Plant Cell Reports (1998) 18: 20–24

S. R. Sikdar · G. Serino · S. Chaudhuri · P. Maliga

Plastid transformation in *Arabidopsis thaliana*

Received: 13 February 1998 / Revision received: 24 April 1998 / Accepted: 5 June 1998

Abstract Plastid transformation is reported in *Arabidopsis thaliana* following biolistic delivery of transforming DNA into leaf cells. Transforming plasmid pGS31A carries a spectinomycin resistance (*aadA*) gene flanked by plastid DNA sequences to target its insertion between *trnV* and the *rps12/7* operon. Integration of *aadA* by two homologous recombination events via the flanking ptDNA sequences and selective amplification of the transplastomes on spectinomycin medium yielded resistant cell lines and regenerated plants in which the plastid genome copies have been uniformly altered. The efficiency of plastid transformation was low: 2 in 201 bombarded leaf samples. None of the 98 plants regenerated from the two lines were fertile.

Key words *aadA* gene · *Arabidopsis thaliana* · Biolistic DNA delivery · Plastid transformation · Spectinomycin resistance

Introduction

The plastid genome is an attractive target for engineering, since proteins in plastids may be expressed at a high level, genes for pathways may be encoded in polycistronic mRNAs, the transgenes are uniformly expressed due to targeted insertion into the plastid genome and the transgenes do not spread via pollen (Maliga 1993; Maliga et al. 1993). A good example for application is expression of the *Bacillus thuringiensis cryIA(c)* gene in tobacco plastids from which the insecticidal protoxin accumulates to 3–5% of the soluble protein in leaves (McBride et al. 1995).

The plastid genome of higher plants is a circular double-stranded DNA molecule of 120–160 kb which may be present in 1,900–50,000 copies per leaf cell (Bendich 1987; Palmer 1991). Stable transformation of the tobacco plastid genome has been achieved through the following steps: (1) introduction of transforming DNA, encoding antibiotic resistance, by the biolistic process (Svab et al. 1990a; Svab and Maliga 1993) or polyethylene glycol treatment (Golds et al. 1993; O'Neill et al. 1993; Koop et al. 1996), (2) integration of the transforming DNA by two homologous recombination events, and (3) selective elimination of the wild-type genome copies during repeated cell divisions on a selective medium. Spectinomycin resistance has been used as a selective marker encoded either in mutant plastid 16S ribosomal RNA genes (Svab et al. 1990a; Staub and Maliga 1992; Golds et al. 1993; O'Neill et al. 1993), or conferred by the expression of an engineered bacterial *aadA* gene (Svab and Maliga 1993). Vectors which utilize *aadA* as a selectable marker gene and target the insertion of chimeric genes into the repeated region of the tobacco plastid genome are available (Zoubenko et al. 1994). Selection of plastid transformants by kanamycin resistance, based on the expression of neomycin phophotransferase (*kan* gene), is also feasible (Carrer et al. 1993; Carrer and Maliga 1995).

Plastid transformation in higher plants has so far been reported only for tobacco (reviewed in Maliga 1993; Maliga et al. 1993). We report here an extension of this technology to *Arabidopsis thaliana*, a model species for plant research (Meyerowitz and Sommerville 1994).

Materials and methods

Construction of vector pGS31A

The *Arabidopsis* plastid transformation vector pGS31A is a pBluescript KS(+) phagemid vector (Stratagene) derivative. The vector

Communicated by E. D. Earle

S. R. Sikdar[1] · G. Serino · S. Chaudhuri[2] · Pal Maliga (✉)
Waksman Institute, Rutgers, The State University of New Jersey
190 Frelinghuysen Road, Piscataway, NJ 08854-8020, USA
Fax: +1-732-4455735
e-mail: maliga@waksman.rutgers.edu

Present addresses:
[1] Bose Institute, Plant Molecular and Cellular Genetics Section, Centenary Building, P1/12, C. I. T. Scheme VIII-M, Calcutta, 700-054, India
[2] Calgene, Inc., 1920 Fifth Street, Davis, CA 95616, USA

carries a 2-kb *Hin*dIII-*Eco*RI *Arabidopsis* ptDNA fragment containing the 5′-end of the 16S rRNA gene, *trnV* and part of the *rps12/7* operon. During construction of the pGS31A plasmid, the *Hin*dIII site has been removed. The vector carries a selectable spectinomycin resistance (*aadA*) gene, which was excised as a *Sac*I/*Bsp*HI fragment from plasmid pZS179 (Svab and Maliga 1993) and cloned into the *Hin*cII site of the cloned ptDNA fragment. In vector pGS31A, the *aadA* coding region is transcribed from a synthetic promoter consisting of the promoter of the tobacco rRNA operon fused with a synthetic ribosome binding site (P*rrn*). The *aadA* mRNA is stabilized by transcriptionally fusing sequences downstream of the coding region with the 3′-untranslated region of the tobacco plastid *psbA* gene (T*psbA*). The sequence of plastid transformation vector pGS31A has been deposited in GenBank under accession number AF061065.

Tissue culture media

The tissue culture protocols were adopted from Marton and Browse (1991) and Czako et al. (1993). The *Arabidopsis* tissue culture media (ARM) are derivatives of the Murashige and Skoog (1962) MS medium. ARM medium: MS salts, 3% sucrose, 0.8% TC agar, 2 ml/l of the vitamin solution (100 mg myo-inositol, 5 mg vitamin B1, 0.5 mg vitamin B6, 0.5 mg nicotinic acid, 1 mg glycine, and 0.05 mg biotin per milliliter). ARMI medium: ARM medium containing 3 mg indolacetic acid, 0.15 mg 2,4-dichlorophenoxyactetic acid, 0.6 mg benzyladenine and 0.3 mg isopentenyladenine per liter. ARMIIr medium: ARM medium supplemented with 0.2 mg/l naphthaleneacetic acid and 0.4 mg/l isoperntenyladenine. *Arabidopsis* shoot induction (ASI-N1B1) medium: ARM medium supplemented with 1 mg/l naphthaleneacetic acid and 1 mg/l benzyladenine. The *Arabidopsis* shoots were rooted on ARM medium. *Arabidopsis* seed culture (ARM5) medium: ARM medium supplemented with 5% sucrose. The stocks of plant hormones were filter sterilized, and added to media cooled to 45 °C after autoclaving.

Selective media contained 500 mg/l spectinomycin HCl and/or streptomycin sulfate. The antibiotics (filter sterilized) were added to media cooled to 45 °C after autoclaving.

Cultivation of *Arabidopsis* plants in sterile culture

As the recipient for transformation, we have used the *Arabidopsis* ecotype RLD, which was reported to regenerate readily in culture (Marton and Browse 1991). For surface sterilization, seeds (25 mg) were treated with 1 ml of commercial bleach (5.25% sodium hypochlorite) in an Eppendorf tube for 5–7 min with occasional mixing (Vortex). The seeds along with the bleach were poured into a 15-ml conical centrifuge tube containing 10 ml 90% ethanol and incubated for 5–7 min. The ethanol-bleach mix was decanted, and the seeds were washed four times with 10 ml autoclaved deionized water and finally suspended in sterile water (~150 seeds/ml). Two milliliters seed suspension was poured in each 10-cm diameter (10 mm high) petri dish containing 50 ml ARM5 medium. The seeds were spread evenly by swirling the suspension. The water was then removed from the plates by pipetting. The seeds germinated after a 10- to 15-day incubation at 24 °C. The plates were illuminated for 8 h using cool-white fluorescent tubes (2,000 lux).

To grow plants with larger leaves, seedlings were individually transferred to ARM5 plates (ten plants per 10-cm petri dish) and were illuminated for 8 h with cool-white fluorescent bulbs (2,000 lux; 21 °C day and 18 °C night temperature). The thick, dark green leaves, 1–2 cm in size, were harvested for bombardment after 5–6 weeks.

Transformation and selection of spectinomycin-resistant lines

Leaves (15–30 mm in length) for plastid transformation were harvested from aseptically grown plants. To cover an area 4–5 cm in diameter, 12–18 leaves were placed on agar-solidified ARMI medium. The pGS31A vector DNA was introduced into leaf chloroplasts by the biolistic process on the surface of microscopic (1 μm) tungsten particles using a helium-driven PDS1000/He biolistic gun (Maliga 1995). Fresh leaves were bombarded at 450 psi (target placed at 9 cm from rupture disk; position no. 3 from top in the biolistic gun). Leaves, cultured for 4 days on ARMI medium were bombarded at 1,100 psi (target placed at 12 cm from rupture disk; position no. 4 from top in the biolistic gun).

Leaf bombardment was performed on ARMI medium. Following bombardment, the leaves were incubated for two additional days on ARMI medium. After this, the leaves were stamped with a stack of ten razor blades to create parallel incisions 1 mm apart. The stamped leaves were transferred onto the same medium (ARMI) containing 500 mg/l spectinomycin. After 8–10 days, the leaf strips were transferred onto selective ARMIIr medium containing 500 mg/l spectinomycin for the selection of spectinomycin-resistant clones. On the selective ARMIIr medium, green, resistant calli were obtained. Plants from the green calli were regenerated on ASI-N1B1 medium containing spectinomycin (500 mg/l) or spectinomycin and streptomycin (500 mg/l each). The shoots were transferred to ARM medium for rooting and shoot elongation. The plants were then transferred to soil.

DNA gel blot analysis of total cellular DNA

Total cellular DNA was isolated according to Mettler (1987). Restriction-enzyme-digested DNA was electrophoresed in 0.7% agarose gels and transferred to nylon membrane (Amersham) using the PosiBlot transfer apparatus (Stratagene). Blots were probed by using Rapid Hybridization Buffer (Amersham) with ^{32}P-labeled probes generated by random priming (Boehringer-Mannheim).

Results

Plastid vector pGS31A

In tobacco, homologous targeting of chimeric genes between the *trnV* and *rps12/7* genes has yielded stable transformants at a high efficiency (Zoubenko et al. 1994). The tobacco and *Arabidopsis* plastid genomes are colinear (Palmer et al. 1994). Therefore, an *Arabidopsis* ptDNA fragment containing the *trnV* and *rps12/7* intergenic region has been cloned and sequenced. Since this region in the two species differs in several small deletions and insertions (not shown), an *Arabidopsis* vector was constructed by ligating a selectable spectinomycin resistance (*aadA*) gene in the *trnV-rps12/7* intergenic region (Fig. 1A; GenBank accession number AF061065). In transformation vector pGS31A, *aadA* is flanked by approximately 1-kb plastid targeting sequences on both sides.

Transformation and selection of spectinomycin-resistant cell lines

Leaves for plastid transformation were harvested from aseptically grown plants and were ~15–30 mm long. Twelve to 18 leaves were placed on agar-solidified ARMI medium to cover an area 4–5 cm in diameter. The pGS31A vector DNA was introduced into leaf chloroplasts by the biolistic process, on the surface of microscopic (1 μm) tungsten particles using a helium-driven PDS1000 biolistic gun. After 2 days, the leaves were stamped with a stack

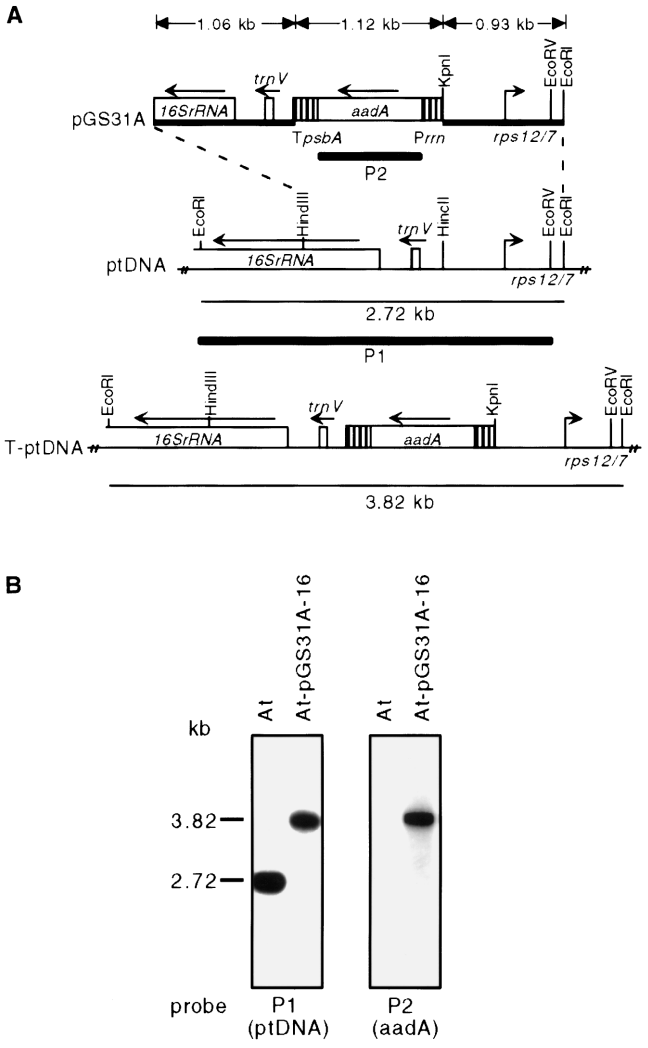

Fig. 1A, B Integration of *aadA* into the *Arabidopsis* plastid genome (ptDNA) after transformation with plasmid pGS31A. **A** Map of the transformation vector pGS31A, the ptDNA region containing the integrated spectinomycin resistance (*aadA*) gene (T-ptDNA) and the cognate region of the wild-type ptDNA. *16SrRNA*, *rps12/7*, and *trnV* are plastid genes (Shinozaki et al. 1986). P1 and P2 (*heavy lines*) mark regions used as targeting ptDNA and *aadA* probes. **B** Southern blot hybridization of *Eco*RI-digested DNA confirms integration of *aadA* in the plastid genome. The *P1* targeting sequences hybridize to a 2.72-kb fragment in the wild-type (*At*) plants and to a larger, 3.82-kb fragment in the transplastomic line (*At-pGS31A-16*). Note absence of the wild-type fragment in transplastomic line. The *aadA* probe, *P2*, hybridizes only to the larger transplastomic fragment

of razor blades to create a series of parallel incisions 1 mm apart. We have found that mechanical wounding was essential to induce uniform callus formation in the leaf blades. The stamped leaves were transferred onto the same medium (ARMI) containing spectinomycin (500 μg/ml; Svab and Maliga 1993) to facilitate preferential replication of plastids containing transformed ptDNA copies. The ARMI medium induces division of the leaf cells and formation of colorless, embryogenic callus. After 7–10 days selection on ARMI medium, spectinomycin selection was continued on the ARMIIr medium which normally induces

Table 1 Recovery of spectinomcyin resistant (*Spc*^{r}) lines after bombardment of *Arabidopsis thaliana* with plasmid pGS31A. The control plates were not bombarded (*psi* pounds per square inch, the value of the rupture disk)

DNA	Number of plates	psi	Number of Spc^r lines	Shoot regeneration	Plastids transformed
Control (none)	100		1	0	
pGS31A	40	1,100	8	6	1
pSG31A	151	450	11	8	1

greening. Since spectinomycin prevents greening of wild-type cells, only spectinomcyin-resistant cells formed green calli. Visible green cell clusters appeared within 21–70 days on the selective ARMIIr medium (Fig. 2a).

In 201 bombarded samples, 19 spectinomcyin-resistant lines were obtained (Table 1). Plant regeneration was attempted in 14 spectinomycin-resistant lines, and succeeded for 10 of them. Shoots from the green calli regenerated on the ASI-N1B1 medium, and were rooted on ARM medium.

While the 164 plants all flowered, none of them set seed after selfing, or after fertilization with pollen from wild-type plants. Included among these were 98 plants regenerated from the two lines in which spectinomcyin resistance was due to plastid transformation (Fig. 2b), and 66 plants regenerated from 8 additional spectinomcyin-resistant lines which were either spontaneous mutants or incorporated the *aadA* gene in their nucleus.

Southern probing to identify transplastomic lines

Plastid transformants were identified by Southern analysis, after digestion with the *Eco*RI restriction enzyme. When using the targeting ptDNA as a probe, in lines At-pGS31A-2 and At-pGS31A-16, the 3.82-kb transgenic fragment was the only fragment detected, indicating that the wild-type ptDNA copies have been diluted out during cell divisions on the selective medium. The same transgenic fragment also hybridized with the *aadA* probe (Fig. 1B). The remaining 17 spectinomycin-resistant lines had a wild-type fragment when the blots were hybridized with the targeting ptDNA probe. Therefore, spectinomycin resistance of the lines could have been due to integration of *aadA* into the nucleus (Svab et al. 1990b) or spontaneous mutation (Fromm et al. 1987; Svab and Maliga 1991).

Discussion

We report here plastid transformation in the model species *A. thaliana*. We have found that a chimeric *aadA* gene, when inserted in the *Arabidopsis* ptDNA targeting cassette, was suitable to recover plastid transformants following biolistic delivery of the transforming DNA. However, the

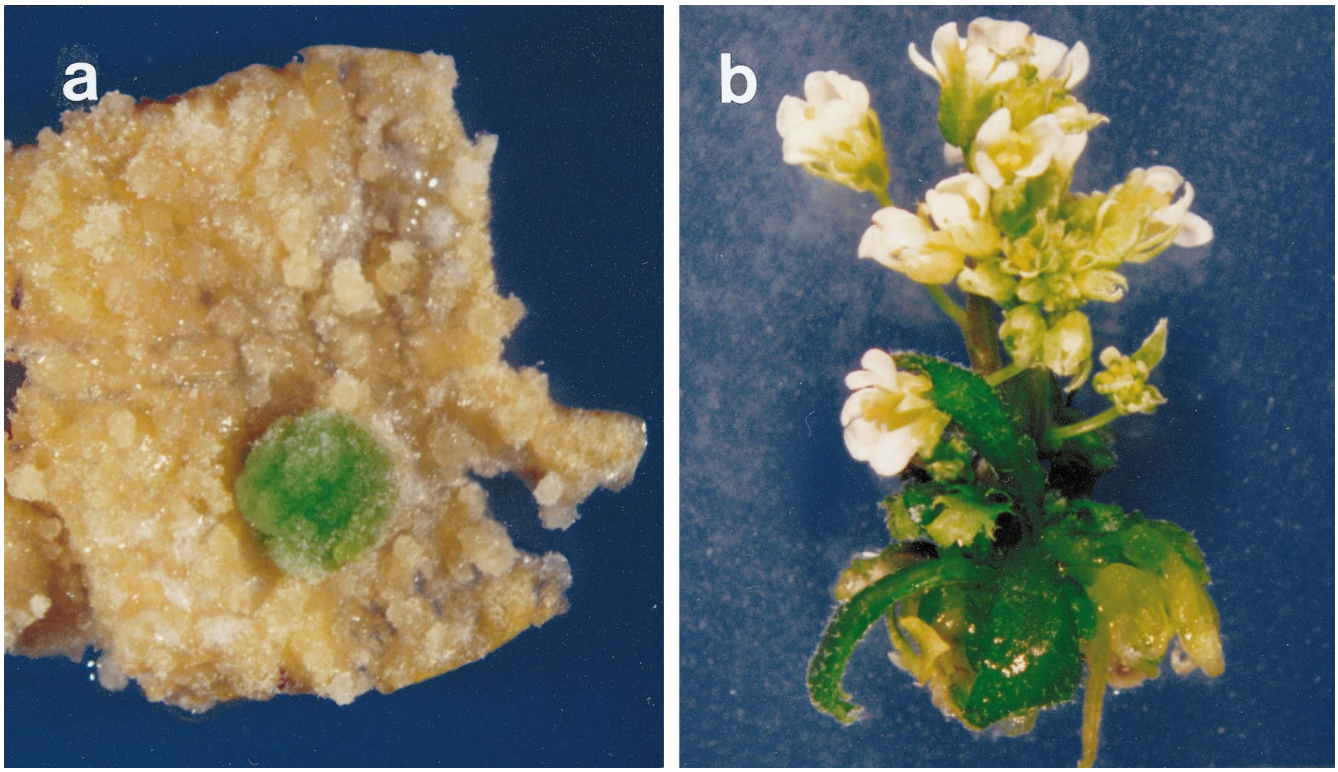

Fig. 2a, b *Arabidopsis* transplastomic lines. **a** A green, spectinomycin-resistant callus is apparent on the background of white, wild-type leaf callus. **b** A flowering At-pGS31A-16 plant

frequency of plastid transformation in *Arabidopsis* was significantly lower than in tobacco. In *Arabidopsis*, we recovered about 1 transplastomic line in 100 bombarded samples, whereas in tobacco, on average, 1 plastid transformation event was obtained in each sample (Svab and Maliga 1993).

There may be multiple reasons for the relatively low transformation efficiency in *Arabidopsis*. One reason could be a relatively inefficient homologous recombination system in *Arabidopsis* chloroplasts. Another could be the relatively short plastid targeting sequence (1 kb) flanking the *aadA* gene in the *Arabidopsis* vector pGS31A. In the tobacco vector pZS197, *aadA* is flanked by 1.56 kb and 1.29 kb of ptDNA. Transformation with vector pZS197 yields about one transplastomic line per bombardment (Svab and Maliga 1993). In plasmid pRB15, also a tobacco vector, *aadA* is flanked by larger targeting segments, 1.56 kb and 3.6 kb of ptDNA. Transformation with vector pRB15 yields about five transplastomic lines per bombardment (Bock and Maliga 1995). Therefore, the efficiency of plastid transformation in *Arabidopsis* may be significantly improved by increasing the size of the targeting ptDNA fragment.

Most of the plants regenerated from tobacco leaves are fertile. Therefore, we were surprised to find that none of the 164 regenerated *Arabidopsis* plants set seed. Lack of fertility is likely due to extensive polyploidy of the leaf tissue (Galbright et al. 1991; Melaragno et al. 1993). In contrast to the aerial parts of the plant, *Arabidopsis* roots are diploid, remain diploid in culture, and readily regenerate fertile plants (Valvekens et al. 1988; Marton and Browse 1991; Czako et al. 1993). Our tissue culture protocol, when applied for *Agrobacterium*-mediated transformation of the nucleus in roots and employing spectinomycin selection (Hajdukiewicz et al. 1994), also yielded fertile plants. Therefore, exposure of *Arabidopsis* to spectinomycin in culture is not responsible for the lack of fertility.

The initial step of extending plastid transformation to *Arabidopsis* has been achieved. Efforts have now to be focused on defining a tissue culture system which facilitates spectinomycin selection while allowing regeneration of fertile *Arabidopsis* plants.

Acknowledgements We thank Zora Svab for preparing the Southern blot shown in Fig. 1. This research was supported by the National Science Foundation Grant MCB 93-05037 to P. M.

References

Bendich AJ (1987) Why do chloroplasts and mitochondria contain so many copies of their genome? Bioessays 6:279–282

Bock R, Maliga P (1995) In vivo testing of a tobacco plastid DNA segment for guide RNA function in *psbL* editing. Molec Gen Genet 247:439–443

Carrer H, 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

Carrer H, Hockenberry TN, Svab Z, Maliga P (1993) Kanamycin resistance as a selectable marker for plastid transformation in tobacco. Mol Gen Genet 241:49–56

Czako M, Wilson J, Marton L (1993) Sustained root culture for generation and vegetative propagation of transgenic *Arabidopsis thaliana*. Plant Cell Rep 12: 603–606

Fromm H, Edelman M, Aviv D, Galun E (1987) The molecular basis of rDNA-dependent spectinomycin resistance in *Nicotiana* chloroplasts. EMBO J 6: 3233–3237

Galbright DW, Harkins KR, Knapp S (1991) Systemic endopolyploidy in *Arabidopsis thaliana*. Plant Physiol 96: 985–989

Golds T, Maliga P, Koop HU (1993) Stable plastid transformation in PEG-teated protoplasts of Nicotiana tabacum. Biotechnology 11: 95–97

Hajdukiewicz P, Svab Z, Maliga P (1994) The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant Mol Biol 25: 989–994

Koop HU, Steinmüller K, Wagner H, Rössler C, Eibl C, Sacher L (1996) Integration of foreign sequences into the tobacco plastome via PEG-mediated protoplast transformation. Planta 199: 193–201

Maliga P (1993) Towards plastid transformation in flowering plants. Trends Biotechnol 11: 101–106

Maliga P (1995) Biolistic transformation of tobacco cells with nuclear drug resistance genes. In: Maliga P, Klessig D, Cashmore A, Gruissem W, Varner J (eds) Methods in plant molecular biology – a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 37–54

Maliga P, Carrer H, Kanevski I, Staub J, Svab Z (1993) Plastid engineering in land plants: a conservative genome is open to change. Phil Trans R Soc Lond B 341: 449–454

Marton L, Browse J (1991) Facile transformation of *Arabidopsis thaliana*. Plant Cell Rep 10: 235–239

McBride KE, Svab Z, Schaaf DJ, Hogan PS, Stalker DM, Maliga P (1995) Amplification of a chimeric *Bacillus* gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. Biotechnology 13: 362–365

Melaragno JE, Mehrotra B, Coleman AW (1993) Relationship between endopolyploidy and cell size in epidermal tissue of *Arabidopsis*. Plant Cell 5: 1661–1668

Mettler IJ (1987) A simple and rapid method for minipreparation of DNA from tissue cultured plant cells. Plant Mol Biol Rep 5: 346–349

Meyerowitz EM, Somerville CR (1994) *Arabidopsis*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol Plant 15: 493–497

O'Neill C, Horvath GV, Horvath E, Dix PJ, 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

Palmer JD (1991) Plastid chromosomes: structure and evolution. In: Bogorad L, Vasil IK (eds) The molecular biology of plastids. Cell culture and somatic cell genetics of plants, vol. 7 A. Academic Press, San Diego, pp 5–53

Palmer JD, Downie SR, Nugent JM, Brandt P, Unseld M, Klein M, Brennicke A, Schuster W, Borner T (1994) Chloroplast and mitochondrial DNAs of *Arabidopsis thaliana*: conventional genomes in an unconventional plant. In: Meyerowitz EM, Somerville CR (eds) *Arabidopsis*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 37–62

Shinozaki K, Ohme M, Tanaka M, Wakasugi T, Hayashida N, Mitsubayashi T, Zaita N, Chunwongse J, Obokata J, Yamaguchi-Shinozaki K, Ohto C, Torazawa K, Meng BY, Sugita M, Deno H, Kamogashira T, Yamada K, Kusuda J, Takaiwa F, Kata A, Todoh N, Shimada H, Sugiura M (1986) The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. EMBO J 5: 2043–2049

Staub J, Maliga P (1992) Long regions of homologous DNA are incorporated into the tobacco plastid genome by transformation. Plant Cell 4: 39–45

Svab Z, Maliga P (1991) Mutation proximal to the tRNA binding region of the *Nicotiana* plastid 16S rRNA confers resistance to spectinomycin. Mol Gen Genet 228: 316–319

Svab Z, Maliga P (1993) High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. Proc Natl Acad Sci USA 90: 913–917

Svab Z, Hajdukiewicz P, Maliga P (1990a) Stable transformation of plastids in higher plants. Proc Natl Acad Sci USA 87: 8526–8530

Svab Z, Harper EC, Jones JDG, Maliga P (1990b) Aminoglycoside-3″-adenyltransferase confers resistance to spectinomycin and streptomycin in *Nicotiana tabacum*. Plant Mol Biol 14: 197–205

Valvekens D, Van Motagu M, Van Lijsebettens M (1988) *Agrobacterium tumefaciens*-mediated transformation of *Arabidopsis thaliana* root explants using kanamycin selection. Proc Natl Acad Sci USA 85: 5536–5540

Zoubenko OV, Allison LA, Svab Z, Maliga P (1994) Efficient targeting of foreign genes into the tobacco plastid genome. Nucleic Acids Res 22: 3819–3824