile 1000 ml flask to create a mating mix.
8. Allow the cells to mate for 2 hours by leaving the flask undisturbed in low light (~10 µE/m^2/s) (*see* **Note 6)**.

9. Remove cells and debris from the mating mix by centrifugation using 50 ml tubes spun at 4000 x g for 10 minutes.
10. Filter the supernatant containing the gametolysin through a 0.22 μm filter attached to a 50 ml syringe into sterile plastic tubes as 10 ml aliquots.
11. Store the tubes at -80 ^{o}C.

3.2 Preparing Cells for Transformation

1. Re-streak the chosen recipient strain on TAP plates several times over a period of about 1 week to ensure that the alga is healthy and actively growing. Incubate the plates at 25 ^{o}C in the light (*see* **Note 7**).
2. Use a large loopful of cells to inoculate 25 ml of TAP medium in a 50 ml flask, and grow the culture for 2-3 days under continuous light (*see* **Note 7**) with shaking (100 rpm).
3. Use 4 ml of this starter culture to inoculate 400 ml of TAP in a 1000 ml flask and grow under continuous light to mid-log phase (1–2 x 10^6 cells/ml), determining cell concentration as above (*see* **Note 8**).
4. Pellet the cells by centrifuging at 4000 x *g* at room temperature for 5 minutes.
5. Discard the supernatant and re-suspend the cells in either fresh TAP medium (for cell wall-deficient strains) or gametolysin solution (walled strains) to a final concentration of ~2 x 10^8 cells/ml (*i.e.* add ~ 3ml of medium). Re-suspend the pellet by gently drawing up and down using a sterile pipette.
6. For cells in gametolysin solution, incubate at room temperature for 1 hour prior to transformation to allow cell wall digestion.

3.3. Glass Bead Transformation

1. Transfer 300 µl of the cell suspension to 10 test tubes, each containing 0.3 g of sterile glass beads (*see* **Note 3**).
2. Add 10 µg of plasmid DNA to each of eight tubes, leaving the remaining two as 'no DNA' controls.
3. Agitate the cell/glass bead/DNA suspension for 15 seconds at top speed using a Vortex Genie-2 (Scientific Industries).
4. After vortexing all the tubes, add 3.5 ml of 0.5 % (w/v) agar that has been kept molten by incubation at 42 ^{o}C and quickly pour the mix onto the surface of selection medium plates (*see* **Note 9**). Gently tip the plates to allow the molten agar to spread across the whole plate.
5. Allow the agar to set for approximately 20 min (cover in foil or black cloth to prevent phototactic migration of cells). Invert the plates and seal with Parafilm.
6. Incubate the plates at 25 ^{o}C in very dim light (1-5 µE/m^2/s) overnight then move to higher light (40-50 µE/m^2/s) and leave for approximately 2-3 weeks until green colonies appear.

3.4 Isolating Homoplasmic Transformants (*see* **Note 10**).

1. Pick individual colonies using an inoculating loop or sterile toothpicks and restreak to single colonies on fresh plates containing selective medium. Incubate plates as before until single colonies are visible.
2. Repeat step 1 once more and then check for homoplasmy by extracting genomic DNA for PCR analysis using the following method take from ref. 17:
3. Pick a single colony and resuspend in 10 µl sterile water.

4. Add 10 µl of absolute ethanol and incubate for 1 minute at room temp.

5. Add 100 µl of a 5% (w/v) suspension of Chelex 100 resin (Bio-Rad).

6. Vortex briefly then place in a boiling water bath for 5 minutes. Cool briefly on ice.

7. Pellet cell debris and resin with a 2 minute spin in a microfuge and transfer the supernatant containing genomic DNA to a fresh tube.

8. Set up a standard PCR reaction using 1 µl of the genomic DNA (*see* **Note 4**).

9. Once homoplasmic lines have been obtained, then further studies to confirm the expression of the transgene can be carried out as illustrated in Fig. 3.

4 Notes

1. The choice of mating type (mt) as recipient strain is important. During the *Chlamydomonas* sexual cycle, the chloroplast DNA is inherited uniparentally from the mt+ parent [1]. If you plan to subsequently transfer the genetically engineered chloroplast DNA into different nuclear backgrounds you should use an mt+ recipient. Alternatively, use of an mt– recipient increases biological containment of any foreign DNA since escaped transgenic lines will not transmit the DNA to interfertile species within the environment.
2. TAP medium contains acetate as an exogenous source of fixed carbon and is used for heterotrophic growth in the dark or mixotrophic growth in the light. HSM medium contains no fixed carbon and is used for phototrophic growth.

3. We use small (5 ml) glass test tubes capped with a loose aluminium cap. Weighing and dispensing 0.3 g of glass beads into these tubes can be tedious and frustrating because the small beads readily pick up electrostatic charge. We have found that a simple measuring scoop can be made by cutting off the bottom ~8 mm of a standard microfuge tube with a razor blade and heat-fusing it to the end of a 1 ml pipette tip. A standard amount of beads can then be rapidly dispensed into each tube. The rack of tubes is then wrapped in foil and autoclaved.
4. When using plasmid constructs based on pSRSapI, pWUCA2 or pWUCA4 for transformation of TN72, a three-primer method can be employed to confirm both integration of the foreign gene into the chloroplast genome, and homoplasmy (i.e. all ~80 copies of the polyploid genome in the organelle have the gene). Primer F1 (5'-GTCATTGCGAAAATACTGGTGC-3') is designed to a region immediately downstream of *trnE2 – i.e.* outside the 0.8 kb chloroplast DNA region that is the left flanking element in the vectors (*see* Fig. 2). When used in conjunction with primer R1 (5'-CATGGATTTCTCCTTATAATAAC-3'), which is designed to the opposite strand of the *psaA-1* element, a 1.1 kb (pSRSapI) or 1.4 kb (pWUCA2 and pWUCA4) PCR product is generated only if the expression cassette has integrated correctly into the genome. A third primer, R2 (5'-CGGATGTAACTCAATCGGTAG-3') designed to the 3' UTR of the *rbcL* element within the *aadA* expression cassette, yields a 0.88 kb band in conjunction with primer F1 if the cassette has failed to integrate. Consequently, a PCR analysis of putative transformants using the three primers together will give three possible outcomes – the 1.1/1.4 kb band only (cassette integrated and genome

homoplasmic), both 1.1/1.4 kb and 0.88 kb bands (cassette integrated, but genome still heteroplasmic), 0.88 kb band only (untransformed TN72 strain or cassette has failed to integrate at the correct locus). For PCR analysis of transformants carrying the *aadA* cassette [15], the following primer pair can be used to generate a product of 1.1 kb as illustrated in Fig. 1: atpA.F (5'-CAAGTGATCTTACCACTCAC-3') and rbcL.R (5'-CAAACTTCACATGCAGCAGC-3').

5. The conversion of vegetative cells to sexually competent gametes is achieved by removal of fixed nitrogen from the medium. Depletion of N for 24 hours in the light is sufficient to bring about this physiological switch, allowing mating and gamete fusion once opposite mating types are mixed.
6. Healthy gametes are motile (with two anterior flagella) and appear highly active when viewed under the light microscope. When opposite mating types are mixed, the cells recognise a mating partner through flagellar 'tipping' and rapidly form 'wrestling' pairs that fuse to form a quadriflagellate diploid cell.
7. Whilst wild-type and cell wall-deficient strains are tolerant to light levels as high as 1000 $\mu E/m^2/s$ PAR, and are typically grown in our lab at non-saturating levels of ~50 $\mu E/m^2/s$, photosystem II mutants such as TN72 are sensitive to light stress and should be grown below 10 $\mu E/m^2/s$.
8. Transformation efficiency declines if cells have progressed into the later stages of exponential growth or are at stationary phase.
9. For selection of phototrophic transformants of TN72, both the soft agar and the plates are prepared with HSM medium. For selection of antibiotic-resistant

transformants of CC-400, the soft agar is prepared using TAP medium and the plates with TAP medium containing spectinomycin at 100 μg/ml [15].

10. The *Chlamydomonas* chloroplast contains approximately 80 copies of its genome. During the initial stage of transformation, only one or a few copies of the genome are modified. It is therefore important to maintain the selection for the modification for sufficient generations such that all genome copies eventually possess the modification and a stable homoplasmic state is reached [11]. In practice, this is best achieved by repeated re-streaking on selective medium such that single cells give rise to discrete colonies. For selection based on the rescue of prototrophy we find that a single re-streaking is sufficient to achieve homoplasmy. For antibiotic resistance conferred by the *aadA* marker, three re-streakings on spectinomycin medium are typically required.

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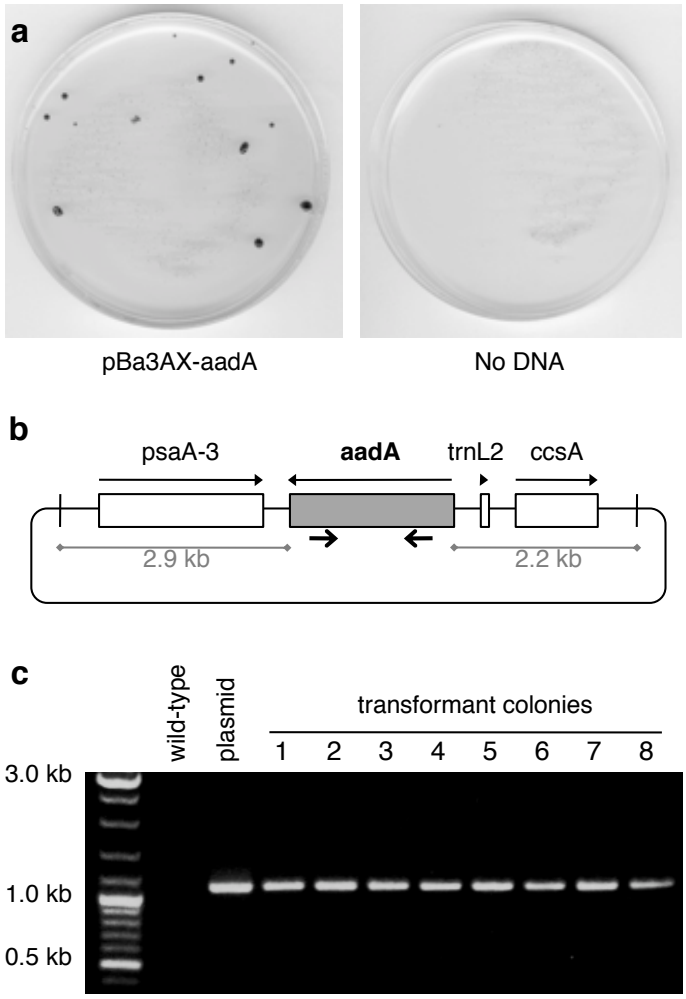

Fig. 1 Glass bead-mediated chloroplast transformation of the cell wall-deficient strain *cw15* using a plasmid carrying the *aadA* cassette that confers spectinomycin resistance. (**a**) A representative plate showing colonies growing on TAP medium containing 100 μg/ml spectinomycin three weeks after vortexing with plasmid pBa3AX-aadA. A control experiment without plasmid DNA yields no colonies. (**b**) Plasmid pBa3AX-aadA containing a 5.1 kb piece of the *Chlamydomonas* chloroplast genome into which the *aadA* cassette has been inserted within the *psaA-3–trnL2* intergenic region. (**c**) PCR confirmation of the presence of the cassette in each transformant line using primers designed to the *aadA* cassette, as indicated in (**b**).

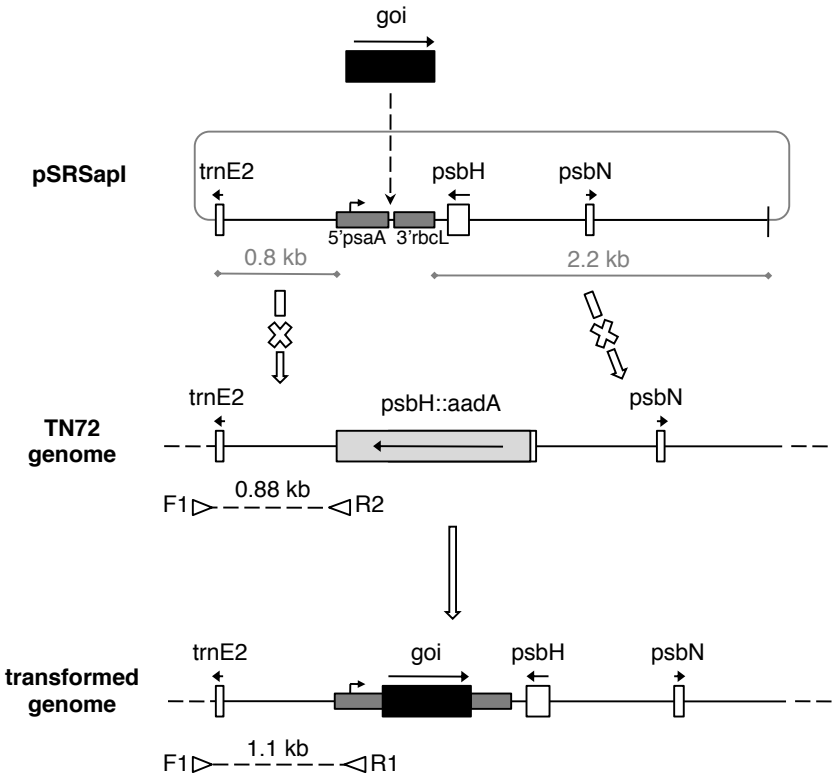

Fig. 2 The pSRSapI vector allows targeted integration and expression of transgenes using the essential photosynthesis gene, *psbH* as a selectable marker. The vector contains an 'expression cassette' within the *psbH–trnE2* intergenic region that comprises: the promoter, 5' untranslated region and start codon of *psaA*; a multiple cloning site for insertion of the coding sequence of the gene-of-interest (goi); the stop codon and 3' untranslated region of *rbcL*. Vectors pWUCA2 and pWUCA4 are identical to pSRSapI except for the presence of the modified tRNA gene $trnW^{UCA}$ immediately upstream of the *psaA* element with pWUCA4 carrying the temperature sensitive variant of $trnW^{UCA}$ [8,16]. Following glass bead transformation of the non-photosynthetic recipient strain (TN72) in which *psbH* has been replaced with the *aadA* cassette, homologous recombination events re-introduce into the wild-type *psbH* together with the expression cassette harbouring the goi. Phototrophic transformants are checked by PCR using a mix

of three primers (F1, R1 and R2: open triangles) where homoplasmic transformants produce a 1.1 kb product only (1.4 kb for pWUCA2 and pWUCA4 transformants), but heteroplasmic transformants produce both the 1.1 kb product and a 0.88 kb product derived from the TN72 genome. Homoplasmy can also be confirmed by scoring transformants for loss of spectinomycin resistance.

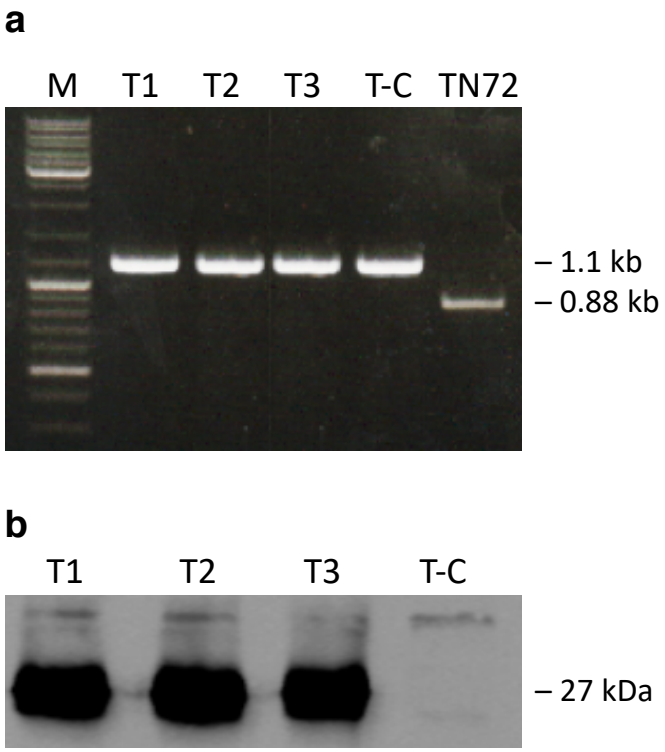

Fig. 3 An example of successful introduction and expression of a transgene achieved using the pSRSapI vector and the glass bead transformation method. Three transformant lines were examined for the presence of a protease gene from *Staphylococcus aureus*, and for the gene product. (**a**) Agarose gel analysis of the products from a 3-primer PCR of the transformant lines (T1-T3), together with a control transformant (T-C) transformed with the empty pSRSapI vector and the untransformed recipient strain TN72. The 1.1 kb band from the transformants confirms the correct integration of the foreign sequence, and the absence of the 0.88 kb band obtained for TN72 indicates that the three lines are homoplasmic. (**b**) Western blot analysis of total soluble protein from the three transformant lines using antibodies to an epitope tag on the recombinant protease. A prominent band of the expected size (27 kDa) is seen in all three transformants, but not in the T-C control (unpublished data, M. Larrea-Alvarez and S. Purton).