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Long Regions of Homologous DNA Are Incorporated into the Tobacco Plastid Genome by Transformation

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We investigated the size of flanking DNA incorporated into the tobacco plastid genome alongside a selectable antibiotic resistance mutation. The results showed that integration of a long uninterrupted region of homologous DNA, rather than of small fragments as previously thought, is the more likely event in plastid transformation of land plants. Transforming plasmid pJS75 contains a 6.2-kb DNA fragment from the inverted repeat region of the tobacco plastid genome. A spectinomycin resistance mutation is encoded in the gene of the 16S rRNA and, 3.2 kb away, a streptomycin resistance mutation is encoded in exon II of the ribosomal protein gene *rps12*. Transplastomic lines were obtained after introduction of pJS75 DNA into leaf cells by the biolistic process and selection for the spectinomycin resistance marker. Homologous replacement of resident wild-type sequences resulted in integration of all, or almost all, of the 6.2-kb plastid DNA sequence from pJS75. Plasmid pJS75, which contains engineered cloning sites between two selectable markers, can be used as a plastid insertion vector.

INTRODUCTION

Application of transgenic technology to study plastid gene function requires gene replacement and insertion by transformation. These objectives have recently been achieved in the unicellular alga Chlamydomonas (Boynton et al., 1988; Blowers et al., 1989, 1990; Goldschmidt-Clermont, 1991; Goldschmidt-Clermont et al., 1991; Kindle et al., 1991, Newman et al., 1991; Przibilla et al., 1991; Roffey et al., 1991; Stern et al., 1991). Replacement by transformation of the gene of the 16S rRNA in tobacco, a land plant, has also been reported by Svab et al. (1990). Transformation involves changing in a cell each of the 5 to 80 genome copies in the one plastid of Chlamydomonas and the total of 500 to 10,000 genome copies in the 10 to 100 plastids of a tobacco cell.

There appeared to be one principal difference in the outcome of the alga and land plant plastid transformation experiments. In Chlamydomonas, complete or nearly complete replacement of a region of the chloroplast genome in the recipient cell by the corresponding region from the donor plasmid was the most common integration event (Newman et al., 1990). In tobacco, all of the markers carried by the transforming DNA could also be recovered in the plastid genome. Each transplastomic line, however, segregated subclones that contained only portions of the transforming DNA (Svab et al., 1990). The previous tobacco transplastomic lines were selected for spectinomycin resistance encoded in the *16SrDNA* gene and scored for the presence of two unselected flanking markers. A PstI site was inserted 520 bp downstream, and a streptomycin resistance mutation was located 278 bp upstream from the selected spectinomycin resistance mutation in the *16SrDNA* gene. Separation of the more closely spaced streptomycin resistance mutation from the selected marker occurred much more frequently than did separation of the PstI restriction site marker. Separation of the physically close markers was not unexpected because an earlier study of a recombinant plastid genome by Fejes et al. (1990) pointed to the existence of an active recombination system in tobacco plastids.

To clarify the reasons for the differences in the recombination frequency of flanking unselected markers, a new study was undertaken. The donor DNA for transformation was cloned from the same (repeat) region of the plastid genome but now contains six newly created restriction fragment length polymorphism (RFLP) markers along a 6.2-kb region in plasmid pJS75. The markers are in different functional domains of the genome, including intragenic and intergenic intracistronic and intercistronic locations. The restriction site markers were created at sites expected not to interfere with normal functioning of the plastid genome.

Based on transformation with plasmid pJS75, we report that, in tobacco, the entire length of the homologous region is likely to be incorporated and retained in the transformed plastid genome.

RESULTS

pJS75 Transformation Vector

The 6.2-kb DNA fragment in vector pJS75, cloned from the repeat region of the plastid genome of tobacco into plasmid pUC119, is shown in Figure 1. The *16SrDNA* gene in this

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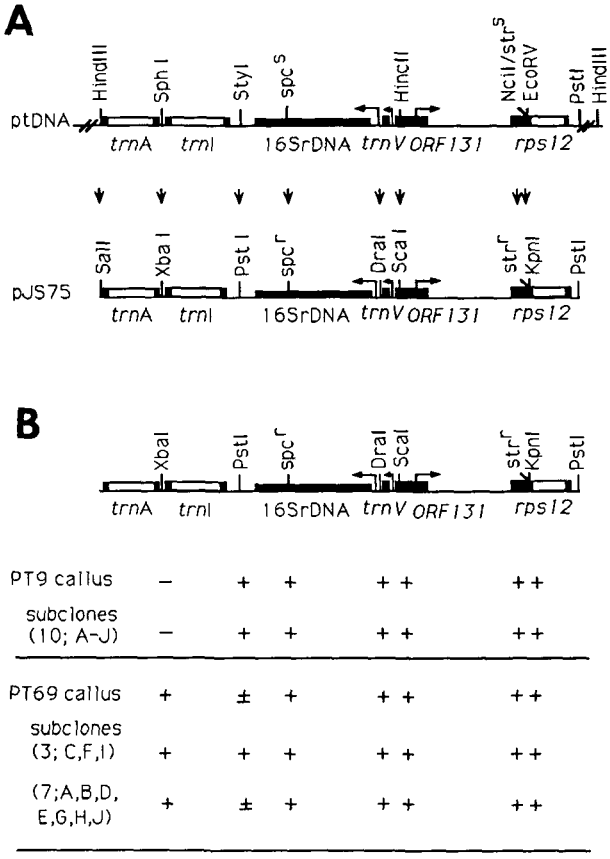

Figure 1. *Plastid DNA in Transformation Vector pJS75 and Regions Incorporated into Transplastomic Lines PT9 and PT69.*

(A) Restriction map of plastid DNA in pJS75 and corresponding region in wild-type plastids (ptDNA). Vertical arrows pinpoint restriction site differences. Horizontal arrows indicate sites of transcription initiation and direction of transcription; coding regions are in black boxes. The genes are designated according to Shinozaki et al. (1986).

(B) Transgenic markers in the PT9 and PT69 transplastomic lines. The SalI site was used to linearize pJS75 DNA before transformation. The HindIII site at this position in ptDNA was present in all clones. Markers: +, present; –, absent; ±, mixed. Note that the lack of an NciI site is the transgenic marker, the presence of which is denoted by a +.

fragment contains the SPC1 mutation that confers resistance to 500 μg/mL of spectinomycin (Svab and Maliga, 1991). Site-directed mutagenesis was used to introduce a streptomycin resistance mutation (Galili et al., 1989) into exon II of the *rps12* gene encoding ribosomal protein S12, which is 1.9 kb apart from the 16S rRNA coding sequence. The distance between the two antibiotic resistance point mutations is 3.2 kb. The nucleotide change required for streptomycin resistance eliminates an NciI restriction site. Additional single base changes were introduced with the same mutagenic oligonucleotide 10 and 13 nucleotides downstream from the streptomycin resistance mutation to create a KpnI restriction enzyme site. An EcoRV restriction site is destroyed by these additional changes. These mutations alter the third base of two consecutive codons and do not result in an amino acid change. The neutral KpnI site was designed to monitor the occurrence of selective elimination of the streptomycin resistance mutation.

Two restriction enzyme sites were created between the antibiotic resistance markers in pJS75. The DraI site is upstream from the rRNA operon promoter (Sun et al., 1989) and is downstream from the sequence required for processing the 3′ end of the *trnV* gene (Gruissem, 1989). The ScaI site is between the divergently transcribed *trnV* and *rps12/7* promoters (Hildebrand et al., 1988). The ScaI oligonucleotide insertion creates a frameshift mutation in ORF131, an open reading frame that is unique to the tobacco plastid genome (Shimada and Sugiura, 1991). Additional RFLP markers PstI and XbaI were introduced into spacer regions in the rRNA operon (Takaiwa and Sugiura, 1982; Tohdoh and Sugiura, 1982). The SalI site at the end of the plastid DNA sequence was used to linearize vector pJS75 before particle bombardment.

Unselected Flanking Markers in Transplastomic Lines

The 40 spectinomycin-resistant cell lines selected in 195 bombarded leaf cultures were tested for the transgenic XbaI, PstI, DraI, ScaI, and KpnI RFLP markers. Thirty-eight of the spectinomycin-resistant lines did not carry any of the transgenic RFLP markers and were classified as spontaneous mutants. Two transplastomic lines, PT9 and PT69, were identified. These lines were evaluated at two stages in their clonal history: in primary callus and in regenerated plants. Primary callus was identified by its green color on bombarded leaf sections on spectinomycin-containing medium. Subclones are plants regenerated from the primary callus. Total cellular DNA from the primary callus and from subclones was analyzed for each of the RFLP markers. Figure 1B shows the results of this analysis.

The PT9 primary callus contained all but one of the transplastomic RFLP markers. The PstI, DraI, ScaI, NciI, and KpnI RFLP markers define the continuous stretch of ptDNA from plasmid pJS75 that was integrated into the plastid genome of the PT9 line. The 10 independent subclones derived from this primary callus also contained the transplastomic markers with no identifiable wild-type fragment present. These data indicate that the PT9 line was homoplasmic for each of these markers already in the primary callus, and these same markers were maintained in the regenerated plants. Primary callus of the PT69 line contained all of the RFLP markers in plasmid pJS75, including the XbaI, PstI, DraI, ScaI, NciI, and KpnI restriction site markers. All of the RFLP markers were homoplasmic in the primary callus except for the introduced PstI site. The RFLP markers that were homoplasmic in the primary callus were also homoplasmic after plant regeneration.

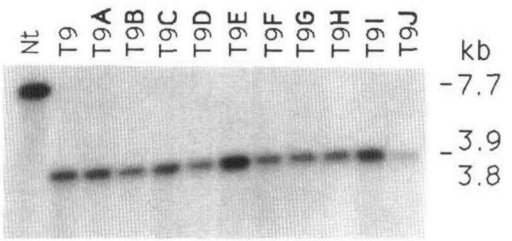

Figure 2. Probing of PT9 DNA Gel Blots Confirms Homoplasmy for the ScaI RFLP Marker.

Total cellular DNA was digested with HindIII and ScaI restriction enzymes and separated on a 0.7% agarose gel. DNA was isolated from wild-type tobacco (Nt); PT9 primary callus (T9); PT9 subclones (T9A to T9J). The 7.7-kb wild-type band is seen in the Nt lane; the transplastomic 3.9- and 3.8-kb bands are not resolved in the T9, and T9A to T9J lanes. The probe was an SalI/DraI DNA fragment covering 6 kb of the plastid DNA in pJS75. Note that the DraI site used to generate the probe is located in the *rps12* gene and is not shown in Figure 1.

The transplastomic PstI marker was recovered in both homoplasmic and heteroplasmic forms in the plants regenerated from the PT69 primary callus.

An example of homoplasmy in the PT9 line is shown in Figure 2 for the ScaI site. In double digests of the tobacco plastid genome from the transplastomic lines with HindIII and ScaI, the wild-type 7.7-kb tobacco DNA fragment lacking the ScaI site is absent and is replaced by the transgenic 3.8- and 3.9-kb doublet formed as a result of incorporation of the additional ScaI site from pJS75. Note that the patterns in the primary PT9 callus and all subclones are identical.

An example of homoplasmy in the PT69 line is shown in Figure 3 for the NciI restriction enzyme site used to monitor the streptomycin resistant *rps12* allele. The wild-type tobacco DNA that does not contain the streptomycin resistance base change has the short 495-bp DNA fragment associated with the sensitive *rps12* allele. The PT69 primary callus and all 10 subclones are homoplasmic for the long 564-bp DNA fragment associated with the resistant *rps12* allele. The fact that this NciI RFLP marker becomes homoplasmic early on is a good indication that the *rps12* streptomycin resistance phenotype is stable.

The only example of heteroplasmy found in this study is shown in Figure 4, where segregation for the PstI marker is demonstrated in the PT69 line. Both the transplastomic 4.4- and 1.8-kb, and wild-type 6.2-kb PstI fragments were recovered in PT69 subclones A, B, D, E, G, H, and J. Subclones C, F, and I have the transplastomic PstI pattern in a homoplasmic form. No PT69 subclone with a pure wild-type PstI pattern was found. Homoplasmic wild-type PstI pattern, however, was obtained in the seed progeny of subclone D (see below).

The PT69 primary callus and some of the subclones contained a small circular DNA molecule that apparently derives from plasmid pJS75. These DNA molecules are unstable and are lost in somatic cells (data not shown).

Antibiotic Resistance in the Seed Progeny

PT9 and PT69 plants were grown to maturity in the greenhouse. Seeds from PT9 plants were collected after selfing and from reciprocal crosses with wild-type tobacco. Viable PT69 seed was obtained after selfing and in crosses with PT69 as the female parent. No seed was obtained in crosses with PT69 as the male parent.

Seeds were germinated on selective media to test transmission of the transplastomic antibiotic resistance traits. All PT9 self and F_1 seed progeny (PT9 is female, and wild type is male in the cross) were uniformly green, indicating resistance to spectinomycin and to streptomycin, as shown in Figure 5 (green appears as black in the figure). In the reciprocal

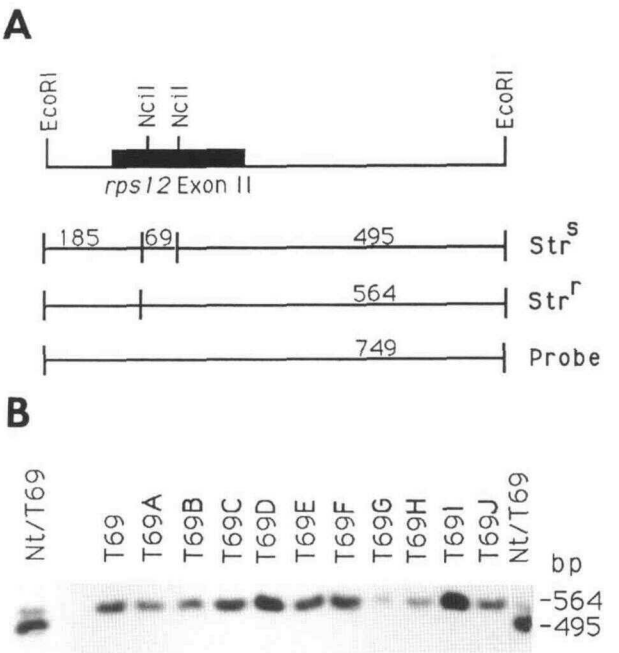

Figure 3. Probing of PT69 DNA Gel Blots Confirms Homoplasmy for the Streptomycin-Resistant *rps12* Allele.

(**A**) The size of DNA fragments after digestion with the EcoRI and NciI restriction enzymes identifies sensitive or resistant genotypes. The streptomycin resistance mutation eliminates an NciI site within the *rps12* coding region, resulting in a diagnostic 564-bp DNA fragment associated with the resistant allele. From the sensitive allele, a 495-bp and a 69-bp DNA fragment are obtained. The 749-bp EcoRI fragment that includes the entire region shown at the top was used to probe the DNA gel blot.

(**B**) Probing of DNA gel blots of PT69 clones for the NciI site. Total cellular DNA was digested with the NciI and EcoRI restriction endonucleases and separated on a 1.1% agarose gel. Control lanes contain mixed wild-type and PT69 total cellular DNA (lane Nt/T69) to show the position of the 564-bp and 495-bp fragments associated with streptomycin resistance and sensitivity, respectively. DNA was isolated from the PT69 primary callus (T69). T69A to T69J, PT69 subclones.

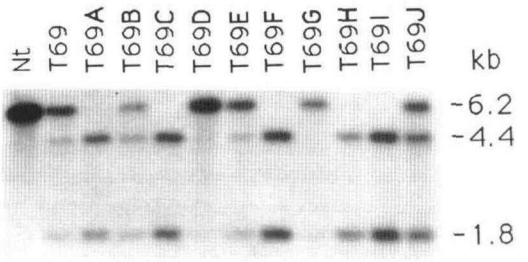

Figure 4. Heteroplasmy for the PstI RFLP Marker in the PT69 Transplastomic Line.

Total cellular DNA was digested with HindIII and PstI restriction enzymes and separated on a 0.7% agarose gel. Lanes containing T69 DNA are designated as in Figure 3. The DNA probe was as in Figure 2.

cross using wild-type plants as the female parent and PT9 as pollen parent, the seedlings were uniformly sensitive (white) to both antibiotics. Inheritance of the antibiotic resistance phenotype was examined in 10 subclones, three capsules each containing >1000 seeds per capsule. The results obtained for the PT69 seed progeny were consistent with those of the PT9 line. The number of seedlings per capsule tested, however, was only ~100. In the absence of seed, no data were obtained on the reciprocal F_1 progeny (PT69 used as male in the cross).

Uniform resistance in the selfed seed progeny and in crosses when the transplastomic lines were used as females, and lack of transmission of resistance through pollen when the transgenic plants were used as male, are as expected for a plastome-encoded trait. Lack of segregation for resistance in the seed progeny indicates homoplasmy for both antibiotic resistance markers.

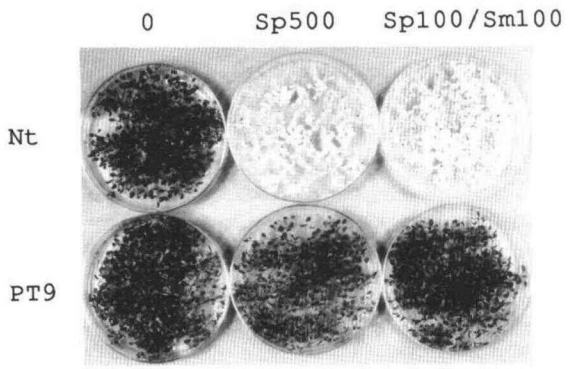

Figure 5. Antibiotic Resistance in PT9 Seedlings.

Selfed PT9 seedlings and wild-type tobacco seedlings (Nt) are shown. Seeds were germinated in the absence of drugs (0) and on selective media containing 500 μg/mL spectinomycin (Sp) or 100 μg/mL each of spectinomycin (Sp) and streptomycin (Sm). Green color (black in photograph) of seedlings on drug-containing media indicates resistance. Bleached (white) seedlings are sensitive to drugs.

RFLP Markers in the Seed Progeny

Plants were raised from selfed seedlings of homoplasmic PT9 and PT69 lines (two seedlings each from three subclones) and checked for the transplastomic RFLP markers. The markers that were present in the regenerated plants were inherited by each of the seed progeny. PT69D seedlings were recovered that are homoplasmic for the XbaI+, PstI−, DraI+, ScaI+, NciI+, and KpnI+ RFLP markers. Therefore, homoplasmy was ultimately obtained for each of transgenic plastid genomes observed in the primary PT69 calli (Figure 1B).

DISCUSSION

Two plastid transformants were identified by RFLP markers among 38 spontaneous spectinomycin-resistant mutants in 195 bombarded leaf cultures. The PT9 line incorporated each of the transgenic markers except the XbaI site at the end of the plastid DNA sequence in plasmid pJS75, whereas the PT69 line incorporated all of the markers (see Figure 1). In this respect, transplastomic lines obtained after transformation with plasmid pJS75 are similar to Chlamydomonas plastid transformants in which incorporation of all or nearly all of the transforming DNA is the most common event (Newman et al., 1990). Recovery of each of the six RFLP markers in plants without an apparent phenotypic consequence confirms that we have succeeded in selecting sites for oligonucleotide insertions and point mutations that will not result in interference with normal functioning of the plastid genome. Expansion of the tightly organized plastid genome by inserting foreign genes should, therefore, be feasible as long as the insertion sites are carefully chosen. The DraI and ScaI sites between the antibiotic resistance genes are such potential insertion sites in plasmid pJS75. Insertion of foreign genes into the Chlamydomonas plastid genome has already been reported by Blowers et al. (1989) and Goldschmidt-Clermont (1991).

In the primary calli, almost all of the pJS75 markers were recovered in homoplasmic form. In the PT9 clone one type and in the PT69 clone two different types of transgenic plastid genomes were found. Formation of these genomes may be explained by transformation of one copy of the repeat region followed by correction of the second copy fully or in part, according to the transgenic template, as shown in Figure 6. Lack of wild-type plastid genome copies in the primary callus means that correction of the second copy of the repeat region immediately followed transformation and that sorting out of the wild-type plastid genome copies has been complete. The only instance of heteroplasmy in this study was the mix of two different transgenic genomes in the PT69 line. Sorting out eventually led to homoplasmy for this marker as well. Heteroplasmy for the PstI marker is most simply explained by differential copy correction (Figure 6B).

Homoplasmy for the RFLP markers in general and the lack of segregation for streptomycin resistance in the seed prog-

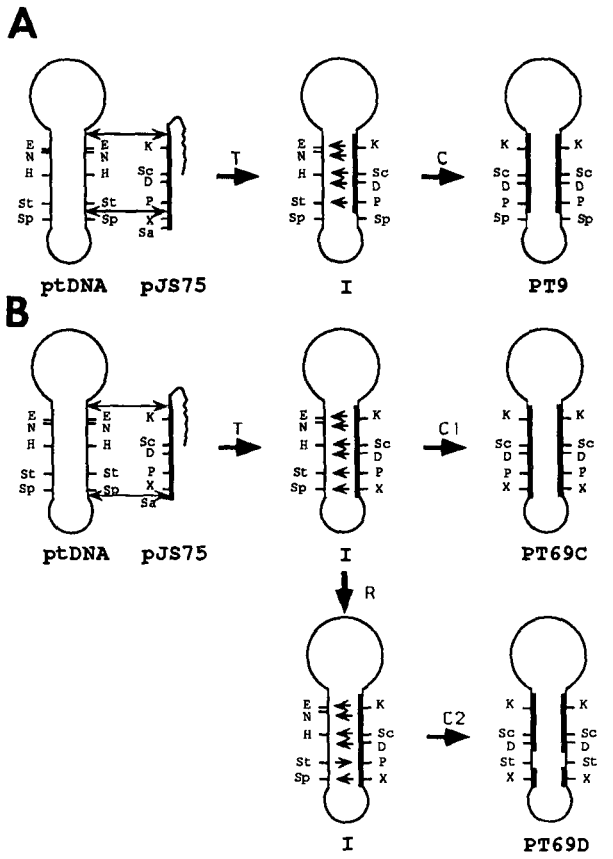

Figure 6. Models To Explain Formation of Transgenic Plastid Genomes.

(**A**) Model for transformation of the PT9 plastid genome. Initial interaction of recipient wild-type plastid genome (ptDNA) with plasmid pJS75 leads to transformation (T) of one of the repeats by replacement of wild-type sequences (thin line) with transforming DNA (bold line). Inverted repeat sequences of ptDNA are paired such that large and small single copy regions form loops. Note that plasmid pJS75 was linearized at the SalI site. In the transformation intermediate (I), copy correction (C) results in two identical copies of the repeat region.
(**B**) Differential copy correction could yield the PT69 plastid genomes. Transformation (T) results in an intermediate (I) with one transgenic and one wild-type copy of the repeated region as for PT9, except that initially all of the markers are incorporated. Copy correction (C1) in one copy of the I genome stabilizes all of the markers yielding the genome of the PT69C subclone. Copy correction (C2) in a second, replicated (R) I genome removes the transgenic PstI (P) site but preserves all of the other transgenic markers yielding the genome of the PT69D subclone.
The template used for correction of RFLP markers is indicated by arrows inside the I genome. (**A**) and (**B**) are not drawn to scale. RFLP markers: D, DraI; E, EcoRV; H, HincII; K, KpnI; N, NciI; P, PstI; Sa, SalI; Sc, ScaI; Sp, SphI; St, StyI; X, XbaI.

eny of pJS75 transplastomic lines are in contrast to the extensive segregation for streptomycin resistance after transformation with plasmid pZS148, as reported by Svab et al. (1990). Streptomycin resistance in plasmid pJS75 is encoded in the *rps12* gene, whereas in plasmid pZS148 it is due to a mutation in the *16SrDNA* gene. We assume, therefore, that there is some degree of incompatibility of the spectinomycin and streptomycin resistance mutations in the pZS148 lines but not in the pJS75 lines. Accordingly, transformation with plasmid pZS148 by Svab et al. (1990) and selection for spectinomycin resistance resulted in incorporation of both of the unselected flanking markers—streptomycin resistance and the PstI RFLP markers. The streptomycin resistance marker was copy corrected in some genomes to the sensitive phenotype, and the spectinomycin-resistant/streptomycin-sensitive molecules were apparently at a selective advantage during the sorting out process.

It is interesting to note that in Chlamydomonas the rates of growth and photosynthesis are adversely affected in a spectinomycin- and streptomycin-resistant transformant resulting from two alterations introduced into the *16SrDNA* gene (Heifetz et al., 1991; P. Heifetz, A. Lers, J. Boynton, N. Gillham, and B. Osmond, personal communication). These effects, detected in quantitative physiological studies of bright light–grown cells of the spectinomycin- and streptomycin-resistant transformant and the standard wild-type strain, were not evident when the two genotypes were grown on agar plates. A subtle mechanism such as this could account for selection against the genomes that carry the *16SrDNA*-encoded spectinomycin and streptomycin resistance mutations in the polyploid genetic system of tobacco plastids, providing an explanation for the frequent loss of the unselected streptomycin resistance marker reported by Svab et al. (1990).

Plasmid pJS75, with cloning sites between antibiotic resistance markers, is a plastid insertion vector. The utility of this vector has been shown by the introduction of a chimeric *uidA* gene into the repeat region of the tobacco plastid genome (J. Staub and P. Maliga, unpublished data).

METHODS

Plant Lines

The recipient for transformation was wild-type *Nicotiana tabacum* cv Petit Havana (tobacco). The *N. tabacum* SPC1 line is a spontaneous spectinomycin-resistant mutant due to an A-to-C change at position 1138 of the plastid 16S rRNA (Svab and Maliga, 1991).

Construction of Vector pJS75

Vector pJS75 contains a 6.2-kb HindIII/PstI DNA fragment derived from the SPC1 line of *N. tabacum* in plasmid pUC119 (Vieira and Messing, 1987). The HindIII site at position 136,330 of the *N. tabacum*

plastid genome was converted to a SalI site by ligation of the synthetic linker 5′-pGGTCGACC-3′ into the blunted HindIII site. The numbering is according to the sequence reported by Shinozaki et al. (1986). The SphI site at position 137,158 was similarly converted to an XbaI site by ligation of the linker 5′-pGCTCTAGAGC-3′. Synthetic oligonucleotide 5′-pGCTGCAGC-3′ encoding a PstI site was ligated into the blunt-ended StyI site as described by Svab et al. (1990). The DraI site was created by looping in 3 bp (underlined) at position 139,968 using synthetic oligonucleotide 5′-pCGACGGCGGGGGAGCTTT-AAATCCAACTAGCAAAAACTCAC-3′ as the template for site-directed mutagenesis (Kunkel, 1985). The ScaI site was created by ligation of oligonucleotide 5′-pAAAGTACTTT-3′ into the HincII site at position 140,219. Mutations (underlined) for streptomycin resistance (C to T, codon 90 of *rps12*, position 141,833) and the KpnI site were created by site-directed mutagenesis using synthetic oligonucleotide 5′-pAA-GGGTTAAGGATTTATCCGGTGTGAGGTACCACATTGTTCGAGGAA-CCC-3′. All constructions were verified by sequencing.

Transformation and Regeneration of Transgenic Plants

Leaves of aseptically grown plants from Murashige and Skoog (MS) media were used for bombardment. The MS medium was agar-supplemented with MS salts (Murashige and Skoog, 1962) and sucrose (30 g/liter). Leaves were placed abaxial side up on RMOP media for bombardment. 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 microprojectiles (1 μm) were coated with DNA according to Gordon-Kamm et al. (1990). Bombardment was performed with the Du Pont PDS1000 gun-powder charge Biolistic gun according to Klein et al. (1988) with the added modification of 1000 and 100 μm wire mesh between the stopping plate and the sample (Gordon-Kamm et al., 1990).

Two days after bombardment, the leaves were cut into sections and transferred to RMOP media containing 500 μg/mL of spectinomycin dihydrochloride. A green callus that formed on a bleached leaf section was termed a "clone." Green calli were subcultured onto the same selective medium for shoot induction. The shoots were rooted individually on MS media to obtain plants. Individual shoots derived from the primary callus were considered independent subclones.

DNA Gel Blots and Probing of Total Cellular DNA

Total cellular DNA was prepared by the method of Mettler (1987). Restriction-enzyme–digested DNA was electrophoresed on 0.7 or 1.1% agarose gels and transferred to nylon membrane (Amersham Corp.) using the PosiBlot Transfer apparatus (StrataGene). DNA gel blot probing was performed using Rapid Hybridization Buffer (Amersham Corp.) with ^{32}P-labeled probes generated by random priming (Boehringer Mannheim).

Testing of Seedling Phenotype

Seedling phenotype was determined by plating surface-sterilized seeds on MS medium. Filter-sterilized antibiotics were added to the medium after autoclaving. On the selective antibiotic-containing media, resistant progeny are green, whereas sensitive progeny are white (Maliga et al., 1990). Spectinomycin alone was used at a concentration of 500 μg/mL, whereas spectinomycin and streptomycin together were used at 100 μg/mL each. Mutation in the *rps12* ribosomal protein was reported to confer resistance to 600 μg/mL streptomycin by Galili et al. (1989). The *rps12* mutation, combined with the SPC1 spectinomycin resistance mutation in the transplastomic seedlings, conferred resistance only to 300 μg/mL streptomycin alone. This is not surprising because mutations in different regions of the rRNA may interact (reviewed by Dahlberg, 1989). Transplastomic seedlings were tested for antibiotic resistance at the lowest level of spectinomycin and streptomycin, 100 μg/mL, that allows distinction from wild-type and SPC1 single spectinomycin-resistant mutant seedlings.

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