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Genetics

Stable transformation of plastids in higher plants

(antibiotic resistance/maternal inheritance/projectile transformation/rRNA genes)

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ABSTRACT **Stable genetic transformation of the plastid genome is reported in a higher plant, *Nicotiana tabacum*. Plastid transformation was obtained after bombardment of leaves with tungsten particles coated with pZS148 plasmid DNA. Plasmid pZS148 (9.6 kilobases) contains a 3.7-kilobase plastid DNA fragment encoding the 16S rRNA. In the 16S rRNA-encoding DNA (rDNA) a spectinomycin resistance mutation is flanked on the 5′ side by a streptomycin resistance mutation and on the 3′ side by a *Pst* I site generated by ligating an oligonucleotide in the intergenic region. Transgenic lines were selected by spectinomycin resistance and distinguished from spontaneous mutants by the flanking, cotransformed streptomycin resistance and *Pst* I markers. Regenerated plants are homoplasmic for the spectinomycin resistance and the *Pst* I markers and heteroplasmic for the unselected streptomycin resistance trait. Transgenic plastid traits are transmitted to the seed progeny. The transgenic plastid genomes are products of a multistep process, involving DNA recombination, copy correction, and sorting out of plastid DNA copies.**

Transgenic plants are widely used to study nuclear gene function and regulation and to improve agronomically important crop plants (1, 2). Production of transgenic plants is achieved routinely by several alternative methods developed specifically for transformation of the nuclear genome of higher plants. Transgenic technology has not, however, been applied yet to genomes of plastids and mitochondria of higher plants.

Introduction and stable integration of exogenous DNA have been reported recently in the plastid genome of a unicellular alga, *Chlamydomonas reinhardtii* (3–5). In this paper, we report stable genetic transformation of the plastid genome in a higher plant, *Nicotiana tabacum*. We propose the term transplastomic for the lines carrying a transgenic plastome. The transplastomic lines were selected by a nonlethal marker, resistance to the antibiotic spectinomycin that allows differentiation by color. On selective media the resistant clones are green and the sensitive clones are white (6). When cells are grown in a selective medium, plastids carrying the resistance genes are preferentially maintained (7). Selection, aided by an active system of recombination (8), was expected to facilitate the recovery of transplastomic clones.

The antibiotic resistance markers were derived from *N. tabacum* SPC2, a line that exhibits a high level of resistance to spectinomycin and streptomycin due to mutations 278 base pairs (bp) apart in the 16S rRNA-encoding DNA (rDNA) (Z.S. and P.M., unpublished results). The 16S rDNA gene is in the repeated region and therefore is present in two copies per plastid genome. The SPC2 16S rDNA clone was further marked by a silent mutation resulting in a new restriction site to facilitate identification of transplastomic clones. Although the efficiency of transformation is low, the results reported in this paper demonstrate the requirements of plastid transformation in higher plants.

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MATERIALS AND METHODS

Plant Lines. Two recipient lines were used: *N. tabacum* cv. Petit Havana with its original cytoplasm, Nt(tbc), and the Nt(pbg) alloplasmic substitution line that has the nucleus of *N. tabacum* plus the cytoplasm of *Nicotiana plumbaginifolia*. The Nt(pbg) line is functionally male sterile due to shortened filaments. Seeds can be obtained, however, by manual pollination. The Nt(pbg) line was obtained from Kevin Vaughn (U.S. Department of Agriculture Delta States Research Center, Stoneville, MS).

The *N. tabacum* SPC2 line has the original *N. tabacum* cytoplasm and is a derivative of the SR1 mutant (9). SPC2 cells exhibit a high level of resistance to streptomycin and spectinomycin. Streptomycin resistance is due to a mutation of a cytidine to an adenosine at position 860 (10) and of a cytidine to a uridine at position 1139 (Z.S. and P.M., unpublished) in the 16S rRNA.

Transformation and Regeneration of Transgenic Plants. For bombardment of leaf tissue, plants were aseptically grown from seed on MS medium. The MS medium was agar supplemented with MS salts (11) and sucrose (30 g/liter). Leaves were placed with abaxial side up on RMOP medium in a Petri dish. The RMOP medium consists of MS salts, N^6-benzyladenine (1 mg/liter), 1-naphthaleneacetic acid (0.1 mg/liter), thiamine (1 mg/liter), inositol (100 mg/liter), agar (6 g/liter) at pH 5.8, and sucrose (30 g/liter).

Tungsten (1 μm) was prepared for transformation by mixing 25 μl of a suspension of 25 mg of tungsten in 500 μl of H_2O, 4 μg of DNA dissolved in 5 μl of $T_{10}E_1$ buffer (10 mM Tris, pH 8/1 mM EDTA), 10 μl of 2.5 M $CaCl_2$, and 2.5 μl of 0.1 M spermidine free base. The particle/DNA mixture was incubated on ice for 2 min and centrifuged for 1 min in an Eppendorf centrifuge. After removing 25 μl of the supernatant, the tungsten was suspended by a brief (1 sec) sonication and applied to the macroprojectiles (2.5 μl per bombardment). The bombardment was performed as described by Klein *et al.* (12).

Two days after bombardment the leaves were cut into sections (5 mm × 5 mm) and transferred to RMOP medium containing 500 μg of spectinomycin dihydrochloride per ml. Green calli formed on the bleached leaves were subcultured onto the same selective medium. These calli formed shoots. The shoots were rooted on MS medium to obtain plants (13).

Leaf and Seedling Assays to Test Resistance Phenotypes. Leaf sections of the regenerated plants were placed on a selective RMOP medium. Spectinomycin dihydrochloride (500 μg/ml) or streptomycin sulfate (500 μg/ml) prevent greening of sensitive leaf callus on RMOP medium. Seedling phenotype was determined by germinating surface-sterilized seeds on MS salts/3% sucrose (13). Antibiotics were filter sterilized and added to the medium after autoclaving in the same concentrations as for the leaf assay.

Abbreviations: Nt(tbc), *Nicotiana tabacum* with its original cytoplasm; Nt(pbg), *N. tabacum* with *Nicotiana plumbaginifolia* cytoplasm; ptDNA, plastid DNA; rDNA, rRNA-encoding DNA.

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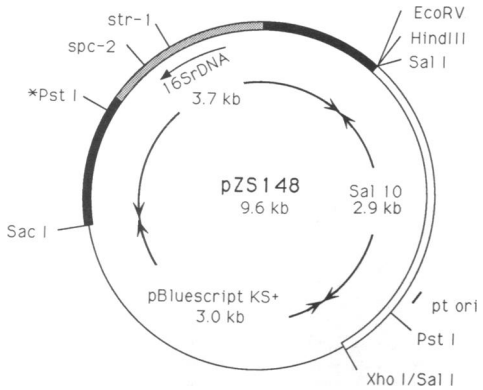

FIG. 1. pZS148 plastid transformation vector. The Bluescript vector contains the 3.7-kilobase (kb) *Sac* I–*Eco*RV fragment from the SPC2 plastid DNA (ptDNA). The 16S rDNA is highlighted, and the relative positions of streptomycin (str-1) and spectinomycin (spc-2) resistance mutations and of the *Pst* I linker (*) are shown. The 2.9-kb *Sal* I fragment includes a region implicated in ptDNA replication (pt ori).

DNA Manipulations. All DNA manipulations were performed according to Maniatis *et al.* (14). Cloning was carried out in the pBluescript I KS+ phagemid vector (Stratagene). Plastid DNA was prepared from leaves of greenhouse plants according to Kolodner and Tewari (15). Designation and position of plastid genes are according to Shinozaki *et al.* (16). Total cellular DNA was prepared from leaves by the method of Fluhr *et al.* (17). DNA for Southern probing was labeled using a random-primed DNA labeling kit (Boehringer Mannheim).

RESULTS

Construction of the pZS148 Plastid Transformation Vector. Plastid transformation vector pZS148 (Fig. 1) is derived from a pUC-based plasmid (18) and carries the 16S rRNA gene cloned as a 3.7-kb *Sac* I–*Eco*RV fragment from the SPC2 ptDNA. The SPC2 line is resistant to streptomycin and spectinomycin due to mutations in the 16S rDNA. An additional marker, a *Pst* I site, was generated 520 bp 3′ to the spectinomycin resistance mutation, in the spacer region between the 16S rDNA and *trn*I genes, by inserting the synthetic deoxyoligonucleotide 5′-pGCTGCAGC-3′ into a *Sty* I site. In addition, plasmid pZS148 carries the 2.9-kb *Sal* I fragment, which is one of the regions implicated in ptDNA replication (19, 20).

Plasmid pZS134 is identical to pZS148 except that it carries the 16S rDNA from the SR1 line that is sensitive to spectinomycin.

Table 1. Selection of plastid transformants in leaf culture

DNA	Line	Bombarded leaves, no.	Spectinomycin-resistant clones, no.	Plastid transformants, no.
pZS134	Nt(pbg)	39	5	0
	Nt(tbc)	11	1	0
pZS148	Nt(pbg)	58	26	2
	Nt(tbc)	90	30	1

Spectinomycin-Resistant Clones After Transformation with pZS148 DNA. Whole leaves of Nt(tbc) and Nt(pbg) plants were bombarded with tungsten particles coated with pZS148 DNA. In a sample of 148 bombarded leaves, 56 spectinomycin-resistant green calli were obtained (Table 1). The calli appeared on different leaf sections and are considered independent clones. Different plants regenerated from the same resistant line are considered subclones and are designated by a capital letter. For example, plants Nt(pbg*)T2B and Nt(pbg*)T2D are derived from the same clone, Nt(pbg*)T2.

To test the frequency of spontaneous spectinomycin-resistant mutants, leaf tissue was bombarded with control pZS134 DNA and cultured on a drug-containing medium as described for the pZS148 DNA. In a sample of 50 bombarded leaves, 6 spectinomycin-resistant calli were recovered independently (Table 1). None of these was resistant to streptomycin (data not shown).

Spectinomycin resistance of the clones may be the result of transformation by the pZS148 DNA or of spontaneous mutation. The regenerated plants were therefore screened for the flanking unselected markers, the *Pst* I site, and streptomycin resistance.

Unselected *Pst* I Marker in the Spectinomycin-Resistant Lines. Plants regenerated from the spectinomycin-resistant clones were screened for the *Pst* I marker by Southern probing of total leaf cellular DNA digested with *Hin*dIII and *Pst* I restriction endonucleases (Fig. 2*A*). In wild-type Nt(tbc) or Nt(pbg) DNA the probe hybridizes to a 6.2-kb fragment. Replacement of the 16S rDNA region with the engineered SPC2 clone introduces a *Pst* I site into this 6.2-kb fragment, which results in the generation of 4.4- and 1.8-kb fragments. Based on the *Pst* I marker three transplastomic clones have been identified (Table 2).

Plants regenerated from the three transplastomic calli contained the *Pst* I marker (Fig. 2*A*). In all but one, Nt(tbc*)T85A, there was no detectable amount of wild-type fragment (Fig. 2*A*). This indicates that the *Pst* I site was present in both rDNA repeats, and the wild-type fragment has been eliminated. In a leaf of one of the plants, Nt(tbc*)T85A, a mixture of rDNA with the wild-type sequence and the engineered *Pst* I site was found. Plants regenerated from the leaf of this plant carry a pure population

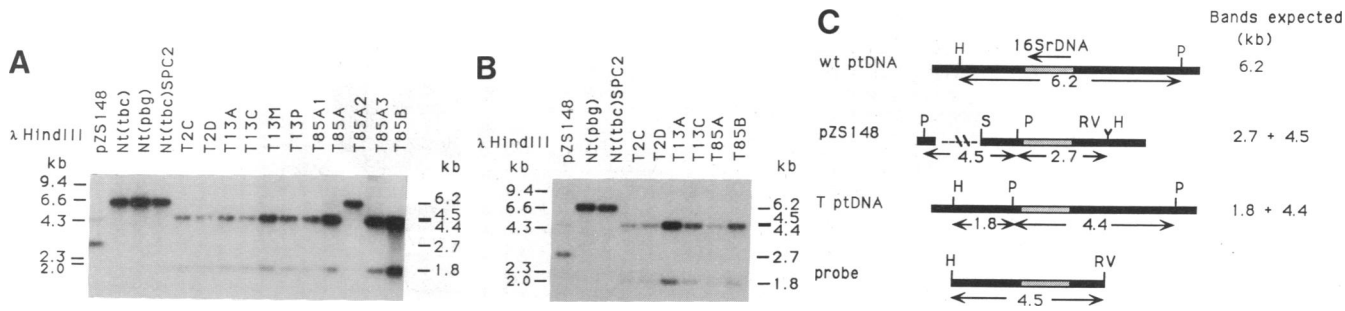

FIG. 2. Southern probing to screen for the *Pst* I marker. Results of probing total cellular DNA from leaves of regenerated plants grown in sterile culture (*A*) and isolated ptDNA from greenhouse plants (*B*) are shown after digestion with *Pst* I and *Hin*dIII restriction endonucleases. As a control, pZS148 plasmid DNA and DNA of the Nt(tbc), Nt(pbg), and Nt(tbc) SPC2 lines were included. (*C*) Physical map of the probed region of wild-type (wt) and transgenic (T) ptDNA and the relative position of the probe and of the 16S rDNA (highlighted) in plasmid pZS148. Expected sizes of hybridizing fragments are listed. Restriction endonucleases: RV, *Eco*RV; H, *Hin*dIII; P, *Pst* I; S, *Sac* I.

Table 2. Flanking markers in plastid transformants

Line	Subclone	Resistance To Sp500	Resistance To Sm500	*Pst* I marker
Nt(pbg*)T2	B	+	+	+
	D	+	+	+
Nt(pbg*)T13	A	+	−	+
	B	+	−	+
	C	+	−	+
	M	+	+	+
	P	+	+	+
Nt(tbc*)T85	A	+	+	+, −
	A1	+	+	+
	A2	+	+	−
	A3	+	+	+
	B	+	+	+
	D	+	+	+

Sp500, 500 μg of spectinomycin dihydrochloride per ml; Sm500, 500 μg of streptomycin sulfate per ml.

of plastids with (T85A1, T85A3) or without (T85A2) the *Pst* I marker. The plant without the *Pst* I marker is also resistant to both antibiotics (Table 2).

ptDNA was isolated from greenhouse-grown plants ≈3 months after the initial screen. At this time ptDNA was probed as described above for total cellular DNA and was found to carry exclusively the *Pst* I marker (Fig. 2*B*). The size of ethidium bromide-stained *Pst* I ptDNA fragments was as predicted if both copies of the wild-type 16S rDNA have been replaced by homologous recombination (Fig. 3 *A* and *B*).

Probing *Hin*dIII/*Pst* I-digested DNA for the *Pst* I site in the 16S rDNA region is suitable to detect a replicating pZS148 plasmid by a 2.7-kb fragment (Fig. 2). This fragment, characteristic of intact pZS148 plasmids, was absent in all samples tested (Fig. 2). Digestion of plasmid pZS148 with *Pst* I yields 5.1-kb (and 4.5 kb) fragments. The 5.1-kb fragment was absent in ethidium bromide-stained gels (Fig. 3), also indicating the absence of unintegrated pZS148 plasmid DNA.

Unselected Streptomycin Resistance Marker in the Spectinomycin-Resistant Lines. The regenerated plants were also tested for the unselected antibiotic resistance marker, streptomycin resistance, in a leaf assay (Fig. 4). Plants regenerated from the Nt(pbg*)T2 and Nt(tbc*)T85 clones were phenotypically resistant to streptomycin. The Nt(pbg*)T13 line yielded streptomycin-resistant and -sensitive plants (Table 2).

Inheritance of Antibiotic Resistance in the Transplastomic Lines. Seeds were collected after selfing, or from reciprocal crosses with wild-type *N. tabacum*. Seeds were germinated

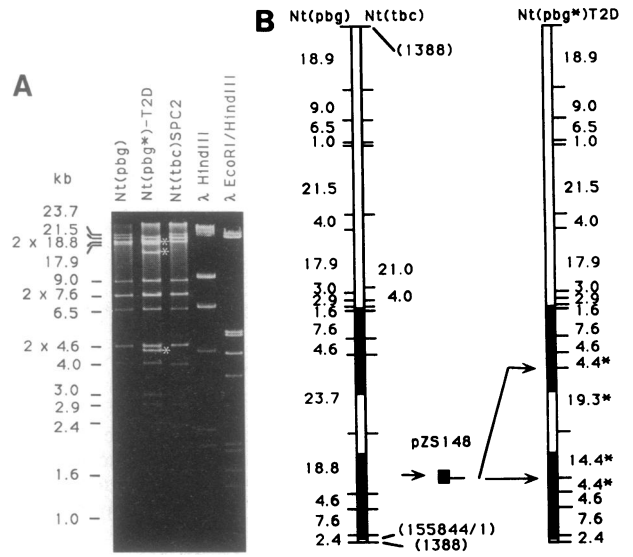

FIG. 3. Nt(pbg*)T2D ptDNA is copy-corrected for the region containing the *Pst* I linker. (*A*) *Pst* I fragment pattern of ptDNA. ptDNA isolated from greenhouse plants was digested, and fragments were separated in agarose gels. λ DNA digested with the *Hin*dIII or the *Hin*dIII/*Eco*RI restriction endonucleases was run alongside to serve as molecular weight standards. Sizes of *Pst* I fragments in the Nt(pbg) recipient are given. Note that the 18.8-kb and 18.9-kb fragments do not separate and are marked as a doublet. The 4.4-kb, 14.4-kb and 19.3-kb fragments in the Nt(pbg*)T2D ptDNA are marked with asterisks. (*B*) *Pst* I map of ptDNA derived from data shown in *A*. The circular map was linearized at base pair 1388 for convenience to include complete *Pst* I fragments. Junctions of base pair 1 and base pair 155,844 are given in parentheses. Sizes of fragments are given in kilobase pairs. The filled bars indicate repeated regions. The map position of the 3.7-kb 16S rDNA fragment, contained in plastid vector pZS148, is shown. Note that the 23.7- and 18.8-kb Nt(pbg) fragments should disappear due to integration of the *Pst* I linker; new 4.4-, 14.4-, and 19.3-kb fragments that form as the result are marked (*).

on selective medium to test seed transmission of the transgenic traits (Fig. 5).

The seed progeny obtained after selfing are uniformly resistant to spectinomycin (Table 3). Lack of segregation indicates that the regenerated plants are homoplasmic for the spectinomycin resistance marker. They carry only one type, the spectinomycin-resistant 16S rDNA. In crosses, the resistance is inherited maternally.

The unselected streptomycin resistance was inherited as expected for a plastome-encoded, heteroplasmic trait. Segre-

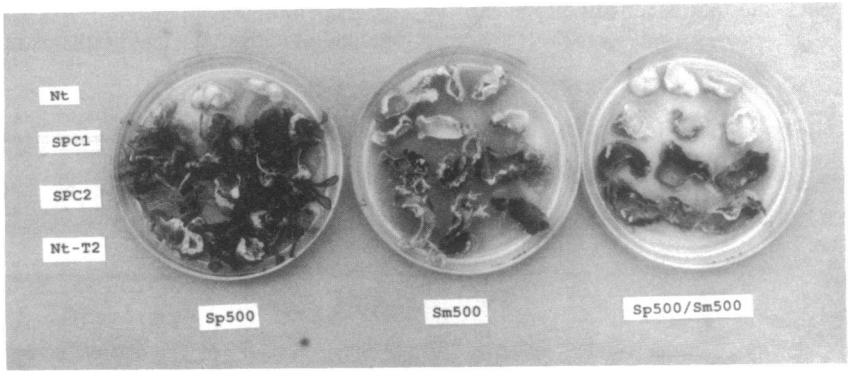

FIG. 4. Antibiotic resistance phenotype in leaf callus. Nt [Nt(tbc)] and SPC2 [Nt(tbc) SPC2] are the wild-type recipients and the source of transforming spectinomycin- and streptomycin-resistant 16S rDNA, respectively. Nt-T2 [Nt(pbg*)T2D] is a transgenic clone; SPC1 [Nt(tbc) SPC1] is a control line, which is resistant to spectinomycin but is sensitive to streptomycin (Z.S. and P.M., unpublished results). Note that resistant leaves form green callus; sensitive leaves form white callus. Plates contain spectinomycin dihydrochloride (Sp500), streptomycin sulfate (Sm500), or both antibiotics (Sp500/Sm500) at 500 μg/ml.

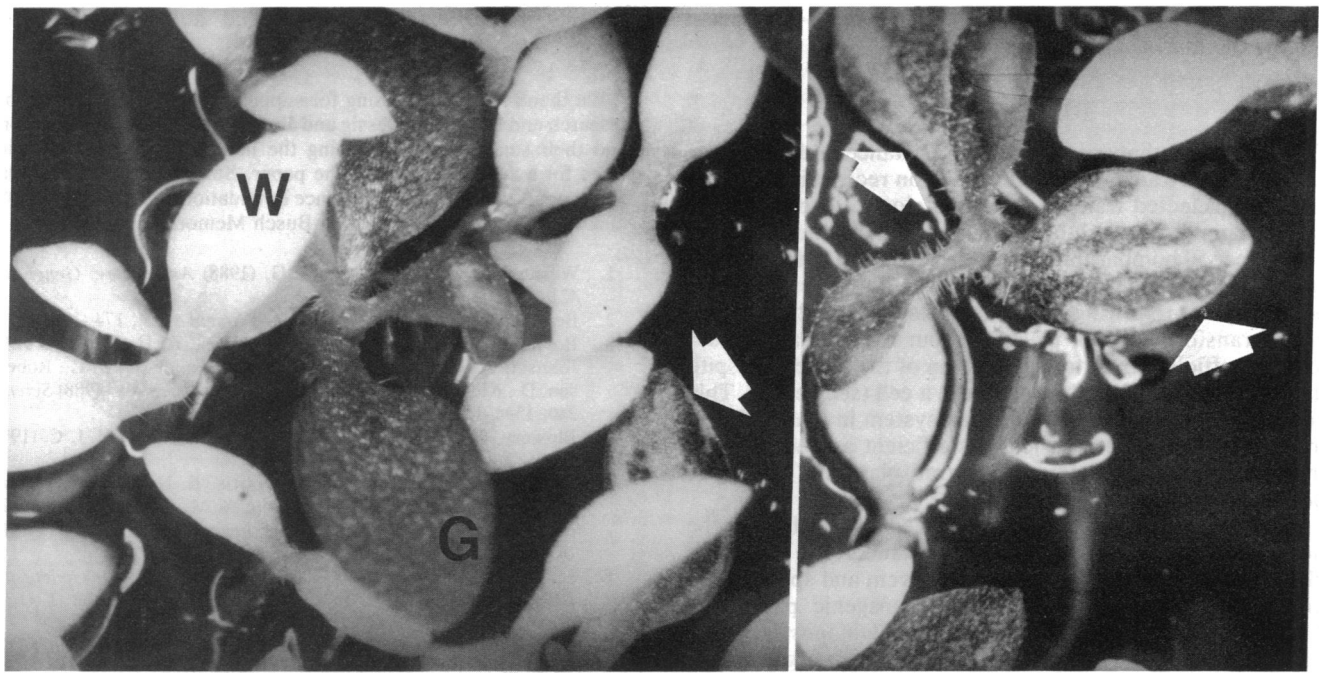

FIG. 5. Antibiotic resistance in the seed progeny of the transplastomic line Nt(pbg*)T2D. Seeds were germinated on a selective medium containing spectinomycin and streptomycin (500 μg/ml each). Note resistant (green; G), sensitive (white; W) and chimeric seedlings with green and white sectors (arrows).

gation for this trait was found in the selfed seed progeny, and in F_1 progeny of all three lines. In addition to resistant (green) and sensitive (white) seedlings, variegated plants were also observed (Fig. 5). Variegation for the streptomycin resistance marker in the progeny indicates that regenerated plants were heteroplasmic for the unselected streptomycin resistance trait. Streptomycin resistance, however, was never transmitted by the pollen parent to any of the RF_1 progeny.

The ratios of resistant to variegated to sensitive seedlings differ in subclones of the same line (e.g., compare the Nt(pbg*)T2D and Nt(pbg*)T2C progenies in Table 3). Note that streptomycin-resistant seedlings were found in the seed progeny of the Nt(pbg*)T13A and T13C plants, although the plants were phenotypically sensitive in the leaf assay (Table 2). Furthermore, the Nt(tbc*)T85A plant was phenotypically resistant to streptomycin in the leaf assay (Table 2) but was segregating mainly streptomycin-sensitive seed progeny. These inconsistencies are expected in heteroplasmic plants and are due to chimerism resulting from random sorting of plastids in the absence of selection.

***Pst* I Marker in the Seed Progeny.** Inheritance of the transgenic *Pst* I marker was confirmed in subclones T2D, T85B (three streptomycin-resistant and three sensitives tested from each subclone), and T13C (two resistant and one

Table 3. Transmission of spectinomycin and streptomycin resistance to the seed progeny

Line	Progeny	No antibiotic			Sp500			Sm500			Sm500/Sp500		
		G	W	G/W	G	W	G/W	G	W	G/W	G	W	G/W
Nt(tbc)		693	0	0	0	709	0	0	689	0	0	629	0
Nt(tbc) SPC2		227	0	0	315	0	0	309	0	0	311	0	0
Nt(pbg*)T2C	Self	670	0	0	866	0	0	3	735	25	0	1090	16
	F_1	774	0	0	1204	0	0	1	1405	20	0	1370	20
	RF_1	355	0	0	0	413	0	0	522	0	0	517	0
Nt(pbg*)T2D	Self	294	0	0	653	0	0	173	511	62	208	557	40
	F_1	522	0	0	477	0	0	297	369	100	283	329	78
	RF_1	421	0	0	0	553	0	0	609	0	0	719	0
Nt(pbg*)T13A	Self	832	0	0	967	0	0	2	1033	1	0	889	8
	F_1	998	0	0	840	0	0	2	1112	11	1	659	12
	RF_1	650	0	0	0	1053	0	0	1030	0	0	1034	0
Nt(pbg*)T13C	Self	580	0	0	766	0	0	4	703	8	4	871	9
	F_1	1194	0	0	1203	0	0	0	1342	7	0	980	3
	RF_1	495	0	0	0	467	0	0	602	0	0	462	0
Nt(tbc*)T85A	Self	497	0	0	556	0	0	0	524	0	0	423	0
	F_1	352	0	0	546	0	0	0	412	0	1	420	3
	RF_1	531	0	0	0	614	0	0	614	0	0	656	0
Nt(tbc*)T85B	Self	592	0	0	635	0	0	179	437	65	274	291	81
	F_1	504	0	0	456	0	0	390	136	31	293	90	30
	RF_1	810	0	0	0	907	(2)*	0	861	0	0	950	0

F_1, cross in which resistant is female; RF_1, cross in which resistant is male; G, green; W, white; G/W, variegated; Sp500, spectinomycin dihydrochloride at 500 μg/ml; Sm500, streptomycin sulfate at 500 μg/ml. The number of seedlings was scored on each antibiotic independently.
*Variegation of these seedlings is probably due to spontaneous mutation.

sensitive seedlings tested). Each of the plants was homoplasmic for the marker (data not shown).

DISCUSSION

The conclusion that spectinomycin resistance in three of the lines is due to transformation is based on recovering the two linked, unselected traits in the same clones. The 148 bombarded samples yielded three transplastomic clones—that is, 1 clone per 50 bombardments (Table 1). Selection for nuclear antibiotic resistance markers yields 2–6 transgenic clones per bombardment following the same protocol (21, 22). Accordingly, transformation of plastids in our hands is about 100-fold less efficient than transformation of the nucleus despite the high number of ptDNA copies in a cell (see below). This contrasts with the *Chlamydomonas* system in which plastid transformation (3–5) is at least as efficient as nuclear gene transformation (23), yielding 2–100 transplastomic clones per bombardment.

Transgenic plastids have been obtained by selection for the nonlethal marker, spectinomycin resistance. The same 16S rDNA markers, resistance to streptomycin and spectinomycin, have been used to select for transgenic plastids in *Chlamydomonas* (5, 31). Since *Chlamydomonas* is grown photoautotrophically, the same markers in that organism are lethal.

We consider nonlethal selection critical in obtaining the transplastomic lines. Higher plants carry a large number of identical plastid genome copies per cell (24, 25). In *N. tabacum* the 3000–12,000 copies (26, 27) are localized in up to 100 plastids (28). This contrasts with the 80 ptDNA copies, carried by a single plastid in *Chlamydomonas* (29). Nonlethal selection in higher plants allows sufficient time for the resistant plastid genome copies to increase in numbers to allow phenotypic expression.

Since plastid transformation is rare, we assume that in a transplastomic clone all plastids derive from the same transformed organelle. In each clone more than one type of transgenic plastid genome was found. Our findings can be explained by assuming a multistep process, involving DNA recombination, copy correction, and sorting out of the transgenic ptDNA copies. Different transgenic plastid genomes may be products of the same initial transformation event or independent recombination events between copies of vector pZS148 and different copies of ptDNA. The initial transformation event is then followed by copy correction, a mechanism that maintains identical DNA sequence in the two repeat regions of ptDNA (16, 30). Copy correction may also generate different transgenic plastid genomes using different copies of the same template. Subsequently, sorting of ptDNA should yield homoplasmic organelles and then homoplasmic cells.

The frequency of spontaneous mutants relative to plastid transformants is high. Three transplastomic clones were recovered from a sample of 56 spectinomycin-resistant lines, of which 53 have been scored as spontaneous mutants. This may underestimate the actual number of transformants. The relative proportion of spectinomycin-resistant lines in the control is one-third of that in the sample bombarded with the spectinomycin-resistant 16S rDNA clone (Table 1). This suggests that some of the clones scored as spontaneous mutants because of loss of the flanking markers were actual transformants in which copy correction eliminated the flanking markers.

Stable genetic transformation reported in this paper opens up the plastid genome of higher plants to experimental modification. This will lead to a better understanding of the regulation of plastid gene expression, the interaction of plastid genes with nuclear genes, and the involvement of plastids in various biochemical and developmental processes.

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1. Weising K., Schell, J. & Kahl, G. (1988) *Annu. Rev. Genet.* **22,** 421–477.
2. Benfey, P. N. & Chua, N. H. (1989) *Science* **244,** 174–181.
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. & Sanford, J. C. (1988) *Science* **240,** 1534–1538.
4. Blowers, A. D., Bogorad, L., Shark, K. B. & Sanford, J. C. (1989) *Plant Cell* **1,** 123–132.
5. Boynton, J. E., Gillham, N. W., Harris, E. H., Newman, S. M., Randolph-Anderson, B. L., Johnson, A. M. & Jones, A. R. (1990) in *Current Research in Photosynthesis*, ed. Baltscheffsky, M. (Kluwer, Amsterdam), Vol. 3, pp. 509–516.
6. Maliga, P., Moll, B. & Svab, Z. (1990) in *Perspectives in Genetic and Biochemical Regulation of Photosynthesis*, ed. Zelitch, I. (Liss, New York), pp. 133–143.
7. Moll, B., Polsby, L. & Maliga, P. (1990) *Mol. Gen. Genet.* **221,** 245–250.
8. Fejes, E., Engler, D. & Maliga, P. (1990) *Theor. Appl. Genet.* **79,** 28–32.
9. Maliga, P., Breznovits, A. & Marton, L. (1973) *Nature (London) New Biol.* **244,** 29–30.
10. Etzold, T., Fritz, C. C., Schell, J. & Schreier, P. H. (1987) *FEBS Lett.* **219,** 343–346.
11. Murashige, T. & Skoog, F. (1962) *Physiol. Plant.* **15,** 473–497.
12. Klein, T. M., Gradziel, T., Fromm, M. E. & Sanford, J. C. (1988) *Bio/Technology* **6,** 559–563.
13. Maliga, P. (1984) in *Cell Culture and Somatic Cell Genetics of Plants*, ed. Vasil, I. K. (Academic, Orlando, FL), Vol. 1, pp. 552–562.
14. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
15. Kolodner, R. & Tewari, K. K. (1975) *Biochim. Biophys. Acta* **402,** 372–390.
16. Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., Yamaguchi-Shinozaki, K., Ohto, C., Torazawa, K., Meng, B. Y., Sugita, M., Deno, H., Kamogashira, T., Yamada, K., Kusuda, J., Takaiwa, F., Kato, A., Tohdoh, N., Shimada, H. & Sugiura, M. (1986) *EMBO J.* **5,** 2043–2049.
17. Fluhr, R., Aviv, D., Galun, E. & Edelman, M. (1984) *Theor. Appl. Genet.* **67,** 491–497.
18. Vieira, J. & Messing, J. (1982) *Gene* **19,** 259–268.
19. Gold, B., Carrillo, N., Tewari, K. K. & Bogorad, L. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 194–198.
20. Carillo, N. & Bogorad, L. (1988) *Nucleic Acids Res.* **16,** 5603–5620.
21. Klein, T. M., Harper, E. C., Svab, Z., Sanford, C., Fromm, M. E. & Maliga, P. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8502–8505.
22. Timmermans, M. C. P., Maliga, P., Vieira, J. & Messing, J. (1990) *J. Biotechnol.* **14,** 333–344.
23. Kindle, K. L., Schnell, R. A., Fernandez, E. & Lefebvre, P. A. (1989) *J. Cell Biol.* **109,** 2589–2601.
24. Palmer, D. J. (1985) *Annu. Rev. Genet.* **19,** 325–354.
25. Possingham, J. V. & Lawrence, M. E. (1983) *Int. Rev. Cytol.* **84,** 1–56.
26. Cannon, G., Heinhorst, S., Siedlecki, J. & Weissbach, A. (1985) *Plant Cell Rep.* **4,** 41–45.
27. Yasuda, T., Kuroiwa, T. & Nagata, T. (1988) *Planta* **174,** 235–241.
28. Thomas, M. R. & Rose, R. J. (1983) *Planta* **158,** 329–338.
29. Harris, E. H. (1989) *The Chlamydomonas Sourcebook* (Academic, San Diego, CA), p. 354.
30. Ohyama, K., Fukuzawa, H., Kochi, T., Shirai, H., Sano, T., Sano, S., Umesono, K., Shiki, Y., Takeuchi, M., Chang, Z., Aota, S., Inokuchi, H. & Ozeki, H. (1986) *Nature (London)* **322,** 572–574.
31. Newman, S. M., Boynton, J. E., Gillham, N. W., Randolph-Anderson, B. L., Johnson, A. M. & Harris, E. H. (1990) *Genetics* **126,** in press.