Stable Plastid Transformation in PEG-treated Protoplasts of *Nicotiana tabacum*

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Plastid engineering currently relies on DNA delivery by the biolistic process. We report here stable plastid transformation in tobacco by an alternate direct transformation protocol that is based on polyethylene glycol (PEG) treatment of leaf protoplasts in the presence of the transforming DNA. Clones with transformed plastid genomes were selected by spectinomycin resistance encoded by a mutant 16S ribosomal RNA gene. Incorporation of the transforming DNA into the plastid genome was confirmed by two unselected markers, streptomycin resistance and a novel PstI site that flank the spectinomycin resistance mutation in plasmid pZS148. Our simple and inexpensive protocol eliminates the dependence on the particle gun for chloroplast transformation and should facilitate applications of plastome engineering in crops.

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To date, stable transformation of plastids in both *Chlamydomonas*, a unicellular alga[1,2] and tobacco, a higher plant[3,4] relies on biolistic DNA delivery through the organelle's double membrane. Mechanical introduction of DNA into *Chlamydomonas* chloroplasts was also achieved by vigorously agitating cell wall-deficient cells in the presence of glass beads and DNA solution[5]. Additional ways to introduce DNA into plastids of higher plants were attempted, including *Agrobacterium*-mediated transformation[6,7], polyethyleneglycol (PEG) treatment[8] and opening temporary holes by a UV laser microbeam[9]. So far there is no definitive report on stable transformation of plastids using these protocols. Previously, we have demonstrated transient expression of a chimeric *uidA* gene (encoding bacterial β-glucuronidase) in isolated plastids after PEG-mediated DNA uptake into protoplasts of *Nicotiana plumbaginifolia*[8]. We report here that PEG can also be used to achieve stable transformation of the plastid genome of a different *Nicotiana* species (*N. tabacum*). Transformation of plastids in leaf protoplasts was carried out with plasmid pZS148 DNA, cloned from the SPC2 mutant in *N. tabacum*[12]. Plasmid pZS148 carries a 16S rRNA gene with a spectinomycin resistant mutation flanked by two additional markers, a streptomycin resistance mutation that is 5′ in the 16S rRNA gene and a novel PstI restriction site 3′, in the intergenic region. The 16S rRNA gene is located in the repeated region of the plastid genome and is therefore present in two copies. Transformation and selection for spectinomycin resistance results in the replacement of one of the sensitive 16S rRNA gene copies by two homologous recombination events, followed by "correction" of the wild-type 16S rRNA gene copy in the second repeat[3-5]. Suitability of the pZS148 spectinomycin resistance marker to select stable plastid transformants has been proven by the biolistic process[3].

Results

Treatment of protoplasts with DNA. Recovery of transplastomic cell lines was attempted after incubation of leaf protoplasts with 12% PEG (Mol. Wt. 6000) in the presence of transforming DNA, a treatment that was suitable for obtaining transient gene expression in plastids[8]. In addition, we investigated the effect of increasing the PEG concentration to 25% on plastid transformation efficiency. Following incubation with PEG, we also used an additional heatshock treatment (50°C, 4 or 10 min) since exposure of PEG-treated protoplasts to higher temperatures was shown to increase the efficiency of nuclear genome transformation[12]. DNA treatment was followed by selection for spectinomycin resistance, a marker carried by plasmid pZS148. The treatments and the results of spectinomycin selection are summarized in Table 1.

Protoplast culture and selection for spectinomycin resistance. Some of the treatments used to introduce plasmid DNA, i.e. 25% PEG and heatshock of 50°C, are severe and significantly reduce protoplast viability. Recovery of colonies from such treatments was possible only if untreated "feeder" protoplasts were also included in the alginate layer (data not shown). The alginate layer was later dissolved, the microcalli washed and selection continued with the microcalli plated on the surface of solidified selection medium containing 0.5 g/l spectinomycin. Protoplast-derived wild-type calli grow slowly and are white, whereas resistant calli grow faster and turn green on this selec-

TABLE 1. Spectinomycin resistant clones after PEG-induced uptake of plasmid pZS148.

DNA	PEG (%)[1]	Heatshock (min)[2]	Number of treated protoplasts	Estimated PE (%)[3]	Number of feeder cells	Number of colonies
Experiment 1						
(a) –	0	0	$1.0.10^5$	80	0	0
(b) +	12	0	$2.5.10^5$	50	0	1
(c) +	25	0	$2.5.10^5$	20	$2.5.10^5$	4
(d) +	12	4	$2.5.10^5$	< 10	$2.5.10^5$	0
(e) +	12	10	$2.5.10^5$	< 10	$2.5.10^5$	0
Experiment 2						
(f) –	0	0	$6.0.10^5$	80	0	3
(g) +	12	0	$6.0.10^5$	50	0	4
(h) +	25	0	$6.0.10^5$	20	$6.0.10^5$	0

[1]Preincubation of protoplasts with DNA for 10 minutes was followed by a 10-minute PEG treatment. [2]Heat-shock treatment (50°C) was applied, where applicable, after incubation with PEG. [3]PE: estimated plating efficiency, given as proportion of the protoplasts developing into macroscopically visible colonies.

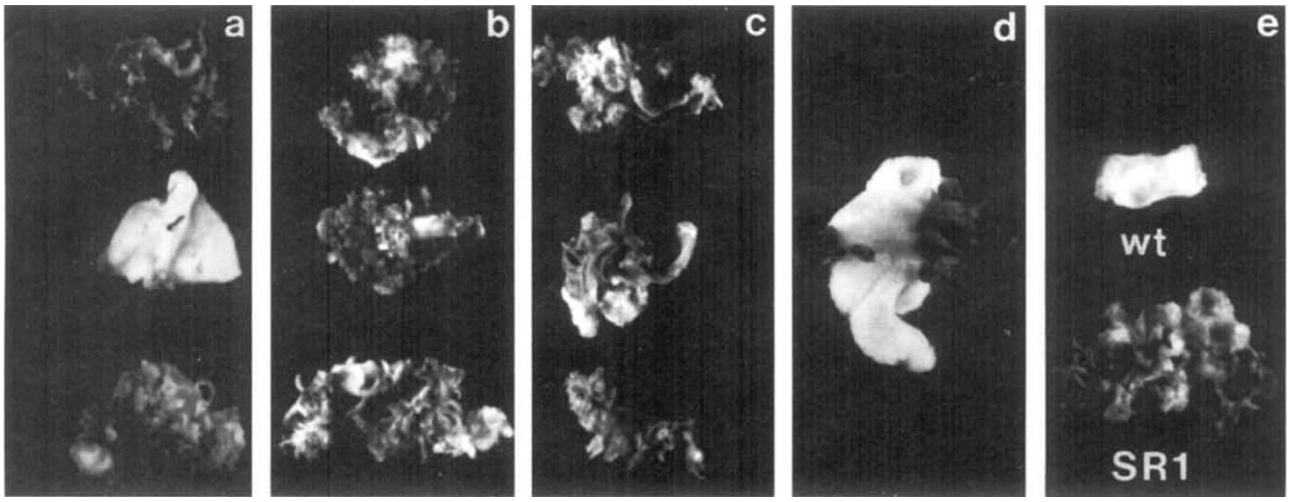

FIGURE 1. Leaf segment assay for streptomycin resistance. (a–c) Panel (a) shows one leaf section each from three plants regenerated from the same resistant clone. Note segregation for resistance in panel (a), and uniform streptomycin resistance in (b,c). (d) Resistant and sensitive sectors within one leaf segment. (e) Assay with sensitive wild-type recipient (wt) and streptomycin resistant mutant SR1. Leaf segments were cultured for 11 weeks on a medium containing 1 g/l of streptomycin sulphate.

tive medium[10]. The culture and selection procedure is outlined in the Experimental Protocol. After DNA treatment, resistant calli were found in both independent experiments (Table 1). The five green calli in Experiment 1, from treatments (b) and (c), were shown to be due to plastid transformation (see below). Since the five calli were recovered in four selection dishes, these five clones represent at least four independent transformation events. From the five colonies a total of 25 plants were regenerated. In Experiment 2, the four clones in sample (g) have not been analyzed to date. In the absence of DNA treatment, resistant clones were obtained only in Experiment 2. Therefore, the three resistant clones in sample (f) should be the result of spontaneous mutation, as reported earlier[3,4] (see also Fig. 2a, lane 5).

Unselected plastid markers in the spectinomycin resistant plants. Spectinomycin resistance of the regenerated plants could be due to spontaneous mutation or incorporation of transforming DNA into the plastid genome. In order to facilitate positive identification of transplastomic lines, two additional markers are included in plasmid pZS148: a streptomycin resistance mutation in the 16S rRNA gene derived from the SPC2 mutant[3,10] and a PstI restriction site created *in vitro*. Streptomycin resistance was confirmed by growing leaf segments in the presence of 1.0 g/l of streptomycin sulphate (Fig. 1). Resistance to streptomycin was indicated by the formation of callus and green shoots in leaf segments from each of the 25 plants regenerated from the five spectinomycin resistant clones in Experiment 1 (Table 1). Two different types of responses were observed. Whereas most (about 85%) of the segments remained green and produced green callus and shoots all over their surfaces (Fig. 1b, c), a few leaf segments turned white, and green regenerants were only formed locally (Fig. 1a, d). Formation of green and white sectors indicates that these leaves were chimeric with respect to streptomycin resistance[3]. Incorporation of the transgenic PstI site from donor plasmid pZS148 was tested by probing total cellular DNA from the spectinomycin resistant lines. The 4.4 kb and 1.8 kb bands, expected only for transplastomic plants, were present in all of the DNA samples (Fig. 2a, lanes 6–10). One plant (lane 8), was still heteroplasmic at the time of probing, since it carried the wild type 6.2 kb as well as the two transgenic ptDNA fragments.

Discussion

Introduction of DNA through the plastid's double membrane, a bottleneck of organelle transformation, has been solved by DNA delivery on the surface of mechanically propelled tungsten or gold particles[1–4]. Transformation, however, was dependent on the availability of a biolistic gun. PEG treatment, a simple, inexpensive protocol, was therefore tried since it is extensively used to introduce DNA into the nuclear genome[13]. Stable transformation of the plastid genome reported here indicates that PEG treatment (Mol. Wt. 6000, 12%) is also suitable for obtaining plastid transformation. Use of a higher PEG concentration (25%) resulted in more resistant colonies in at least one sample (Table 1, c), but no resistant clones were obtained after the same treatment in the second experiment (Table 1, h). No resistant colonies were found after an additional heat shock application either, a treatment that would normally increase the frequency of nuclear gene transformation[12]. The experiments reported here establish the feasibility of plastid transformation by PEG treatment. Further experiments are necessary, however, to optimize the various treatments and obtain reliable data on transformation efficiency. This simple, inexpensive direct plastid transformation protocol should facilitate applications of plastome engineering to crops.

Experimental Protocol

Protoplast isolation and transformation. Growth of *in vitro* shoot cultures of *Nicotiana tabacum* L. cv. Petit Havanna and protoplast isolation followed the procedures described earlier[8,14]. Plasmid pZS148 DNA was isolated from *E. coli* host strain DH5 α and purified using anion-exchange resin columns (Qiagen,Tip-500), following the manufacturer's recommendations. Protoplasts were suspended in 0.4 M mannitol at a density of 2.0×10^6/ml and 125 μl of the protoplast suspension mixed with 85 μl of 0.4 M mannitol and 40 μl of DNA (400 μg/ml plasmid DNA and 1.0 mg/ml sonicated calf thymus carrier DNA[14], in sterile water) and incubated for 10 min at room temperature. PEG solution (250 μl) (Mol. Wt. 6000; Sigma, St. Louis), dissolved at twice the required concentration in 0.4 M mannitol, 30 mM $MgCl_2$, was added, samples were mixed by gentle agitation, incubated for another 10 min, followed (where applicable) with a heat-shock (50°C) of varying duration.

Protoplast culture and selection. Protoplast culture media for *Nicotiana*[15] (PCN) are based on the macro- and micronutrients of the B5 medium[16], additionally supplemented with 200 mg/l $CaCl_2$, 200 mg/l myoinositol, 1.0 mg/l thiamine-HCl, 2.0 mg/l Ca-panthotenate, 2.0 mg/l nicotinic acid, 2.0 mg/l pyridoxine-HCl, 0.02 mg/l biotin, 1.0 mg/l BAP, 0.1 mg/l NAA, 1% Polybuffer74 (LKB-Pharmacia, Uppsala) and are adjusted to a pH of 5.7; PCN1: 0.5mg/l 2,4 D, 0.4 M glucose, 550 mOsm, PCN2: 0.5 mg/l 2,4-D, 0.2 M glucose, 350 mOsm, PCN3: 0.05 mg/l 2,4-D, 0.09 M sucrose (2%), 150 mOsm. The media contained 0.5 g/l spectinomycin throughout. The incubation solution (0.5 ml), containing protoplasts, DNA, PEG, $MgCl_2$ and mannitol was carefully mixed with 2.5 ml of PCN1[14], containing the appropriate amount of spectinomycin to

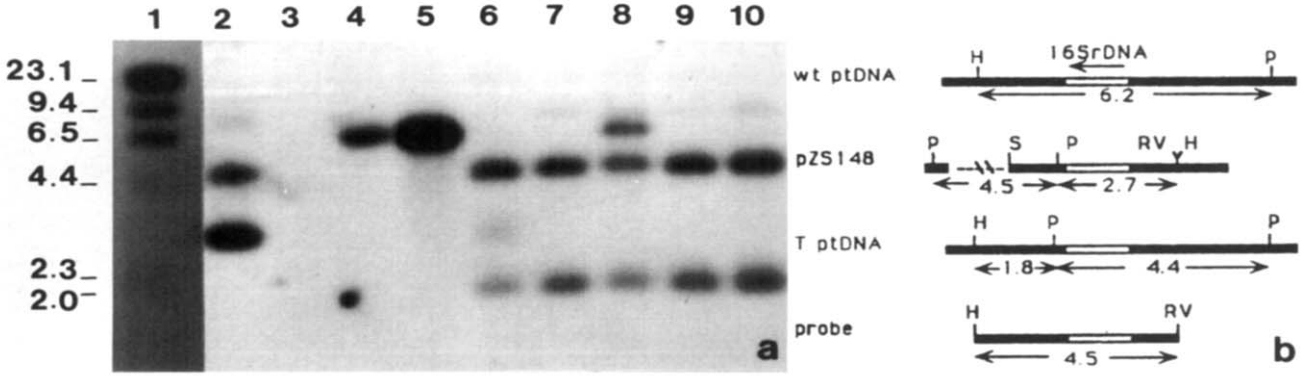

FIGURE 2. Southern analysis to confirm incorporation of the novel PstI restriction-site marker from plasmid pZS148 into the plastid genome. (a) Total cellular DNA was digested with HindIII and PstI restriction enzymes and probed with a 4.5 kb HindII-EcoRV fragment. Lane 1: Lambda DNA digested with HindIII, lane 2: HindIII-PstI digest of plasmid pZS148, lane 4: wild type *N. tabacum*, lane 5: spectinomycin resistant clone derived from a control treatment without plasmid DNA (Table 1, sample f) representing a spontaneous mutation, lanes 6–10: plants regenerated from 5 different clones (Table 1, samples b,c). The 6.2 kb wild-type fragment is cut into 4.4 kb and 1.8 kb fragments if the transgenic PstI site is present. Transgenic and wild-type fragments in lane 8 indicate heteroplasmy. (b) Physical map of the probed region of the wild-type (wt) and transgenic plastid DNA (T ptDNA), plasmid pZS148, and the relative position of the probe are shown. Position of the 16SrDNA gene encoding the 16S ribosomal RNA is highlighted. Restriction endonuclease recognition sites: RV, EcoRV; H, HindIII; P, PstI; S, SacI.

give the required final concentration (0.5 g/l), and with 3 ml of 1.6% sodium alginate (Sigma, St. Louis) dissolved in 0.4 M mannitol. This mixture (6 ml) was equally distributed into five Petri dishes of 5 cm diameter, where solidification occurred within 30 to 60 min. In all transformation experiments involving 25% PEG and/or a heat-shock treatment (Table 1, samples c, d, e, h) untreated protoplasts were included in the alginate layer to compensate for protoplasts not surviving the transformation procedure. Proptoplast culture in alginate was according to Damm and Willmitzer[17]. Culture conditions were 25 ± 1 °C, about 1000lx generated from daylight fluorescent lamps, 16 h light per day. After one week, the alginate slabs were transferred to 9.4 cm Petri dishes and 8 ml of fresh liquid PCN1 medium was added. Half of the liquid medium was replaced with PCN2 medium after a further week of culture to reduce the osmotic value to 450 mOsm. One week later, the alginate slabs were transferred to a centrifuge tube and 10 ml of citric acid (40 mM, and adjusted to 450 mOsm with mannitol) were added to dissolve the gel. Colonies were sedimented by centrifugation (50 g, 5 min), and, following removal of the supernatant, resuspended in PCN3 medium. The calli were then sedimented by centrifugation, resuspended in 0.5 to 1 ml of liquid PCN3 medium and spread onto the surface of 20 ml agar solidified (0.7% Bacto Agar, Difco, Detroit) PCN3 medium. Cultures were incubated without changing the culture medium for five to seven weeks when the resistant green calli became visible.

Plant regeneration and streptomycin assay. Resistant colonies were regenerated to plants on MS medium[18], containing 1.0 mg/l BAP, 0.1 mg/l NAA[14] and 0.5 g/l spectinomycin. Streptomycin resistance assays were performed by excising 1 cm^2 square leaf segments and plating onto agar solidified MS medium[18], containing 0.1 mg/l NAA, 1.0 mg/l BAP and 1.0 g/l streptomycin sulphate[19]. Leaf segments were transferred to fresh medium of the same composition after four weeks and evaluated after a total selection period of 8–12 weeks (Fig. 1).

DNA gel blots. Total cellular DNA was isolated from leaves of *in vitro*-grown plants[20]. DNA samples (2 μg) were digested with HindIII and PstI (Boehringer Mannheim) and electrophoresed (14 h, 10 V) on a 1.0% agarose gel (electrophoresis grade, BRL, Gaithersburg) in TAE running buffer (40 mM Tris-acetate, lmM EDTA pH 8.0). DNA was transferred to a nylon membrane (Hybond-N, Amersham, Braunschweig) using 20 × SSC as a transfer buffer (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate) and bound by U.V. cross-linking. Samples were probed with a 4.5 kb HindIII-EcoRV fragment labeled by random priming according to the manufacturer's recommendations (Boehringer, Mannheim). Hybridization (20 h, 65°C) was performed in 5 × SSC, 5 × Denhardt's solution (100 × Denhardt's solution contains 2% each of bovine serum albumin, ficoll and polyvinylpyrrolidone), 2.5 mM EDTA, 0.6% SDS, 5% dextran sulphate, 25 mM sodium phosphate, pH 7.3, and 100 μg/ml sheared and denatured herring sperm DNA. Washing was for 30 min in 2 × SSC + 0.1% SDS, 1 × SSC + 0.1% SDS and 0.5 × SSC + 0.1% SDS, respectively.

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