Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 1485–1489, March 1985
Genetics

Efficient induction and selection of chloroplast-encoded antibiotic-resistant mutants in *Nicotiana*

(*N*-nitroso-*N*-methylurea/plant cell culture/protoplast fusion/*in organello* protein synthesis/cytoplasmic inheritance)

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Communicated by Charles J. Arntzen, November 1, 1984

ABSTRACT **A high rate of plastome-encoded mutations was induced in *Nicotiana* by exposing seeds to *N*-nitroso-*N*-methylurea. Seeds then were subjected to nutritional and *in vitro* selection procedures for systematic isolation of plastome-dependent antibiotic-resistant plants. Multiple flowering lines resistant to streptomycin, spectinomycin, lincomycin, or chloramphenicol were obtained. A detailed analysis of the streptomycin-resistant lines is presented. Sexual hybridization, cybrid formation following protoplast fusion, and *in organello* protein synthesis were used to rigorously assign the mutations to the chloroplast genome. The efficient rates of mutagenesis combined with the *in vitro* mass-screening procedures described here should facilitate investigation of fundamental aspects of chloroplast genetics in higher plants.**

An extensive body of literature, dating back more than half a century, exists on chloroplast inheritance in higher plants (1). The use of nuclear genes capable of increasing plastome (i.e., chloroplast) mutation rates (2) and studies with exceptional biparentally inherited plastids (3) continue to contribute to our knowledge of organelle inheritance. Likewise, recent advances in molecular analysis of the chloroplast genome (4) and the development of protoplast-fusion methodologies for plants (5) have paved the way to a more thorough understanding of plastome genetics and nuclear-genome/plastome interactions. Nevertheless, hypotheses about the rules governing chloroplast genetics usually are the result of extrapolation from the alga *Chlamydomonas* rather than based on intrinsic advancements with higher plants (1).

Spontaneous plastome-dependent variegations in plants occur at a frequency of about 5×10^{-4}; however, the spontaneous mutation rate for specific traits is estimated to be less than 10^{-9} (2). Consequently, successful isolation of specific mutants probably requires a plastome-targeted mutagenic agent and a selection scheme that allows sorting-out of the multicopy plastome in the somatic dividing cells. Mutagenesis followed by selection for specific plastome-encoded traits in callus culture has been studied in the genus *Nicotiana*. The results to date are one lincomycin- (6) and two streptomycin-resistant (7, 8) cell lines. In this report we evaluate the chemical mutagen *N*-nitroso-*N*-methylurea (NMU), which efficiently causes plastome lesions (9), and cell culture and nutritional procedures that potentiate mutant expression during plastid segregation in the developing plant. The combined application of these procedures has resulted in the isolation of numerous *Nicotiana* mutant cell lines resistant to streptomycin, spectinomycin, lincomycin, or chloramphenicol.

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MATERIALS AND METHODS

Plant Material. *Nicotiana tabacum* cultivar (cv.) Xanthi is a self-fertile line. *N. tabacum* line 92 is a cytoplasmic male-sterile, alloplasmic substitution line containing *N. undulata*-type cytoplasm (10). Clonal line VBW, containing albino plastids (10), is from a variegated *N. tabacum* mutant, Dp1, containing defective plastids in the pale sectors of its leaves (11).

Mutagenesis, Selection, and Growth. A stock solution of 100 mM NMU (Sigma) in 70% ethanol/0.1% acetic acid was diluted 20-fold with water before use. Seeds were soaked in 5 mM NMU for 2 hr at room temperature, surface-sterilized by immersion in 3% (wt/vol) sodium hypochlorite for 20 min, and rinsed thoroughly with sterile water. Seed germination and rooting took place in Nitsch medium (12) containing 1% agar (Nitsch agar) and, for selection, one of the following antibiotics (Sigma): streptomycin (1 mg/ml), spectinomycin (50 μg/ml), or lincomycin (500 μg/ml). For regeneration of cotyledon explants, sections containing green islands of cells (13) were transferred to Murashige–Skoog medium (14) containing 1% agar (MS agar), indole-3-acetic acid (0.8 μg/ml), kinetin (2 μg/ml), and antibiotics as above, except that lincomycin was used at 50 μg/ml. After shoot regeneration from explants, rooting was accomplished on Nitsch agar without antibiotics.

In the case of chloramphenicol selection, seeds were germinated on Nitsch agar without antibiotic. Explants from the resulting cotyledons were then regenerated in the presence of D-*threo*-chloramphenicol (40 μg/ml) on MS agar containing indole-3-acetic acid (0.8 μg/ml) and kinetin (2 μg/ml). Rooting took place on Nitsch agar without antibiotic. All rooted plants were potted in peat moss and autotrophically grown to maturity in the greenhouse.

Protoplast Fusion and Plant Regeneration. See Galun and Aviv (15) for a general discussion. Mesophyll protoplasts were isolated as detailed in ref. 16. Protoplasts (10^5 per ml) from line str-92-7 (see Table 2) were x-irradiated at 5000 rad, whereas VBW protoplasts (10^5 per ml) were incubated in 0.25 mM iodoacetic acid for 30 min. After treatments, the protoplasts were fused and cultured as detailed in ref. 17. Finally, small colonies were transferred to MS agar containing streptomycin (1 mg/ml). Green calli that proliferated were transferred to MS agar containing indole-3-acetic (0.8 μg/ml) and kinetin (2 μg/ml) for shoot regeneration. Shoots were rooted in Nitsch agar (12), and resulting plants were potted in peat moss.

Chloroplast Markers. Isolation of *N. tabacum* whole cell DNA and hybridization with chloroplast DNA probes were

Abbreviation: NMU, *N*-nitroso-*N*-methylurea.

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as described (10). The tentoxin leaf assay is detailed in ref. 18.

***In Organello* Protein Synthesis.** Chloroplasts were isolated at 4°C, according to the procedure of Colijn *et al.* (19). Young leaves (10 g) were deribbed and then homogenized by grinding in 100 ml of buffer with three 5-sec bursts at 15,000 rpm (freely rotating speed) in a Waring Blendor with razor blades for cutting edges. Homogenization buffer contained 300 mM sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $Na_4P_2O_7$, 5 mM L-ascorbic acid, and 50 mM Hepes/NaOH (pH 6.8). The homogenate was filtered in a single step through four layers of gauze and three layers of nylon net (Nytex) of 135, 100, and 30 μm pore sizes and centrifuged at 2500 × *g* for 1 min. The pellet was resuspended in 1 ml of 330 mM sorbitol/50 mM Tricine/KOH (pH 8.3), 200 μCi (7.4 MBq) of [^{35}S]methionine (1000 Ci/mmol; Amersham) was added per ml, and the suspension was incubated at 3000 lux at 25°C for 40 min. Reactions were terminated by adding 0.5 volume of 3× $NaDodSO_4$/PAGE sample buffer (20). Gel electrophoresis and fluorography were performed as described (21).

RESULTS

Selection Schemes for Plastome-Dependent Antibiotic Resistance. Initial tests were conducted to integrate conditions for NMU mutagenesis, seed viability, and chloroplast-genome expression. Viability of *N. tabacum* cv. Xanthi seeds mutagenized with 1–10 mM NMU was not impaired when germination was on a nutrient medium containing sucrose (Nitsch medium). However, viability decreased progressively with mutagen concentration (to 10% for seeds treated with 10 mM NMU) when seeds were germinated autotrophically (in peat moss). Moreover, 95% of the plants mutagenized with 10 mM NMU had variegated first true leaves when grown on Nitsch medium, whereas many of the autotrophically germinated survivors did not. In a preliminary experiment (22), 35 variegated seedlings were grown to sexual maturity after seed mutagenesis in 5 mM NMU and germination on Nitsch agar. Reciprocal crosses revealed that the pigment mutation was maternally inherited in 14 plants and transmitted biparentally in only 4. The mode of inheritance in the remaining 17 plants could not be determined, as variegation in these cases did not extend to the reproductive tissue. These high rates of maternally inherited variegation encouraged us to seek efficient procedures for selecting specific plastome mutations, starting with resistance to various antibiotics.

Our strategy, schematically outlined in Fig. 1, was based on using heterotrophic nutrition to sustain vigorous growth of non-photosynthesizing tobacco seedlings in culture. It was reasoned that expression of photosynthetically competent, antibiotic-resistant tissue would be readily recognized during somatic segregation against a background of bleached cells. Subsequent transfer of the seedlings containing green areas to autotrophic culture would then ensure development and survival of the mutant.

Fig. 2 shows typical results obtained at several stages of the selection and testing regimen, and Table 1 summarizes quantitative aspects of selection for antibiotic resistance in *N. tabacum*. Invariably, when nonmutagenized (control) seeds were incubated in the light on Nitsch agar containing the levels of antibiotic shown in Table 1, the cotyledons and true leaves were fully bleached. However, two phenomena were observed consistently, when NMU-treated seeds were incubated under these conditions: (*i*) 20–90% of the cotyledons (depending on the antibiotic) were spotted with green islands on a pale-green to bleached background (e.g., Fig. 2*A*) and (*ii*) 1–2% of the seedlings showed green sectors in their first true leaves (e.g., Fig. 2*B*).

The Nature of the Green Areas. The green islands were microscopically analyzed and identified as cell clusters containing pigmented chloroplasts. Explant regeneration was used to help establish whether these clusters resulted from the presence of mutated chloroplasts or were caused by transient, epigenetic phenomena. Almost all of the cotyledon segments with green islands regenerated shoots having green or variegated leaves in selective media (Fig. 2 *C* and *D*). Fully bleached cotyledons from control plants showed either no shoot-regenerating capacity (Fig. 2*D*, bottom row) or, oc-

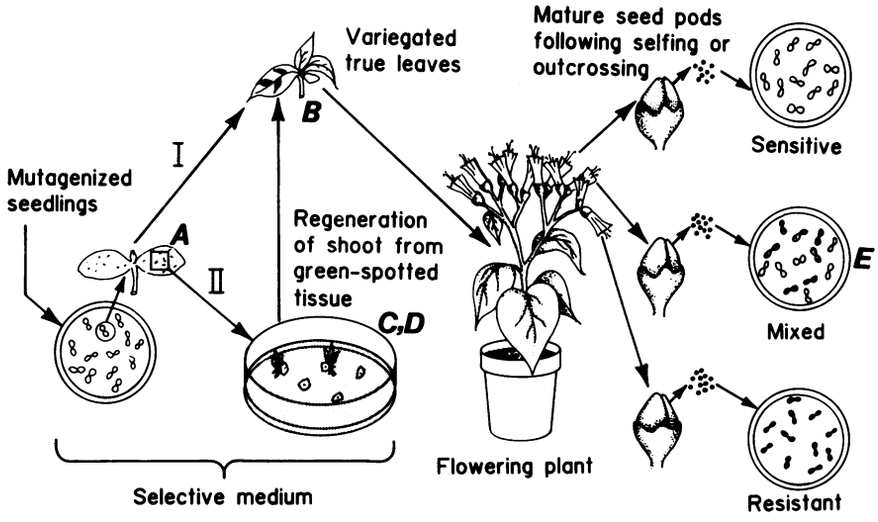

FIG. 1. Schematic outline of selection and testing regimens for antibiotic-resistant plastome mutants. Seeds treated with NMU were germinated in heterotrophic media containing antibiotic (selective medium) to produce mutagenized seedlings. In procedure I, seedlings were grown to the first true-leaf stage. Those plantlets containing variegated true leaves were transferred to heterotrophic, nonselective medium and, when sufficiently grown, were potted to obtain the mature, flowering plant. Seeds were produced by selfing (*N. tabacum* cv. Xanthi) or outcrossing (*N. tabacum* line 92). M_2 seeds from mature pods were germinated in the appropriate selective medium to test for non-Mendelian inheritance patterns and to screen for antibiotic resistance. In procedure II, tissue from cotyledons containing green islands was excised and transferred to shoot-regeneration medium containing antibiotic. Resistant shoots were rooted in nonselective medium and then potted to produce the mature flowering plant as above. Both procedures generally required 4–6 months from mutagenesis to the flowering plant stage. The bold italic letters cross-reference stages pictured in Fig. 2.

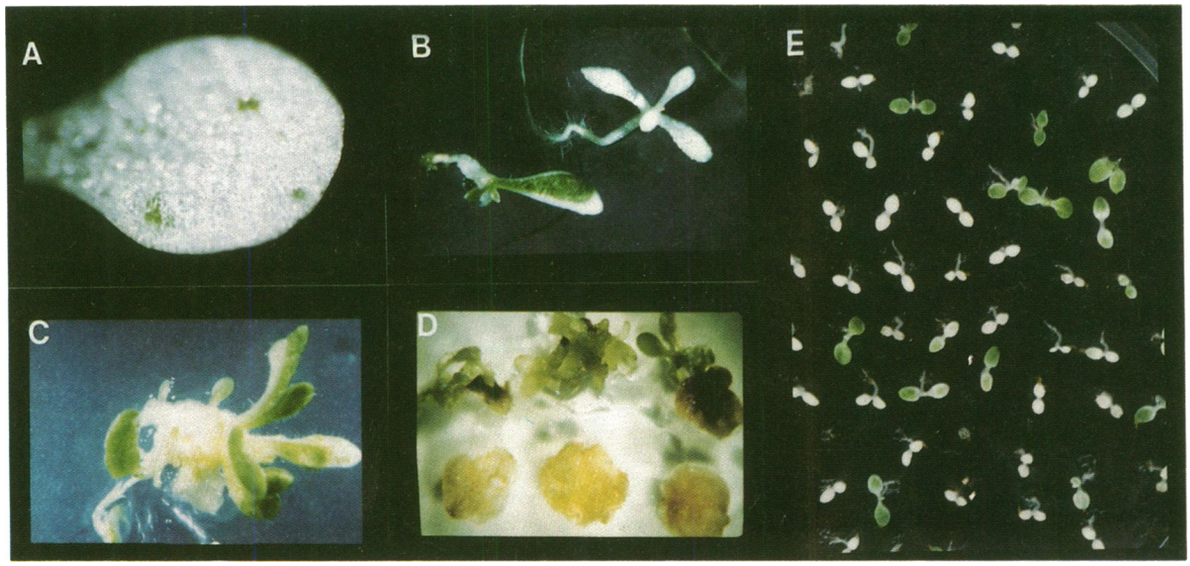

FIG. 2. Stages in selection and propagation of tobacco plastome mutants resistant to antibiotics. Seeds from *N. tabacum* line 92 were treated with 5 mM NMU and plated on Nitsch agar as detailed in *Materials and Methods*. (*A*) Cotyledon from mutagenized seedling grown in streptomycin (1 mg/ml). Green islands are composed of clusters of antibiotic-resistant, chlorophyll-containing cells against a background of bleached cells. (*B*) Pattern of lincomycin resistance in the first true leaves of a mutagenized seedling grown in the presence of antibiotic at 500 μg/ml. Lower seedling shows a typical pattern of green, antibiotic-resistant areas in its true leaves. Upper seedling, from the same experiment, does not exhibit such regions. Green islands were not found in the cotyledonary tissues because the concentration of lincomycin required to effectively inhibit chlorophyll synthesis in true leaves is an order of magnitude higher than that needed for cotyledon bleaching (cf. Table 1). (*C*) Regeneration of green, spectinomycin-resistant shoots from callus tissue that developed on a cotyledonary explant grown in medium containing the antibiotic at 50 μg/ml. (*D*) Regeneration of cotyledonary explants in the presence of D-*threo*-chloramphenicol. Both mutagenized and control seeds were germinated in the absence of antibiotic. After 1 week, cotyledonary explants were plated in shoot-regeneration medium containing antibiotic at 40 μg/ml. Upper row shows calli originating from three independent explants from mutagenized seeds, and lower row, three explants from nonmutagenized seeds. Note absence of regeneration in nonmutagenized explants. (*E*) Seed test shows non-Mendelian inheritance of streptomycin resistance in M_2 seedlings. Putative mutant plant str-92-2 (see Table 2) was outcrossed with normal pollen from *N. tabacum* cv. Xanthi. Mature seeds from a single pod then were germinated on Nitsch agar containing streptomycin at 1 mg/ml. Note the mixture of sensitive, variegated, and resistant seedlings characteristic of plastome mutations.

casionally, chlorotic shoots. Thus, the green tissue in the mutagenized seedling suggested the presence of mutated, antibiotic-resistant cells in the developing plantlet.

Plantlets obtained by procedure I or II (cf. Fig. 1) were transferred to nonselective media, grown to potting stage and brought to flowering in the greenhouse. M_2 seeds from pollinated flowers then were tested in selective media (e.g., Fig. 2*E*) to determine the patterns of inheritance. The analyses used to prove the plastomal origin of the mutations will be illustrated for the streptomycin-resistance character. The results for 10 independently selected and, putatively, streptomycin-resistant plants are summarized in Table 2. The inheritance pattern of antibiotic resistance was cytoplasmic in character. The resistance mutation was maternally transferred to the seed progeny of 7 plants. Among these, 2 pure-line mutants were obtained (str-92-7 and str-92-8) whereas for the other 5, mixed phenotypes were observed, indicating a non-Mendelian character in the process of sorting-out.

Table 1. Antibiotic-resistant areas in cotyledons and first true leaves of mutagenized *N. tabacum* line 92

Selective agent	M_1 cotyledons exhibiting green islands,* %	M_1 first true leaves exhibiting green sectors, %
Streptomycin,† 1000 μg/ml	90	1.0
Spectinomycin,† 50 μg/ml	60	1.2
Lincomycin, 50 μg/ml	60	1.9‡
Chloramphenicol, 40 μg/ml	20	—

Seeds were treated with NMU and plated on Nitsch agar containing the indicated antibiotic, as described in *Materials and Methods*. Antibiotic concentrations chosen were the lowest that caused complete bleaching (<1% chlorophyll) of cotyledons or first true leaves of nonmutagenized seedlings. In the case of chloramphenicol, the lowest concentration that prevented regeneration of nonmutagenized explants was used. One thousand mutagenized seedlings were analyzed with each selective agent, except in the case of chloramphenicol, for which 50 cotyledons were screened. No green islands or sectors were detected in 1000 control seedlings grown in the presence of selective agents.

*Scored under the dissecting microscope as having at least one green island per cotyledon.

†Similar yields were obtained in parallel experiments using *N. tabacum* cv. Xanthi.

‡Resistant sectors in first true leaves were assayed on lincomycin at 500 μg/ml.

Production of Cybrids Through Protoplast Fusion. *N. tabacum* line 92 is cytoplasmic male-sterile. Therefore, reciprocal crosses could not be performed with the plants described in Table 2. To substantiate the genetic site of induced resistance, organelles were transferred from protoplasts of resistant plants to protoplasts of sensitive plants by the "donor–recipient" fusion method (15). In this technique, x-irradiation inhibits nuclear division in the donor, and iodoacetate treatment prevents proliferation of the recipient cells. The fused products that survive are expected to contain nuclear characters of the recipient and cytoplasmic traits of both parental cells. Somatic sorting-out during subsequent cell divisions enables cytoplasmic-marker analysis.

Table 3 presents the results of a fusion experiment between donor protoplasts, isolated from M_2 progeny of line str-92-7, and recipient protoplasts, isolated from line VBW (which contains albino plastids). Five calli greened on media containing streptomycin (1 mg/ml). Each of these was transferred to shoot-regeneration and rooting media, and 40 re-

Table 2. Streptomycin resistance in M_2 seedlings after NMU mutagenesis

Plant designation	Reaction of M_2 cotyledons to streptomycin: No. of seedlings Sensitive (bleached)	Resistant (green)	Mixed (variegated)	% resistance*
str-92-1	274	40	2	13
str-92-2	386	42	21	14
str-92-3	276	0	0	0
str-92-4†	8	171	12	96
str-92-5	50	0	0	0
str-92-6†	45	348	53	90
str-92-7†	0	193	0	100
str-92-8†	0	352	0	100
str-92-9	230	0	0	0
str-92-10	118	3	15	13
Control	400	0	0	0

Seeds of *N. tabacum* line 92 were treated with NMU, germinated in the presence of streptomycin and grown to sexual maturity as outlined in procedure I, Fig. 1. Flowers of plants from 10 independent regeneration events were pollinated with *N. tabacum* cv. Xanthi. The seeds (M_2) from 5–10 pods of each maternal parent then were germinated on Nitsch agar containing streptomycin at 1 mg/ml. Analysis was performed at the cotyledon stage. Seeds of untreated *N. tabacum* line 92 served as control.

*% resistance = (Resistant + Mixed) × 100/total number of tested seedlings.

†Although all seedlings scored as resistant had green cotyledons in streptomycin at 1 mg/ml, two classes of resistance in first true leaves were noted. Leaves from M_3 progeny of str-92-4 and str-92-6 were resistant to streptomycin at 0.6 mg/ml, whereas those from str-92-7 and str-92-8 were resistant to 2.0 mg/ml. Progeny of other mutant plants were not tested.

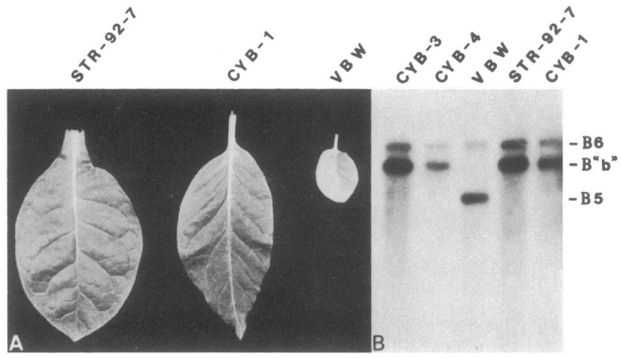

FIG. 3. Characterization of cybrids resulting from fusion of lines str-92-7 and VBW. (*A*) Leaf type. The leaf from str-92-7 is sessile at its base whereas both the small albino leaf from VBW and the normal chlorophyllous leaf from the cybrid cyb-1 are petiolated at their base. (*B*) Chloroplast DNA fragment patterns. Chloroplast DNA fragment *Bam*HI-7 from tobacco (23) is capable of distinguishing between *Bgl* I fragmentation patterns of *N. undulata*-type (e.g., line str-92-7) and *N. tabacum*-type (e.g., line VBW) chloroplast DNA (24). *Bgl* I digests of total DNA extracted from leaves of parental-line plants VBW and str-92-7, cybrid calli cyb-3 and cyb-4, and leaves of cybrid plant cyb-1 were electrophoresed on agarose gel, transferred to nitrocellulose, hybridized with ^{32}P-labeled *Bam*HI-7 from *N. tabacum*, and autoradiographed. *Bgl* I fragment designations for tobacco chloroplast DNA are taken from ref. 24.

sultant plantlets were potted and transferred to the greenhouse. Two of the plants showed some degree of variegation, likely due to the residual presence of albino plastids of line VBW, and were not included in the subsequent marker-segregation analysis. The remaining 38 cybrid plants had petiolated leaves, a nuclear character of line VBW (10), which can be readily distinguished from the sessile leaves of line str-92-7 (Fig. 3*A*). Likewise, 7 out of 8 plants analyzed had 48 somatic chromosomes, the normal diploid number of the parental lines. When plastid markers were tested, the cybrid plants exhibited the chloroplast characteristics of line str-92-7 (the organelle donor). This was true for DNA fragment analyses (Fig. 3*B*), reaction to tentoxin, and leaf pigmentation patterns. An additional parameter, cytoplasmic male sterility, which is probably under mitochondrial control (25), was also determined for the cybrid plants. All cybrids derived from the 5 original calli turned out to be male-fertile, a characteristic associated with line VBW, the recipient parent. This agrees with our previous findings (26) of independent transfer of chloroplasts and mitochondria by the donor–recipient fusion method. In summary, the data in Table 3 indicate that true cybrids were obtained and that streptomycin resistance cotransferred with plastomal rather than chromosomal or mitochondrial traits.

Antibiotic Resistance in Isolated Chloroplasts. Direct evidence linking NMU-induced streptomycin resistance with the chloroplast was obtained from protein synthesis experiments *in organello*. Fig. 4 shows the major polypeptides synthesized by isolated chloroplasts from line 92 (streptomycin sensitive) and line str-92-7 (streptomycin resistant): these are the α and β subunits of the proton ATPase, the large subunit (LS) of ribulosebisphosphate carboxylase/oxygenase, and the 32-kDa photosystem II membrane protein. Protein synthesis was cycloheximide-resistant, D-*threo*-chloramphenicol-resistant, and streptomycin-sensitive.

Table 3. Analysis of cybrids obtained by the donor–recipient fusion method

Plant line	Genetic markers: Streptomycin test*	Leaf morphology†	Plastid-DNA fragment pattern‡	Tentoxin test§	Plastid pigmentation	Fertility
str-92-7 (organelle donor)	Resistant	Sessile	*undulata*	Sensitive	Green	Sterile¶
VBW‖ (organelle recipient)	Sensitive	Petiolated	*tabacum*	Resistant	White	Fertile
Cybrids (5 independent lines)	Resistant	Petiolated	*undulata*	Sensitive	Green	Fertile

Protoplasts from *N. tabacum* lines str-92-7 and VBW were fused, cybrids were cultured, and plants were grown as described in *Materials and Methods*. Cybrid calli and plants were compared to the parental lines with respect to several genetic markers. At least one plant from each of five independent cybrid calli was tested.

*Performed on M_2 and M_3 seeds as described in the legend to Table 2. Line str-92-7 was pollinated by *N. tabacum* cv. Xanthi, and cybrid lines, by selfing.

†Leaf morphology (see Fig. 3*A*) is a nuclear genetic marker (10).

‡See Fig. 3*B*.

§Performed as detailed in ref. 18 on both leaves and seedlings of line str-92-7 and on plants derived from the five independent cybrid lines.

¶Flowers from M_2 plants of line str-92-7 had femalized anthers. Their pistils were pollinated with *N. tabacum* cv. Xanthi and the inheritance pattern of male sterility in the M_3 progeny was determined to be non-Mendelian.

‖Albino line VBW was derived from the green, streptomycin-sensitive, tentoxin-resistant, fertile line *N. tabacum* var. Samsun, in which these markers were tested (ref. 10 and unpublished results).

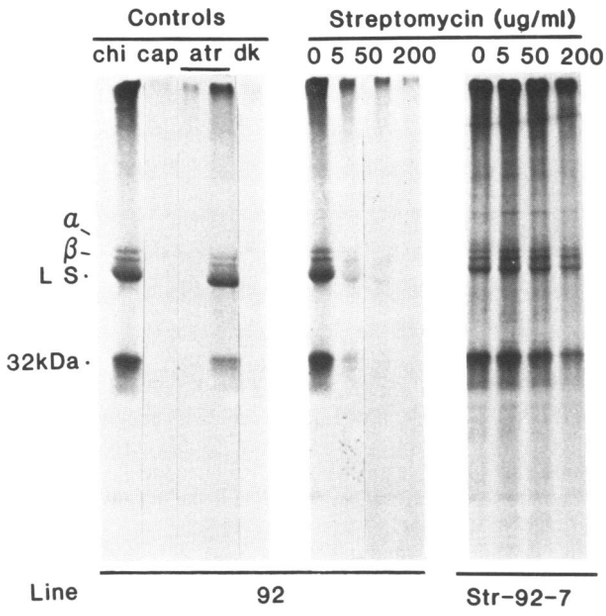

FIG. 4. Protein synthesis in isolated chloroplasts from streptomycin-sensitive and streptomycin-resistant plants. Chloroplasts from young leaves were isolated and incubated in the light with [^{35}S]methionine as described in *Materials and Methods*. Chloroplasts from streptomycin-sensitive line 92 were incubated with the following additions (from left to right): cycloheximide (chi), 20 μg/ml; D-*threo*-chloramphenicol (cap), 100 μg/ml; atrazine (atr), 10^{-5} and 10^{-7} M; no light (dk); or streptomycin at 0, 5, 50, or 200 μg/ml. Chloroplasts from streptomycin-resistant line str-92-7 were incubated with streptomycin at 0, 5, 50, or 200 μg/ml. After incubation, chloroplasts were lysed and equal amounts of starting material were applied to all lanes of a NaDodSO$_4$/10–20% polyacrylamide gradient gel for electrophoresis and fluorography. Positions are indicated for the α and β subunits of the proton ATPase, the large subunit (LS) of ribulosebisphosphate carboxylase/oxygenase, and the 32-kDa photosystem II polypeptide.

col- and atrazine (a photosystem II inhibitor)-sensitive, and light-dependent, indicating translation within intact organelles. Protein synthesis in isolated chloroplasts from line 92 was sensitive to streptomycin at 1/50th the concentration required for equivalent inhibition in isolated chloroplasts from progeny of the resistant line str-92-7. An average IC_{50} for each cell line was obtained by densitometric scanning of protein bands from several experiments (data not shown). Line 92 chloroplasts had an IC_{50} of 4 μg/ml for streptomycin, whereas chloroplasts from NMU-mutated line str-92-7 had an IC_{50} of 200 μg/ml.

DISCUSSION

We have developed a method that combines efficient plastome-mutant induction with sensitive *in vitro* selection procedures and allows systematic isolation of chloroplast-dependent antibiotic-resistant mutants in *N. tabacum*. The streptomycin-resistant mutants have been most completely characterized genetically and biochemically. In addition, using procedure I or II (as illustrated in Fig. 2 *B–D*), we have isolated several stable mutant lines (M_3 generation) resistant to spectinomycin or lincomycin. Putative chloramphenicol-resistant plants have been obtained as well. Proof of the plastomal origin of these mutations will be presented elsewhere. In this work, two classes of streptomycin-resistant plants have been identified: class I, resistant to 0.6 mg/ml, and class II, resistant to 2.0 mg/ml (see explanatory note to Table 2). Four classes of streptomycin resistance previously have been assigned to the chloroplast genetic-linkage map of *Chlamydomonas* by recombination analysis (27). The availability of variant resistant strains and several different antibiotic markers now expands the opportunities for seeking chloroplast recombination in *Nicotiana* via somatic fusion.

As a step in this direction, we recently have obtained results that indicate that tobacco plants resistant to more than one antibiotic can readily be produced by a two-stage procedure. Thus, seeds from streptomycin-resistant line str-92-7 were mutagenized with NMU and germinated in the presence of spectinomycin to yield pure-line streptomycin/spectinomycin double-mutant plants (data to be presented elsewhere). The efficient rates of mutagenesis and mass-screening procedures described in this work suggest that sufficiently large numbers of seedlings can be processed to enable testing for simultaneous selection of such double mutants. Thus, an experimental approach to such fundamental questions in chloroplast genetics as dominance, mutagenic target number, and target size may now be feasible in *Nicotiana*. Finally, our integrated approach is likely to be applicable to other angiosperms, as NMU has been found to be an effective plastome mutagen for seeds of several crop and ornamental plants (9), a number of which are amenable to cell culture techniques. Note, however, that mutagen efficiency may be influenced by the rate of plastome assortment and plastome copy number in each case.

In addition to the antibiotic resistant mutants, we have also isolated a plastome mutant blocked in the assembly of ribulosebisphosphate carboxylase/oxygenase, by using partial chlorophyll deficiency as a marker and screening for altered protein patterns by polyacrylamide gel electrophoresis (22).

The assistance of S. Bleichman and M. Chemla is gratefully acknowledged. This work was supported in part by a grant from the Israeli National Council for Research and Development.

1. Gillham, N. W. (1978) *Organelle Heredity* (Raven, New York).
2. Epp, M. D. (1973) *Genetics* **75**, 465–483.
3. Stubbe, W. & Herrmann, R. G. (1982) in *Methods in Chloroplast Molecular Biology*, eds. Edelman, M., Hallick, R. B. & Chua, N.-H. (Elsevier, Amsterdam), pp. 149–165.
4. Whitfeld, P. R. & Bottomley, W. (1983) *Annu. Rev. Plant Physiol.* **34**, 279–310.
5. Galun, E. (1982) in *Plant Improvement and Somatic Cell Genetics*, eds. Vasil, I. K., Frey, K. J. & Scowcroft, W. R. (Academic, New York), pp. 205–210.
6. Cseplo, A. & Maliga, P. (1982) *Curr. Genet.* **6**, 105–109.
7. Maliga, P., Sz-Breznovits, A. & Marton, L. (1975) *Nature (London)* **255**, 401–402.
8. Umiel, N. (1979) *Z. Pflanzenphysiol.* **92**, 295–301.
9. Hagemann, R. (1982) in *Methods in Chloroplast Molecular Biology*, eds. Edelman, M., Hallick, R. B. & Chua, N.-H. (Elsevier, Amsterdam), pp. 119–127.
10. Fluhr, R., Aviv, D., Galun, E. & Edelman, M. (1984) *Theor. Appl. Genet.* **67**, 491–497.
11. Burk, L. G., Stewart, N. & Derman, H. (1964) *Am. J. Bot.* **51**, 713–724.
12. Nitsch, J. P. (1969) *Phytomorphology* **19**, 389–404.
13. Radin, D. N. & Carlson, P. S. (1978) *Genet. Res.* **32**, 85–89.
14. Murashige, T. & Skoog, F. (1962) *Physiol. Plant.* **15**, 473–497.
15. Galun, E. & Aviv, D. (1983) in *Handbook of Plant Cell Culture*, eds. Evans, D. A., Sharp, W. R., Ammirato, P. V. & Yamada, Y. (Macmillan, New York), Vol. 1, pp. 358–392.
16. Aviv, D., Fluhr, R., Edelman, M. & Galun, E. (1980) *Theor. Appl. Genet.* **56**, 145–150.
17. Aviv, D. & Galun, E. (1985) *J. Hered.*, in press.
18. Galun, E. (1982) in *Methods in Chloroplast Molecular Biology*, eds. Edelman, M., Hallick, R. B. & Chua, N.-H. (Elsevier, Amsterdam), pp. 139–148.
19. Colijn, C. M., Kool, A. J. & Nijkamp, H. J. J. (1982) *Planta* **155**, 37–44.
20. Laemmli, U. (1970) *Nature (London)* **227**, 680–685.
21. Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **76**, 83–88.
22. Fluhr, R. (1984) Dissertation (The Weizmann Institute of Science, Rehovot, Israel).
23. Tassopulu, D. & Kung, S. D. (1984) *Theor. Appl. Genet.* **67**, 185–193.
24. Fluhr, R. & Edelman, M. (1981) *Nucleic Acids Res.* **24**, 6841–6853.
25. Galun, E., Arzee-Gonen, P., Fluhr, R., Edelman, M. & Aviv, D. (1982) *Mol. Gen. Genet.* **186**, 50–56.
26. Fluhr, R., Aviv, D., Edelman, M. & Galun, E. (1983) *Theor. Appl. Genet.* **65**, 289–294.
27. Harris, E. H., Boynton, J. E., Gillham, N. W., Tingle, C. L. & Fox, S. B. (1977) *Mol. Gen. Genet.* **155**, 249–265.