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Mutation proximal to the tRNA binding region of the *Nicotiana* plastid 16S rRNA confers resistance to spectinomycin

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Summary. *Nicotiana tabacum* lines carrying maternally inherited resistance to spectinomycin were obtained by selection for green callus in cultures bleached by spectinomycin. Two levels of resistance was found. SPC1 and SPC2 seedlings are resistant to high levels (500 μg/ml), SPC23 seedlings are resistant to low levels (50 μg/ml) of spectinomycin. Lines SPC2 and SPC23 are derivatives of the SR1 streptomycin-resistant plastome mutant. Spectinomycin resistance is due to mutations in the plastid 16S ribosomal RNA: SPC1, an A to C change at position 1138; SPC2, a C to U change at position 1139; SPC23, a G to A change at position 1333. Mutations similar to those in the SPC1 and SPC2 lines have been previously described, and disrupt a conserved 16S ribosomal RNA stem structure. The mutation in the SPC23 line is the first reported case of a mutation close to the region of the 16S rRNA involved in the formation of the initiation complex. The new mutants provide markers for selecting plastid transformants.

Key words: *Nicotiana tabacum* – Plastid mutants – 16S ribosomal RNA – Spectinomycin resistance – Streptomycin resistance

Introduction

Given the large number of plastid genomes in higher plant cells, the ability to select for the transformed genome in culture is the key to recovery of plastid transformants (Svab et al. 1990). Resistance to inhibitors of protein synthesis, conferred by mutations in the plastid 16SrDNA and 23SrDNA genes, are the most readily available markers for transformation in higher plants. The list of markers in *Nicotiana* species includes resistance to streptomycin (Maliga et al. 1973; Etzold et al. 1987; Fromm et al. 1989), spectinomycin (Fromm et al. 1987) and lincomycin (Cseplo and Maliga 1984; Cseplo et al. 1988). These mutants have been characterized genetically, after plant regeneration, and at the DNA sequence level. Similar mutants are available in *Nicotiana plumbaginifolia* (To et al. 1989), *Solanum nigrum* (McCabe et al. 1989) and *Lycopersicon peruvianum* (Jansen et al. 1990). Higher plant cells in photoheterotrophic culture respond to these drugs by bleaching and retarded growth, but not cell death. Bleaching in cell culture is not lethal because the culture medium containing sucrose dispenses with the requirement for photosynthesis. Resistant mutants are identified by their green color in the selective medium (reviewed in Maliga et al. 1990).

The use of double mutants for plastid transformation is desirable because the frequency of spontaneous mutants is relatively high. The frequency of spontaneous lincomycin-resistant clones is 10^{-4} in protoplast-derived colonies (Cseplo and Maliga 1984); the frequency of streptomycin-resistant clones is 10^{-5} (To et al. 1989). Simultaneous selection for two closely linked markers should essentially eliminate the possibility of selecting spontaneous mutants in transformation experiments, as shown in the unicellular alga *Chlamydomonas* (Newman et al. 1990). In this paper, *Nicotiana tabacum* mutants with two plastome-encoded antibiotic resistance markers are reported. The double mutants were obtained by selection for spectinomycin resistance in SR1, a previously characterized streptomycin-resistant line.

Materials and methods

Cell culture. Mutant selection was carried out in cultures of *Nicotiana tabacum* cv. Petit Havana, and its streptomycin-resistant derivative, the SR1 mutant (Maliga et al. 1973, 1975; Etzold et al. 1987). Protoplasts were isolated from leaves, and cultured in K3 medium as described (Maliga et al. 1988). Callus was induced from leaf sections on RMO medium (Maliga 1984). The RMO medium is the Murashige and Skoog basal medium containing 3% sucrose, indoleacetic acid (2 mg/l) and benzyladenine (0.5 mg/l). Plants from the selected clones were

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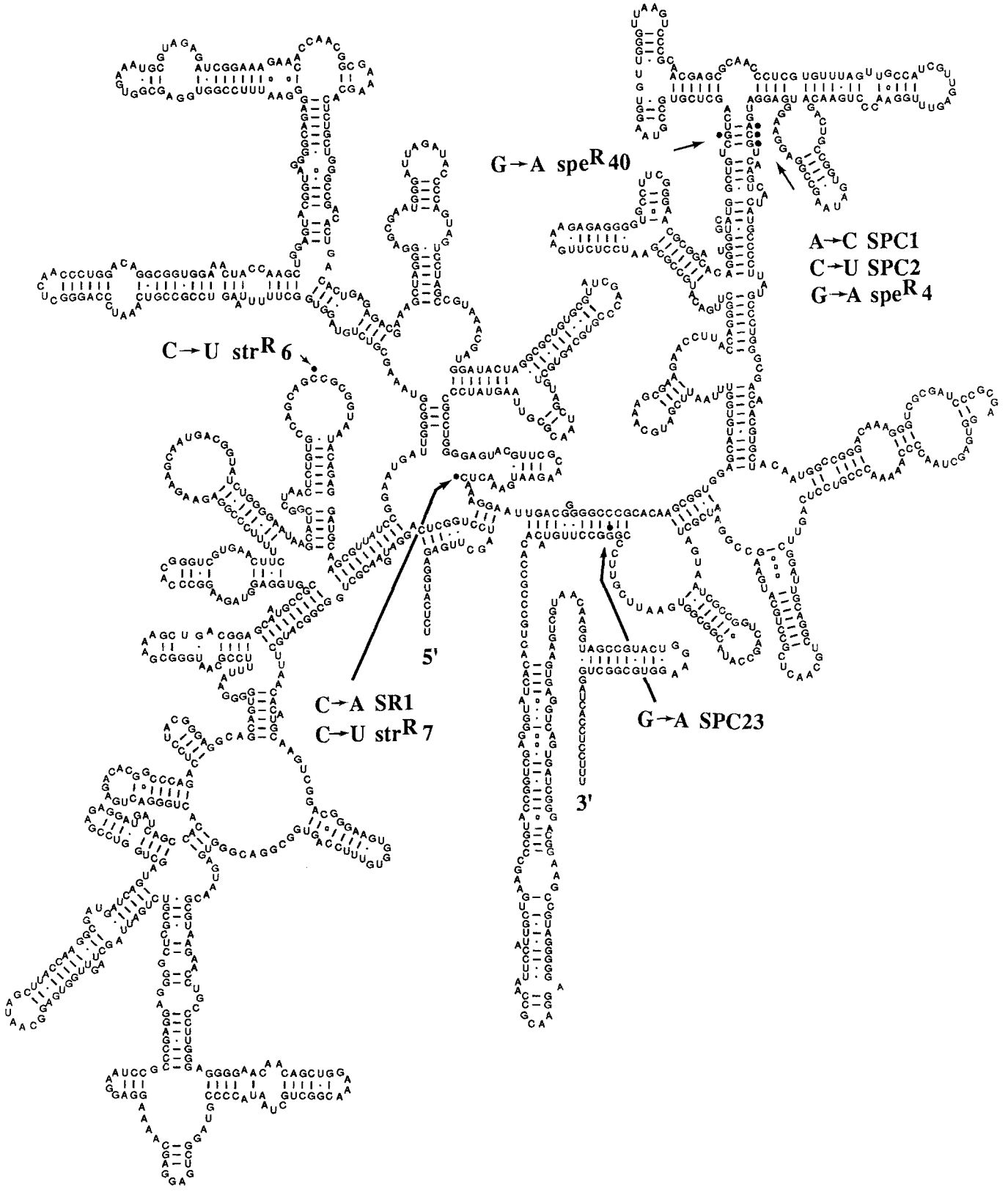

Fig. 1. Secondary structure of 16S chloroplast rRNA of *N. tabacum* (Robin Gutell, unpublished), with mutations for streptomycin and spectinomycin resistance. 16S rDNA gene sequence is from Shinozaki et al. 1986. Spectinomycin resistance mutations in lines SPC1 (nucleotide 1138), SPC2 (1139), SPC23 (1333) are reported in this paper; data on lines *spe*^{R}4 (1140) and *spe*^{R}40 (1012) are from the paper of Fromm et al. 1987. The streptomycin resistance mutation in the SR1 line (860) was reported by Etzold et al. 1987; mutations in the *str*^{R}6 (472) and *str*^{R}7 (860) lines were described by Fromm et al. 1989

obtained by rooting the shoots formed on the RMO medium. Roots formed in sterile culture on a medium containing the Murashige and Skoog salts and 3% sucrose.

Classification of seed progeny. Resistance phenotype of seedlings was determined by germinating surface-sterilized seedlings on MS salts, 1% sucrose (Maliga 1984). The antibiotics spectinomycin dihydrochloride and streptomycin sulfate were filter-sterilized, and added to autoclaved culture medium.

DNA sequencing. Plastid DNA was isolated from greenhouse-grown plants (Kolodner and Tewari 1975). The 16S rDNA was cloned in plasmids pUC118 and pUC119 (Vieira and Messing 1987), and the DNA sequence was determined by the dideoxynucleotide chain termination method using appropriate oligonucleotide primers, and the Sequenase kit (United States Biochemical Corporation).

Results

Isolation of spectinomycin-resistant lines

Callus formed on RMO medium, from leaves or from protoplasts, is green. Spectinomycin, at a concentration of 500 to 1000 μg/ml, prevents greening. Mutant lines SPC1 and SPC2 were identified as green sectors in leaf callus. The SPC1 line was found in a wild-type culture, on a medium containing 500 μg/ml spectinomycin. The SPC2 line was found in the leaf culture of the SR1 mutant, on a medium containing 1000 μg/ml spectinomycin. The SPC23 line was identified among protoplast-derived calli of the SR1 mutant, on a medium containing 500 μg/ml spectinomycin. No attempt was made to determine the frequency of forward mutations to spectinomycin resistance.

Inheritance of spectinomycin resistance

Seed was collected after selfing the regenerated plants, and from crosses with wild-type *N. tabacum*, and tested for antibiotic resistance. Resistant seedlings were green, whereas sensitive seedlings were white on selective media. Selfed SPC1 and SPC2 seedlings are resistant to 500 μg/ml spectinomycin, whereas the SPC23 seedlings are resistant only to 50 μg/ml of the drug. The SPC23 seedlings are somewhat resistant at the 500 μg/ml level since they are yellow rather than white. In crosses, the spectinomycin resistance phenotype is inherited maternally (data not shown). It should be noted that pollen fertility of the SPC23 line is low. Low pollen fertility may be due to the plastome mutations or, more probably, may be the result of independent mutations induced in cell culture.

The two SR1 derivatives, SPC2 and SPC23, were tested for expression of streptomycin resistance. There is no interference between the streptomycin and spectinomycin resistance mutations as judged by the resistance phenotype.

Localization of mutations in the 16S rDNA

The plastid 16SrDNA gene was cloned from the mutants as a 3.2 kb *Bam*HI-*Eco*RV fragment. The entire 16S rDNA sequence was determined, and was compared to the SR1 and wild-type *N. tabacum* 16S rDNA genes. In the 16S rRNA of the SPC lines the following mutations were found: SPC1, an A to C change at position 1138; SPC2, a C to U change at position 1139; SPC23, a G to A change at position 1333 (Fig. 1).

Discussion

The antibiotic spectinomycin interacts with specific regions of the 16S ribosomal RNA and inhibits protein synthesis (Moazed and Noller 1987). Mutations in the SPC1 and SPC2 lines are adjacent, at bp 1138 and 1139, and disrupt a conserved 16S rRNA stem structure. Similar mutations have been described in *Nicotiana* (Fromm et al. 1987; Fig. 1) and in *Chlamydomonas reinhardtii* and *Escherichia coli* (reviewed in Harris et al. 1989). The SPC23 mutation is proximal to the conserved region of the 16S rRNA involved in binding tRNA (Dahlberg 1989). Spectinomycin has been known to inhibit initiating ribosomes (Wallace et al. 1974; reviewed in Dahlberg 1989). However, the SPC23 mutation is the first 16S rRNA mutation located close to the region known to be involved in the formation of the initiation complex. This site of interaction between spectinomycin and the 16S rRNA, the G at position 1333 in the *N. tabacum* 16S rRNA, corresponding to nucleotide 1386 in *E. coli*, was not predicted by chemical footprinting (Moazed and Noller 1987). The SPC23 line has two 16S rRNA mutations: one at position 860 (C to A) that confers streptomycin resistance (Etzold et al. 1987) and a second mutation at position 1333 (G to A) that confers spectinomycin resistance. The streptomycin resistance mutation may be required for the second mutation to confer spectinomycin resistance, since mutations in different regions of the 16S rRNA may interact (reviewed in Dahlberg 1989).

Mutations in the plastid 16S rDNA of the SPC1 and SPC2 lines confer resistance to 500 μg/ml spectinomycin. Resistance is also shown by the SPC23 seedlings, but only to 50 μg/ml of the drug. It is noteworthy that the SPC23 line was isolated by resistance to bleaching at 500 μg/ml of spectinomycin. Apparently, there is no correlation between the levels of resistance shown in culture and in the seedling assay.

Recombination in chloroplasts with a mixed plastome population is extensive (Fejes et al. 1990) and may result in separation of genetic markers upon transformation. The streptomycin and spectinomycin resistance mutations are close together (278 bp apart) in the 16S rDNA gene of the SPC2 line. This line exhibits a high level of resistance to both spectinomycin and streptomycin

and has provided suitable markers for selecting plastid transformants (Svab et al. 1990).

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