Chapter 2

Engineering the Plastid Genome of *Nicotiana sylvestris*, a Diploid Model Species for Plastid Genetics

Pal Maliga and Zora Svab

Abstract

The plastids of higher plants have their own ~120–160-kb genome that is present in 1,000–10,000 copies per cell. Engineering of the plastid genome (ptDNA) is based on homologous recombination between the plastid genome and cloned ptDNA sequences in the vector. A uniform population of engineered ptDNA is obtained by selection for marker genes encoded in the vectors. Manipulations of ptDNA include (1) insertion of transgenes in intergenic regions; (2) posttransformation excision of marker genes to obtain marker-free plants; (3) gene knockouts and gene knockdowns, and (4) cotransformation with multiple plasmids to introduce nonselected genes without physical linkage to marker genes. Most experiments on plastome engineering have been carried out in the allotetraploid *Nicotiana tabacum*. We report here for the first time plastid transformation in *Nicotiana sylvestris*, a diploid ornamental species. We demonstrate that the protocols and vectors developed for plastid transformation in *N. tabacum* are directly applicable to *N. sylvestris* with the advantage that the *N. sylvestris* transplastomic lines are suitable for mutant screens.

Key words: Plastid transformation, *Nicotiana sylvestris*, Tobacco, *aadA*, Spectinomycin resistance, Streptomycin resistance

1. Introduction

The plastid genome (ptDNA) of higher plants is highly polyploid. The number of plastids per cell and the number of ptDNA per plastid is dependent on the species and the cell type. For example, an *Arabidopsis thaliana* leaf cell contains about 120 chloroplasts, the green plastid type differentiated for photosynthesis, and these harbor 1,000-1,700 copies of the 154,478-bp plastid genome (1). In comparison, *Nicotiana tabacum* leaf cells contain about 100 chloroplasts harboring ~10,000 copies of the 155,939-bp ptDNA (2). Transformation of the plastid genome was first accomplished

James A. Birchler (ed.), *Plant Chromosome Engineering: Methods and Protocols*, Methods in Molecular Biology, vol. 701,
DOI 10.1007/978-1-61737-957-4_2, © Springer Science+Business Media, LLC 2011

in *Chlamydomonas reinhardtii*, a unicellular alga (3), followed by plastid transformation in this laboratory in *N. tabacum*, a multicellular flowering plant (4). Plastid transformation since has been extended to *Porphyridium*, a unicellular red algal species (5) and the mosses *Physcomitrella patens* (6) and *Marchantia polymorpha* (7). In higher plants, plastid transformation is reproducibly performed in *N. tabacum* (8), tomato (9), soybean (10), lettuce (11, 12), and cabbage (13). Monocots as a group are still recalcitrant to plastid transformation.

Plastid transformation in each of these systems is based on homologous recombination. Plastid transformation vectors are *Escherichia coli* plasmid derivatives that do not replicate in plastids, so that the marker genes encoded in the vectors are expressed only if they integrate in the plastid genome via cloned ptDNA fragments flanking the marker gene (1–2 kb each side). In some of the plastid transformation vectors the restriction sites have been removed so that the plastid vectors can be used for cloning in *E. coli* (9, 14–16). The transforming DNA is introduced into plastids by the biolistic process (4, 8) or by polyethylene glycol treatment (17, 18), followed by selection for the marker gene. The marker genes employed for selective amplification of transformed ptDNA are plastid small rRNA genes with point mutations conferring spectinomycin resistance (4, 17, 18); or chimeric genes that confer resistance to spectinomycin and streptomycin (*aadA*)(8), kanamycin (*neo* or *aph(3′)IIa*) (19, 20) (21), or the amino acid analogues 4-methylindole (4MI) and 7-methyl-DL-tryptophan (7MT) (*ASA2*) (22). If the transforming DNA is introduced into protoplasts, the DNA delivery is by PEG treatment (17, 18). DNA delivery into leaf chloroplasts (4, 8) and the plastids of tissue culture cells (10, 23) is by the biolistic approach.

Engineering of the plastid genome includes four types of manipulations. The first type of manipulation is insertion of the marker gene and the gene of interest in intergenic regions. If mutations are included in the plastid-targeting region, the mutation may or may not be introduced into the plastid gene dependent on the site of recombination as described (24–26). The second type of manipulation is posttransformation marker gene excision. When we contemplate posttransformation excision of the marker genes, we flank the marker gene by recombinase target sites in the transformation vector. Excision of the marker gene is accomplished by a plastid-targeted recombinase expressed from a nuclear gene introduced by Agrobacterium-mediated transformation or by crossing. The example we show for the insertion of a transgene in Fig. 1 is transformation of the *N. sylvestris* plastid genome with the pCK2 plastid transformation vector that carries a selectable spectinomycin resistance (*aadA*) marker gene and the aurea bar^{au} gene of interest. Recombination between the pCK2 vector and ptDNA (dashed lines) yielded Ns-pCK2 ptDNA

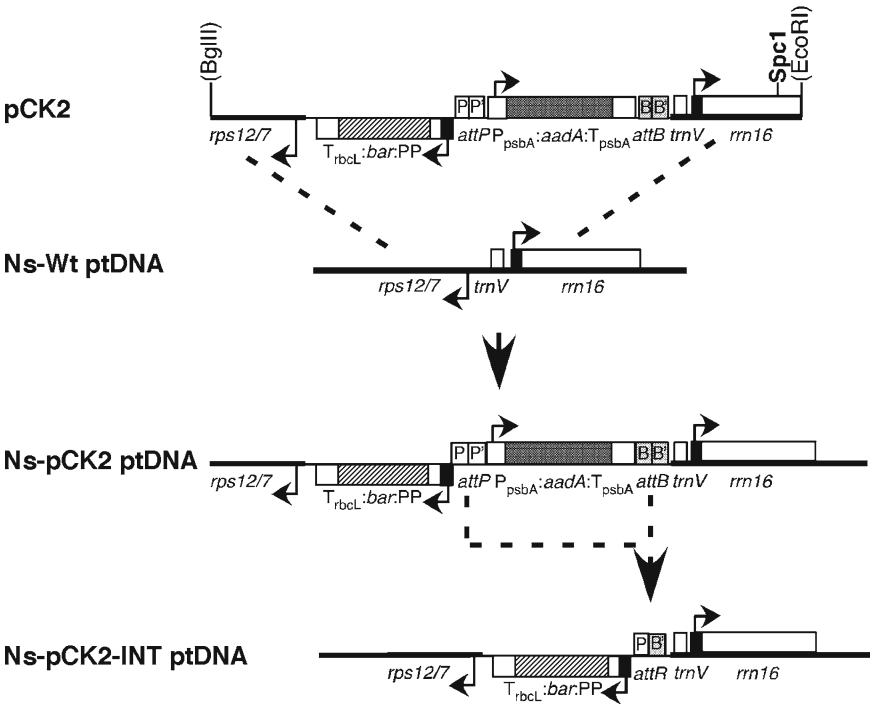


Fig. 1. Construction of transplastomic *Nicotiana sylvestris* plants carrying the aurea *bar*^{au} gene in their plastid genome. Recombination between the pCK2 vector and wild-type ptDNA (*dashed lines*) yields the Ns-pCK2 transplastome carrying the *bar*^{au} gene and the selectable spectinomycin resistance (*aadA*) gene. Note that *aadA* is flanked with the phiC31 phage recombinase *attB* (BB') and *attP* (PP') target sites. Excision of *aadA* by Int leaves behind a recombinant PB′ target site and the *bar*^{au} gene in the Ns-pCK2-INT ptDNA. For further details see reference on the construction of transplastomic *bar*^{au} *N. tabacum* plants (41).

carrying both transgenes. Excision of *aadA* by Int, the phiC31 phage site-specific recombinase, via the *attP* and *attB* target sites left behind only the aurea *bar*^{au} gene and a recombinant target site in the Ns-pCK2-INT ptDNA.

The third type of plastid genome engineering is knocking out plastid genes to query their function. The knockout lines are obtained by replacement of endogenous genes with marker genes by homologous recombination. The first examples of plastid gene knockouts were lacking the plastid *rbcL* (27) and *rpoB* (28) genes. By now at least 25 plastid gene knockout plants have been described (29). The example we show in Fig. 2 is a knockdown variant, in which introduction of an editing site in the *psbF* gene yielded an editing-dependent, functionally impaired version of the *psbF* gene. Transformation of the *N. sylvestris* plastid genome with vector pRB8, dependent on the site of recombination, yielded two types of transplastomic plants carrying *aadA*: editing-dependent, slow-growing plants with reduced chlorophyll content (T1-ptDNA) and plants with a wild-type phenotype (T2-ptDNA).

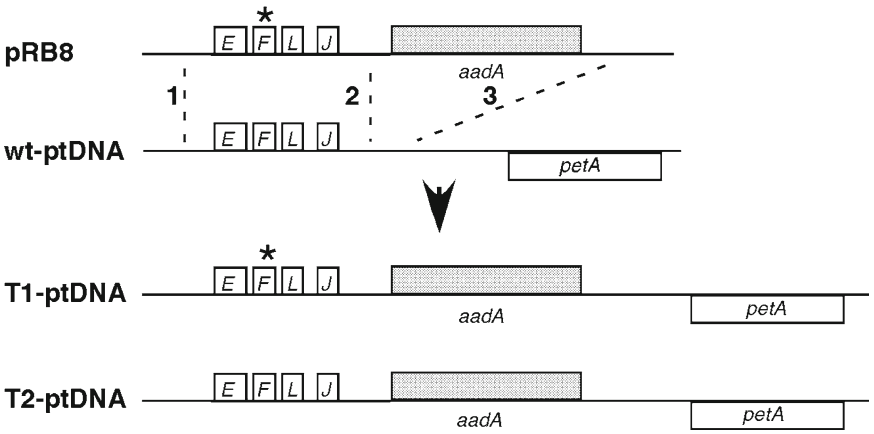


Fig. 2. Construction of *psbF* knockdown plants in *Nicotiana sylvestris* by transformation with the pRB8 plastid vector. The pRB8 plasmid carries a selectable spectinomycin resistance (*aadA*) gene targeting the insertion of *aadA* gene in the *psbJ-petA* intergenic region. The targeting region contains the tobacco *psbE* operon (*psbE*, *psbF**, *psbL,* and *psbJ* genes) with the engineered spinach *psbF* editing site marked by an asterisk (24). Recombination (*dashed lines*) between sites 1 and 3 yielded pigment-deficient knockdown plants (T1 ptDNA), whereas recombination between sites 2 and 3 yielded normal green plants (T2 ptDNA).

The fourth type of manipulation is cotransformation carried out with a mix of two plastid transformation vectors. First, the transplastomic clones are selected by the marker in vector one; then these transplastomic clones are screened for sequences from the second vector that does not carry a selectable marker. Cotransformation by the two plasmids is relatively frequent: plastid genomes recovered after transformation with the mixed vector carry the second, nonselected modification in ~20% of the clones (30, 31). Independent transformation of ptDNA copies with vectors targeted to alternative sites and subsequent sorting could also be used to obtain marker-free transplastomic plants carrying the nonselected genes (32). No cotransformation experiments have been carried out in *N. sylvestris* yet. In Fig. 3 we schematically show introduction of a nonselected marker by cotransformation in *N. tabacum* (30). For reviews on the applications of plastome engineering in basic science and biotechnology, see refs. (14, 29, 33–37).

Most experiments on plastid genome engineering have been carried out in *N. tabacum*, an allotetraploid species. Because of our interest in the identification of nuclear genes that control plastid inheritance, we were looking for a diploid *Nicotiana* species in which a mutant screen can be carried out. We report here protocols for plastid transformation in *Nicotiana sylvestris*. *N. sylvestris* shares all the advantages of *N. tabacum*: facile tissue culture regeneration, transformation of nuclear and plastid genomes, numerous progeny (greater than million) and short generation time (4 months) with the additional advantage that it has a

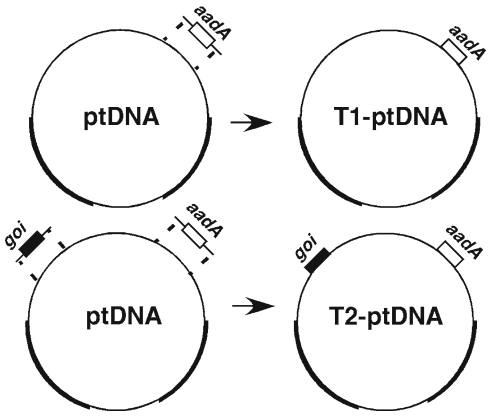


Fig. 3. Cotransformation with mixed plasmids to insert nonselected sequences in the plastid genome without physical linkage to the selectable marker. The nonselected sequence may be a gene (30) or a protein tag (31). Plastids contain multiple copies of the plastid genome (ptDNA). The selectable spectinomycin resistance (*aadA*) gene (*open box*) and nonselected gene-of-interest (*goi*; *filled box*) are targeted to different regions of ptDNA. Clones carrying *aadA* genes (T1-ptDNA and T2-ptDNA) are selected by spectinomycin resistance and distinguished from spontaneous mutants by streptomycin/spectinomycin resistance (see text). Approximately 20% of the transplastomic clones carries both *aadA* and the nonselected *goi* (T2-ptDNA). Based on ref. (30, 31).

diploid nuclear genome ($n=12$; 2,600 Mb; (38)). Because the diploid *N. sylvestris* is the maternal progenitor of the allotetraploid *N. tabacum*, the plastid genomes of the two species are very similar (39). We report here that vectors pRB8 (24), pMSK56 (40), and pCK2 (41) originally developed for *N. tabacum* can be used for plastid transformation in *N. sylvestris*. We currently use the transplastomic *N. sylvestris* lines in a genetic screen for mutations in plastid inheritance.

2. Materials

2.1. Cleaning of Gold Particles

1. 0.6 μm gold microcarrier available from Bio-Rad.
2. Ice-cold 70% ethanol.
3. Ice-cold sterile distilled water.
4. 50% glycerol, sterilized by autoclaving.

2.2. Coating of Gold Particles with Plasmid DNA

1. Plasmid DNA prepared using the QIAGEN Maxi Kit (Qiagen Inc., Valencia, CA).
2. 2.5 M $CaCl_2$ solution.
3. 0.1 M spermidine free base (Sigma, St Louis, MO).

4. 70% ethanol.
5. Ice-cold 100% ethanol.

2.3. DNA Delivery into Chloroplasts by the Biolistic Process

1. Bio-Rad PDS1000/He biolistic gun (Bio-Rad Laboratories, Hercules, CA).
2. Macrocarrier disk for PDS1000/He biolistic gun (Bio-Rad).
3. Rupture disk for PDS1000/He biolistic gun (Bio-Rad).
4. Vacuum pump for the gun ThermoSavant VLP285 (ThermoSavant, Holbrook, NY).
5. Pressurized helium in tank, 99.999% pure, moisture free.
6. Laminar flow hood for bombardment and tissue culture.
7. Whatman No. 4 filter paper disks sterilized by autoclaving in glass Petri dish.
8. Sterile *Nicotiana sylvestris* TW137 plants in Magenta boxes (see Note 1) grown on RM plant maintenance medium (see Note 2).
9. Petri plate (100×15 mm) containing solid RMOP medium (20 ml) for leaf bombardment (see Note 3).
10. Deep (100×20 mm) Petri plates with 50 ml selective RMOP containing 500 mg/L Spectinomycin dihydrochloride pentahydrate (Sigma, St Louis, MO) (see Note 4).
11. 70% ethanol.
12. Plastic wrap (strips) that is permeable to gas exchange, for example, Glad ClingWrap.

2.4. Identification of Transplastomic Plants

1. Deep (100×20 mm) Petri dishes containing RM plant maintenance medium (see Note 2).
2. Deep (100×20 mm) Petri dishes containing a selective spectinomycin (500 mg/L; Sigma, St Louis, MO) RMOP plant regeneration medium (see Note 3).
3. Deep (100×20 mm) Petri dishes containing 500 mg/L each of spectinomycin (Sigma, St Louis, MO; Catalog No. S9007) and streptomycin (Sigma, St Louis, MO) in the RMOP plant regeneration medium (see Note 4).
4. Magenta boxes containing selective spectinomycin (500 mg/L; Sigma, St Louis, MO) RM plant maintenance medium (see Note 2).
5. Deep (100×20 mm) Petri dishes containing RM plant maintenance medium (see Note 2).
6. Captan (Bonide Products Inc., Oriskany, NY), a fungicide; active ingredient *N*-Trichloromethylthio-4-cyclohexane-1, 2-dicarboximide.
7. Plastic trays (10″×20″) with holes on bottom with garden soil and transparent plastic domes.

3. Methods

The protocol we describe here was used to obtain transplastomic *N. sylvestris* after bombardment of tissue culture grown leaves with the pRB8 (24), pMSK56 (40) and pCK2 (41) plastid transformation vectors. The Ns-pRB8 and Ns-pMSK56 plants carry a selectable spectinomycin resistance (*aadA*) gene. The *aadA* gene in the Ns-pMSK56 plasmids is translationally fused with the green fluorescent protein. The Ns-pCK2 plants carry an aurea bar^{au} gene linked to *aadA*. In the Ns-pCK2-INT derivatives the *aadA* gene has been excised with Int, the phiC31 phage site-specific integrase. The protocol we describe here for *N. sylvestris* complements two other protocols published on plastid transformation in *N. tabacum* from this laboratory: one describing plastid transformation and marker gene excision by the CRE-*loxP* site-specific recombination system (42); the second on the applications of plastid transformation to study mRNA editing (43).

3.1. Cleaning of Gold Particles

This protocol was modified from Bio-Rad Bulletin 9075.

1. Weigh out 30 mg 0.6 μm gold microcarrier in a 1.5 ml Eppendorf tube and add 1 ml ice-cold 70% ethanol. Place tube in a Vortex microtube holder and shake vigorously for 5 min. Let the particles settle in the tube for 15 min at room temperature (20–25°C).
2. Spin in microcentrifuge at 3,000 rpm (600 × *g*) for 1 min to compact gold. Remove the ethanol with a pipette and add 1 ml ice-cold sterile distilled water. Vortex the tube to suspended the particles. Allow the gold particles to settle at room temperature for 10 min.
3. Sediment the gold particles by spinning in a microcentrifuge at 3,000 rpm (600 × *g*) for 1 min. Remove water with pipette and add 1 ml ice-cold sterile distilled water.
4. Repeat washing the gold particles with water by repeating steps 2 and 3.
5. Suspend the gold particles by vortexing and store the tube at room temperature for 10 min to allow the particles to settle.
6. Spin the tube in a microcentrifuge at 5,000 rpm (1,700 × *g*) for 15 s, then remove water completely. Add 500 μl 50% glycerol and vortex for 1 min to resuspended particles. Gold concentration will be 60 mg/ml. The clean gold can be stored for 2 weeks at room temperature.

3.2. Coating of Gold Particles with Plasmid DNA

This protocol was modified from Bio-Rad Bulletin 9075.

1. Place Eppendorf tube containing gold in a Vortex microtube holder and shake at setting 3. While tube is shaking, remove

50 µl aliquots of gold and pipette into ten 1.5 ml Eppendorf tubes in a rack (see Note 5). While tubes are shaking, add 5 µl plasmid DNA (1 µg/µl), 50 µl 2.5 M $CaCl_2$, and 20 µl 0.1 M spermidine free base. Make sure to add the components in this order and that the contents are thoroughly mixed before adding the next component.

2. Shake tubes on Vortex at setting 3 for 5 min.
3. Sediment gold by spinning in microcentrifuge at 3,000 rpm (600×*g*) for 1 min. Remove the supernatant and add 140 µl 70% ethanol to each tube. Tap tube lightly until the pellet just starts to come into solution to make sure pellet is not tightly packed. If gold does not go into solution by gently tapping the tube, break up pellet by pipetting up and down.
4. Sediment gold by spinning in a microcentrifuge at 3,000 rpm for 1 min. Remove supernatant and add 140 µl ice-cold 100% ethanol to each tube. Lightly tap tube until the pellet just starts to come into solution.
5. Sediment gold by spinning in a microcentrifuge at 5,000 rpm (1,700×*g*) for 15 s. Resuspend coated gold pellet in 50 µl 100% ethanol by gently tapping tube. Pellet should easily enter solution. Shake tubes at setting 3 while waiting to use them for bombardment. If tubes are sitting for a long period of time before bombardment, replace ethanol in tube with fresh 100% ethanol.

3.3. DNA Delivery into Chloroplasts by the Biolistic Process

The protocol we describe here is for biolistic transformation of *N. sylvestris* leaves collected from plants grown in sterile culture. Bombardment of 20–30 leaves with the Bio-Rad PDS1000/He gun yields 10–40 transplastomic clones. The Hepta-adaptor version of the gun (which is simultaneously using seven macrocarriers) is more efficient; bombardment of five leaves is sufficient to obtain 5–25 transplastomic clones.

1. Place leaves for biolistic transformation abaxial side up on two sterile Whatman No. 4 filter papers on top of solid RMOP medium (20 ml) in a 100×15 mm Petri plate. Use more than one leaf if necessary to cover the central area of the Petri plate (see Note 6).
2. Set up the biolistic gun in a sterile laminar flow hood. Before bombardment, wipe off main chamber, rupture disk retaining cap, microcarrier launch assembly, and the target shelf of the gun with a cloth soaked in 70% ethanol.
3. Sterilize rupture disks (1,100 psi), macrocarriers, macrocarrier holders, and stopping screens by soaking in 100% ethanol (5 min) then air dry them in tissue culture hood in open container.

4. Turn on helium tank and set pressure in regulator (distal to tank) for 1,300 psi (200–300 psi above rupture disk value).
5. Turn on vacuum pump and gene gun. Set the vacuum rate on the gene gun to 7 and the vent rate to 2.
6. Prepare DNA-coated gold particles as described above. Pipette 10 µl of DNA-coated gold onto macrocarrier or "flying disk" in holder and let the disk air dry for 5 min. Five samples may be made up at one time if bombardment is carried out with one macrocarrier (or seven, if Hepta-adaptor is used).
7. Place rupture disk into retaining cap and screw in place tightly.
8. Put stopping screen and flying disk (face down) in microcarrier launch assembly and place in chamber just below rupture disk. For description see Bio-Rad Bulletin 9075.
9. Place leaf on thin RMOP plate (100 × 15 mm) into chamber 9 cm (fourth shelf from top) below the microcarrier launch assembly and close the door (see Note 7).
10. Press vacuum button to open valve. When vacuum reaches 28 inches of mercury (Hg) hold down fire button until the pop from the gas breaking the rupture disk is heard (see Note 8).
11. Immediately release the vacuum and remove the leaf sample.
12. Repeat steps 6–11 until all leaf samples are bombarded. When finished, turn off helium tank, and release pressure by holding down the fire button while vacuum is on. Turn off vacuum pump.
13. Place clear plastic sleeves over plates containing bombarded leaf samples and incubate in culture room. Incubation allows time for marker gene expression before selection is started.
14. After 2 days, cut bombarded leaves into small (1 cm square) pieces and place abaxial side up in deep plates (100 × 20 mm) containing a selective RMOP medium with 500 mg/L spectinomycin. Place only seven pieces per plate as the leaf pieces will grow and expand (see Note 9).
15. Seal each plate with plastic wrap that is permeable to gas exchange and incubate plates in culture room for 4–12 weeks.

3.4. Identification of Transplastomic Plants

1. The bombarded green leaf sections turn pale on the selective spectinomycin medium and expand. Cells carrying transformed spectinomycin-resistant plastids turn green and overcome the inhibition of shoot regeneration by spectinomycin. As a result, transplastomic cells form green shoots on the pale leaf sections. However, mutations in the 16S rRNA also confer spectinomycin resistance and yield green shoots (8).

Dissect green shoots that appear 4–12 weeks after bombardment onto RM plates for rooting and classification as a transplastomic clone or a spontaneous mutant. Each shoot at a distinct location derives from an independent event; therefore, they should be treated as independently derived clones. We mark the independent clones by a serial number.

2. To distinguish transplastomic clones from spontaneous mutants, transfer small (1 cm^2) leaf sections of the regenerated shoots (Subheading 3.4, step 1) onto (a) selective streptomycin–spectinomycin RMOP medium and (b) spectinomycin RMOP medium. Transgenic clones carrying an *aadA* gene are resistant to both spectinomycin and streptomycin, whereas spontaneous spectinomycin-resistant mutants are resistant only to spectinomycin (8). Resistance is manifested as formation of green calli with regenerating shoots; sensitivity is indicated by the bleaching of leaf sections and the absence of shoot regeneration. Classify mutants on the streptomycin–spectinomycin medium; pick shoots of the transplastomic clones from spectinomycin and transfer them onto RM plant maintenance medium (see Note 10). Carry out DNA gel blot analyses in leaves regenerated on spectinomycin medium to confirm plastid transformation and to test the uniformity of ptDNA population (42). At this stage we identify the transplastomic clones by the initials of the plant species and the transforming plasmid name, for example, Ns-pCK2-6A for *N. sylvestris* transformed with plasmid pCK2, serial number 6 and one or more letters of the alphabet to indicate the number of shoot regeneration cycles on selective medium. Always test plants from three to four independently transformed clones because ~10% of plants regenerated in tissue culture are sterile due to somaclonal variation.
3. Because the plastid genome is highly polyploid, the plant may be chimeric even if it looks homoplastomic on the gel blots. Therefore, we repeat plant regeneration on the selective medium and verify the uniformity of ptDNA by gel blot analyses. Plants regenerated 2× on selective spectinomycin medium are normally homoplastomic and are marked by the addition of two letters of the alphabet, for example, Ns-pCK2-6AB.
4. Transfer rooted transplastomic shoots to greenhouse to obtain seed. Gently break up the agar; wash roots to remove the agar-solidified RM medium; drench the soil after planting with Captan to fend off fungal infection; and cover plantlets with a plastic dome to prevent desiccation. Grow plants in shade for about a week, and then remove plastic dome and expose the plants to full sunlight.

5. Collect mature seedpods from transplastomic plants and let the seed dry on the lab bench at room temperature for 1 week. Remove moisture for long-term storage by a 2-week storage in seed drier.
6. Germinate surface-sterilized seeds on RM-spectinomycin (500 mg/L) medium (see Note 2). Seedlings carrying transformed ptDNA will be dark green, whereas sensitive seedlings will be white. One hundred percent green seedlings confirm uniform population of transformed ptDNA in the transplastomic plants.

4. Notes

1. *Nicotiana sylvestris* TW137 plants in Magenta boxes. The *Nicotiana sylvestris* ecotype TW137 was obtained from the USDA seed collection at NC State University. The fertile TW137 was converted to a cytoplasmic male sterile (CMS) form by repeated pollination of the *N. tabacum* CMS92 line with *N. sylvestris* TW137 pollen. We obtained the CMS92 *N. tabacum* line with the *Nicotiana undulata* cytoplasm from Ezra Galun (44). The seeds are vapor sterilized by storing them for 3 h in an open 1.5 ml Eppendorf tube in a closed desiccator over the mix of 100 ml Clorox bleach and 3 ml concentrated (37.8%) HCl. The seed is germinated in deep (100 × 20 mm) Petri dishes on RM plant maintenance medium (60 ml/dish; see below). The seedlings are grown to plants in Magenta boxes containing 80 ml RM medium.
2. The RM plant maintenance medium (MS medium, ref. (45)) per liter contains: 100 ml 10× macronutrients, 10 ml 100× micronutrients, 5 ml 1% Fe–EDTA, 30 g sucrose, pH 5.6–5.8 with 1 M KOH; 7 g agar). RM Medium 10× macronutrient solution per liter contains: 19 g KNO_3, 3.7 g $MgSO_4 \cdot 7H_2O$, 4.4 g $CaCl_2 \cdot 2H_2O$, 1.7 g KH_2PO_4, 16.5 g $(NH_4)NO_3$. RM Medium 100× micronutrient solution per liter contains: 169 mg $MnSO_4 \cdot H_2O$, 62 mg H_3BO_3, 86 mg $ZnSO_4 \cdot 7H_2O$, 8.3 mg KI, 2.5 ml $Na_2MoO_4 \cdot 2H_2O$ (1 mg/ml), 2.5 ml $CuSO_4 \cdot 5H_2O$ (1 mg/ml), 0.25 ml $CoCl_2 \cdot 6H_2O$ (1 mg/ml). Agar, plant tissue culture tested (Sigma, St Louis, MO).
3. RMOP shoot regeneration medium (46) per liter contains: 100 ml RM medium 10× macronutrients, 10 ml RM medium 100× micronutrients, 5 ml 1% Fe–EDTA, 1 ml thiamine (1 mg/ml), 0.1 ml alpha-naphthaleneacetic acid (NAA 1 mg/ml in 0.1 M NaOH), 1 ml 6-benzylaminopurine (BAP 1 mg/ml in 0.1 M HCl), 0.1 g myo-inositol, 30 g sucrose, pH 5.8 with 1 M KOH, 7 g agar.

4. The selective RMOP medium contains filter-sterilized 500 mg/L spectinomycin dihydrochloride (Sigma, St Louis, MO), or 500 mg/L streptomycin sulfate (Sigma, St Louis, MO), or both. The composition of RMOP medium is described above.
5. With one DNA construct 20–30 single leaves are bombarded. Each tube (30 mg) of gold is sufficient for 50 bombardments (two DNA constructs). One may use freshly prepared gold particles, or stored gold. If using stored gold, vortex tube for 5 min before coating with DNA.
6. If you are using greenhouse leaves, you need to surface sterilize the leaves and cut out a segment that covers the plate. Greenhouse leaves may be surface sterilized by rinsing the leaves in water containing a drop of surfactant (Tween 80 or liquid soap) to remove dirt; dipping the leaves in 70% ethanol; and placing them in diluted commercial bleach (tenfold diluted Clorox; final sodium hypochlorite concentration is ~0.6%) for 3 min. The bleach should be thoroughly removed by rinsing the leaves in sterile distilled water 5×.
7. Leaves should be prepared in advance of transformation and may be stored on top of the filter paper in a "ready-to-shoot" state for at least a half day.
8. If gun is fired at a lower vacuum pressure, the DNA-coated particles will lack the momentum to penetrate cells and no transplastomic lines will be obtained. If you have no experience with biolistic transformation, we recommend that you test particle coating and DNA delivery using transient expression of a nuclear *uidA* gene that encodes the enzyme β-glucuronidase, the activity of which can be readily detected by histochemical staining (47, 48).
9. Leaf pieces from 1 leaf fit on 3–5 plates. If the leaf sections are too large, there will be insufficient nutrient in the medium to support growth for up to 12 weeks, the time frame within which transplastomic clones appear. Diagnostic sign of overcrowding is absence of spontaneous spectinomycin-resistant mutants and transplastomic clones. Overcrowding may be also caused by less than the desired 50 ml culture medium in a deep plate.
10. We prefer to work with shoots regenerated on spectinomycin, because streptomycin is a mutagen.

Acknowledgments

This work was supported by grants from the USDA Biotechnology Risk Assessment Research Grant Program Award No. 2005-33120-16524 and 2008-03012.

References

1. Zoschke, R., Liere, K., and Borner, T. (2007) From cotyledon to mature plant: Arabidopsis plastidial genome copy number, RNA accumulation and transcription are differentially regulated during leaf development, *Plant J* **50**, 710–722.
2. Shaver, J. M., Oldenburg, D. J., and Bendich, A. J. (2006) Changes in chloroplast DNA during development in tobacco, *Medicago truncatula*, pea, and maize, *Planta* **224**, 72–82.
3. Boynton, J. E., Gillham, N. W., Harris, E. H., Hosler, J. P., Johnson, A. M., Jones, A. R., Randolph-Anderson, B. L., Robertson, D., Klein, T. M., Shark, K. B., and Sanford, J. C. (1988) Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles, *Science* **240**, 1534–1538.
4. Svab, Z., Hajdukiewicz, P., and Maliga, P. (1990) Stable transformation of plastids in higher plants, *Proc Natl Acad Sci USA* **87**, 8526–8530.
5. Lapidot, M., Raveh, D., Sivan, A., Arad, S. M., and Shapira, M. (2002) Stable chloroplast transformation of the unicellular red alga Porphyridium species, *Plant Physiol* **129**, 7–12.
6. Sugiura, C., and Sugita, M. (2004) Plastid transformation reveals that moss trnR-CCG is not essential for plastid function, *Plant J* **40**, 31–321.
7. Chiyoda, S., Linley, P. J., Yamato, K. T., Fukuzawa, H., Yokota, A., and Kohchi, T. (2007) Simple and efficient plastid transformation system for the liverwort *Marchantia polymorpha* L. suspension-culture cells, *Transgenic Res* **16**, 41–49.
8. Svab, Z., and Maliga, P. (1993) High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene, *Proc Natl Acad Sci USA* **90**, 913–917.
9. Ruf, S., Hermann, M., Berger, I. J., Carrer, H., and Bock, R. (2001) Stable genetic transformation of tomato plastids: foreign protein expression in fruit, *Nat Biotechnol* **19**, 870–875.
10. Dufourmantel, N., Pelissier, B., Garcon, F., Peltier, G., Ferullo, J. M., and Tissot, G. (2004) Generation of fertile transplastomic soybean, *Plant Mol Biol* **55**, 479–489.
11. Lelivelt, C., McCabe, M., Newell, C., de Snoo, B., Van Dunn, K., Birch-Machin, I., Gray, J. C., Mills, K., and Nugent, J. M. (2005) Plastid transformation in lettuce (*Lactuca sativa* L), *Plant Mol Biol* **58**, 763–774.
12. Kanamoto, H., Yamashita, A., Asao, H., Okumura, S., Takase, H., Hattori, M., Yokota, A., and Tomizawa, K. (2006) Efficient and stable transformation of *Lactuca sativa* L. cv. Cisco (lettuce) plastids, *Transgenic Res* **15**, 205–217.
13. Liu, C. W., Lin, C. C., Chen, J. J., and Tseng, M. J. (2007) Stable chloroplast transformation in cabbage (*Brassica oleracea* L. var. *capitata* L.) by particle bombardment, *Plant Cell Rep* **26**, 1733–1744.
14. Lutz, K. A., Azhagiri, A. K., Tungsuchat-Huang, T., and Maliga, P. (2007) A guide to choosing vectors for transformation of the plastid genome of higher plants, *Plant Physiol* **145**, 1201–1210.
15. Sinagawa-Garcia, S. R., Tungsuchat-Huang, T., Paredes-Lopez, O., and Maliga, P. (2009) Next generation synthetic vectors for transformation of the plastid genome of higher plants, *Plant Mol Biol* **70**, 487–498.
16. Zhou, F., Karcher, D., and Bock, R. (2007) Identification of a plastid intercistronic expression element (IEE) facilitating the expression of translatable monocistronic mRNAs from operons, *Plant J* **52**, 961–972.
17. Golds, T., Maliga, P., and Koop, H. U. (1993) Stable plastid transformation in PEG-treated protoplasts of *Nicotiana tabacum*, *Biotechnology* **11**, 95–97.
18. O'Neill, C., Horvath, G. V., Horvath, E., Dix, P. J., and Medgyesy, P. (1993) Chloroplast transformation in plants: polyethylene glycol (PEG) treatment of protoplasts is an alternative to biolistic delivery systems, *Plant J* **3**, 729–738.
19. Carrer, H., Hockenberry, T. N., Svab, Z., and Maliga, P. (1993) Kanamycin resistance as a selectable marker for plastid transformation in tobacco, *Mol Gen Genet* **241**, 49–56.
20. Huang, F. C., Klaus, S. M. J., Herz, S., Zuo, Z., Koop, H. U., and Golds, T. J. (2002) Efficient plastid transformation in tobacco using the *aphA-6* gene and kanamycin selection, *Mol Genet Genomics* **268**, 19–27.
21. Lutz, K., Corneille, S., Azhagiri, A. K., Svab, Z., and Maliga, P. (2004) A novel approach to plastid transformation utilizes the phiC31 phage integrase, *Plant J* **37**, 906–913.
22. Barone, P., Zhang, X. H., and Widholm, J. M. (2009) Tobacco plastid transformation using the feedback-insensitive anthranilate synthase [alpha]-subunit of tobacco (ASA2) as a new selectable marker, *J Exp Bot* **60**, 3195–3202.
23. Langbecker, C. L., Ye, G. N., Broyles, D. L., Duggan, L. L., Xu, C. W., Hajdukiewicz, P. T., Armstrong, C. L., and Staub, J. M. (2004)

High-frequency transformation of undeveloped plastids in tobacco suspension cells, *Plant Physiol* **135**, 39–46.

24. Bock, R., Kössel, H., and Maliga, P. (1994) Introduction of a heterologous editing site into the tobacco plastid genome: the lack of RNA editing leads to a mutant phenotype, *EMBO J* **13**, 4623–4628.
25. Whitney, S. M., and Andrews, T. J. (2001) Plastome-encoded bacterial ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) supports photosynthesis and growth of tobacco, *Proc Natl Acad Sci USA* **98**, 14738–14743.
26. Sharwood, R. E., von Caemmerer, S., Maliga, P., and Whitney, S. M. (2008) The catalytic properties of hybrid rubisco comprising tobacco small and sunflower large subunits mirror the kinetically equivalent source Rubiscos and can support tobacco growth, *Plant Physiol* **146**, 83–96.
27. Kanevski, I., and Maliga, P. (1994) Relocation of the plastid *rbcL* gene to the nucleus yields functional ribulose-1,5-bisphosphate carboxylase in tobacco chloroplasts, *Proc Natl Acad Sci USA* **91**, 1969–1973.
28. Allison, L. A., Simon, L. D., and Maliga, P. (1996) Deletion of *rpoB* reveals a second distinct transcription system in plastids of higher plants, *EMBO J* **15**, 2802–2809.
29. Maliga, P. (2004) Plastid transformation in higher plants, *Ann Rev Plant Biol* **55**, 289–313.
30. Carrer, H., and Maliga, P. (1995) Targeted insertion of foreign genes into the tobacco plastid genome without physical linkage to the selectable marker gene, *Biotechnology* **13**, 791–794.
31. Rumeau, D., Becuwe-Linka, N., Beyly, A., Carrier, P., Cuine, S., Genty, B., Medgyesy, P., Horvath, E., and Peltier, G. (2004) Increased zinc content in transplastomic tobacco plants expressing a polyhistidine-tagged Rubisco large subunit, *Plant Biotechnol J* **2**, 389–399.
32. Ye, G. N., Colburn, S., Xu, C. W., Hajdukiewicz, P. T. J., and Staub, J. M. (2003) Persistance of unselected transgenic DNA during a plastid transformation and segregation approach to herbicide resistance, *Plant Physiol* **133**, 402–410.
33. Lutz, K. A., and Maliga, P. (2007) Construction of marker-free transplastomic plants, *Curr Opin Biotechnol* **18**, 107–114.
34. Bock, R. (2001) Transgenic plastids in basic research and plant biotechnology, *J Mol Biol* **312**, 425–438.
35. Bock, R. (2007) Plastid biotechnology: prospects for herbicide and insect resistance, metabolic engineering and molecular farming, *Curr Opin Biotechnol* **18**, 100–106.
36. Daniell, H., Chebolu, S., Kumar, S., Singleton, M., and Falconer, R. (2005) Chloroplast-derived vaccine antigens and other therapeutic proteins, *Vaccine* **23**, 1779–1783.
37. Bock, R., and Warzecha, H. (2010) Solar-powered factories for new vaccines and antibiotics, *Trends Biotechnol* **28**(5), 246–252.
38. Leitch, I. J., Hanson, L., Lim, K. Y., Kovarik, A., Chase, M. W., Clarkson, J. J., and Leitch, A. R. (2008) The ups and downs of genome size evolution in polyploid species of *Nicotiana* (Solanaceae), *Ann Bot* **101**, 805–814.
39. Yukawa, M., Tsudzuki, T., and Sugiura, M. (2006) The chloroplast genome of *Nicotiana sylvestris* and *Nicotiana tomentosiformis*: complete sequencing confirms that the *Nicotiana sylvestris* progenitor is the maternal genome donor of *Nicotiana tabacum*, *Mol Genet Genomics* **275**, 367–373.
40. Khan, M. S., and Maliga, P. (1999) Fluorescent antibiotic resistance marker to track plastid transformation in higher plants, *Nat Biotechnol* **17**, 910–915.
41. Kittiwongwattana, C., Lutz, K. A., Clark, M., and Maliga, P. (2007) Plastid marker gene excision by the phiC31 phage site-specific recombinase, *Plant Mol Biol* **64**, 137–143.
42. Lutz, K. A., Svab, Z., and Maliga, P. (2006) Construction of marker-free transplastomic tobacco using the Cre-*loxP* site-specific recombination system, *Nat Protocols* **1**, 900–910.
43. Lutz, K. A., and Maliga, P. (2007) Transformation of the plastid genome to study RNA editing, *Methods Enzymol* **424**, 501–518.
44. Galun, E., Arze-Gonen, P., Fluhr, R., Edelman, M., and Aviv, D. (1982) Cytoplasmic hybridization in *Nicotiana*: mitochondrial DNA analysis in progenies resulting from fusion between protoplasts having different organelle constitutions, *Mol Gen Gent* **186**, 50–56.
45. Murashige, T., and Skoog, F. (1962) A revised medium for the growth and bioassay with tobacco tissue culture, *Physiol Plant* **15**, 473–497.
46. Sidorov, V., Menczel, L., and Maliga, P. (1981) Isoleucine-requiring *Nicotiana* plant deficient in threonine deaminase, *Nature* **294**, 87–88.
47. Jefferson, R. A., Kavanagh, T. A., and Bevan, M. W. (1987) GUS fusions: beta-glucuronidase as a sensitive and versatile gene fusion marker in higher plants, *EMBO J* **6**, 3901–3907.
48. Gallagher, S. R., (Ed.) (1992) *GUS protocols: using the GUS gene as a reporter of gene expression*, Academic Press, San Diego.



http://www.springer.com/978-1-61737-956-7

Plant Chromosome Engineering
Methods and Protocols
Birchler, J.A. (Ed.)
2011, XI, 329 p., Hardcover
ISBN: 978-1-61737-956-7
A product of Humana Press