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The molecular basis for rRNA-dependent spectinomycin resistance in *Nicotiana* chloroplasts

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The chloroplast genes coding for the 16S ribosomal RNA from several spectinomycin-resistant *Nicotiana* mutants were analyzed. Two classes of mutants were identified. In one class, a G to A base transition is found at position 1140 of the tobacco-chloroplast 16S rRNA gene, which eliminates an *Aat*II restriction endonuclease site. This base transition is proximal to a mutation previously described for spectinomycin resistance in *Escherichia coli*. In the other class, a novel G to A transition is found at position 1012 of the 16S rRNA gene. Although the mutations in the two classes are 128 nucleotides apart, the secondary structure model for 16S rRNA suggests that the two mutated nucleotides are in spatial proximity on opposite sides of a conserved stem structure in the 3′ region of the molecule. Phylogenetic evidence is presented linking this conserved stem with spectinomycin resistance in chloroplasts. Perturbation of the stem is proposed to be the molecular-genetic basis for rRNA-dependent spectinomycin resistance.

Key words: antibiotic resistance/chloroplast mutation/16S ribosomal RNA

Introduction

Spectinomycin is an aminoglycoside antibiotic that blocks protein synthesis on prokaryotic-like ribosomes (Anderson, 1969). Mutants resistant to this antibiotic have been isolated from a wide range of bacteria (Davies *et al.*, 1965; Goldthwaite *et al.*, 1970; Yamada and Davies, 1971), and from the alga *Chlamydomonas* (Gillham, 1965, 1969; Sager and Ramanis, 1971). In *Chlamydomonas*, resistance to this antibiotic is maternally inherited and linked to the chloroplast (Schlanger and Sager, 1974). Maternally-inherited spectinomycin-resistant mutants were also recently reported in the higher plant genus *Nicotiana* (Fluhr *et al.*, 1985).

In bacteria (Davies *et al.*, 1965), and in chloroplasts of *Chlamydomonas* (Schlanger and Sager, 1974), spectinomycin-resistant mutants are altered in the 30S subunit of the ribosome. Two mechanisms were reported to account for spectinomycin resistance in bacteria. In the more common type of mutant, ribosomal protein S5 of the 30S particle (Bollen *et al.*, 1969) had single amino acid substitutions within a restricted region (Funatsu *et al.*, 1971). In a rarer type of mutant (Sigmund *et al.*, 1984), a transition from C to T at position 1192 of a 16S ribosomal RNA gene was detected. This change was sufficient to confer co-dominant or dominant spectinomycin resistance.

Chloroplast ribosomes resemble their bacterial counterparts in monomer and subunit sedimentation coefficients, RNA primary- and secondary-structural characteristics, and sensitivity to several antibiotics (Edelman, 1981). A remarkable resemblance also exists with respect to the amino acid sequence of several ribosomal proteins (Wittmann-Leibold, 1986). However, a gene coding for ribosomal protein S5 has not been identified in the fully sequenced chloroplast genome of *Nicotiana tabacum* (Shinozaki *et al.*, 1986). On the other hand, a sequence of 21 nucleotides (nucleotides 1186-1206; Brosius *et al.*, 1981) surrounding the rRNA mutation site conferring spectinomycin resistance in *Escherichia coli* (Sigmund *et al.*, 1984), is present in the chloroplast 16S rRNA genes of *N. tabacum* (nucleotides 1133-1153; Shinozaki *et al.*, 1986, revised from Tohdoh and Sugiura, 1982). We therefore analyzed the chloroplast 16S rRNA genes from several spectinomycin-resistant mutants of *Nicotiana* in an attempt to pinpoint the molecular basis for this resistance. Our results provide molecular-genetic and phylogenetic evidence for a link between a conserved stem structure in the 3′ region of 16S rRNA and spectinomycin resistance.

Results and discussion

Spectinomycin-resistant mutants are altered in the chloroplast 16S rRNA gene

The two copies of the 16S rRNA gene from chloroplasts of *Nicotiana* reside within *Xho*I fragment 11 in the large inverted repeat region of the genome (Fluhr *et al.*, 1983). The nucleotide sequence of these genes from *N. tabacum* (Shinozaki *et al.*, 1986) showed the presence of a unique *Aat*II recognition sequence (5′ GACGTC 3′) at positions 1137-1142. Since a mutation in the analogous region of a 16S rRNA gene was found in spectinomycin-resistant *E. coli* strains (Sigmund *et al.*, 1984), we analyzed the *Aat*II fragmentation pattern of the spectinomycin-resistant *Nicotiana* mutants.

Chloroplast DNA was isolated from wild-type lines *N. tabacum* var. Xanthi (X/wild-type) and line 92 (92/wild-type), and mutant lines resistant to either streptomycin (92/*str*^{R}7), spectinomycin (92/*spe*^{R}4, 92/*spe*^{R}6, X/*spe*^{R}40), or both antibiotics (92/*str*^{R}7/*spe*^{R}1). DNA from each line was then digested with endonuclease *Xho*I (Figure 1, lanes 1) or *Xho*I + *Aat*II (Figure 1, lanes 2). Digestion with *Xho*I generated the same fragment pattern for all lines. However, digestion with *Xho*I followed by digestion with *Aat*II yielded two classes of patterns. In lines 92/wild-type, X/wild-type, streptomycin-resistant control line 92/*str*^{R}7, and spectinomycin-resistant lines 92/*spe*^{R}6 and X/*spe*^{R}40, *Xho*I fragment 11 (arrow) is digested by *Aat*II. In contrast, in spectinomycin-resistant lines 92/*spe*^{R}4 and 92/*str*^{R}7/*spe*^{R}1, *Xho*I fragment 11 is not digested by *Aat*II. In these two mutants *Xho*I fragment 11 remains as a double intensity band after digestion with *Aat*II. This indicates that both copies of the 16S rRNA gene have been altered at the *Aat*II site. Thus, based upon restriction endonuclease analysis, the spectinomycin-resistant mutants could be classified as those which were altered at the *Aat*II site versus others which preserved the *Aat*II site within the 16S rRNA gene.

Spectinomycin resistance in *Nicotiana* was further analyzed by determining the nucleotide sequence of the 16S rRNA gene from the wild-types and representatives of the two classes of

mutants. The 16S rRNA genes from line 92/wild-type, 92/*str*^{R}7, 92/*str*^{R}7/*spe*^{R}1 and X/*spe*^{R}40 were completely sequenced, while those from line 92/*spe*^{R}4 and X/wild-type were partially sequenced. We determined that the nucleotide sequence for the 16S rRNA gene from line 92/wild-type is identical to that published for the 16S rRNA of *N. tabacum* (Shinozaki *et al.*, 1986). Nucleotide changes were found in the mutant lines as described in Figure 2. The base changes on the strand complementary to the 16S rRNA are shown. A change of C to T, which corresponds to a G to A change at position 1012 of the 16S rRNA gene, was found in line X/*spe*^{R}40 (Figure 2a). A change of C to T, which corresponds to a G to A change at position 1140, was found in the two independently selected lines 92/*str*^{R}7/*spe*^{R}1 and 92/*spe*^{R}4 (Figure 2b). Line 92/*str*^{R}7/*spe*^{R}1 carries, in addition, a mutation at nucleotide C860 (equivalent to nucleotide C912 in *E. coli* 16S rRNA), as does its parent control line, 92/*str*^{R}7 (H.Fromm, unpublished). Mutation of 16S rRNA at nucleotide C860 confers streptomycin resistance, as has been reported for *Euglena* chloroplasts (Montandon *et al.*, 1985). Table I summarizes the data for all of the lines studied.

Spectinomycin resistance revealed in wild-type maize based on its 16S rRNA gene sequence

The nucleotide sequences 5′-UGGCUGUCGUCAG-3′ and 5′-AUGACGUCAAGUCA-3′ are conserved in the 16S rRNA genes from *E. coli* (positions 1056–1068 and 1189–1201, Brosius *et al.*, 1981), chloroplasts of *Chlamydomonas* (positions 1001–1013 and 1132–1145, Dron *et al.*, 1982), and *Nicotiana* chloroplasts (positions 1004–1016 and 1135–1148, Shinozaki *et al.*, 1986). These sequences, which contain the spectinomycin

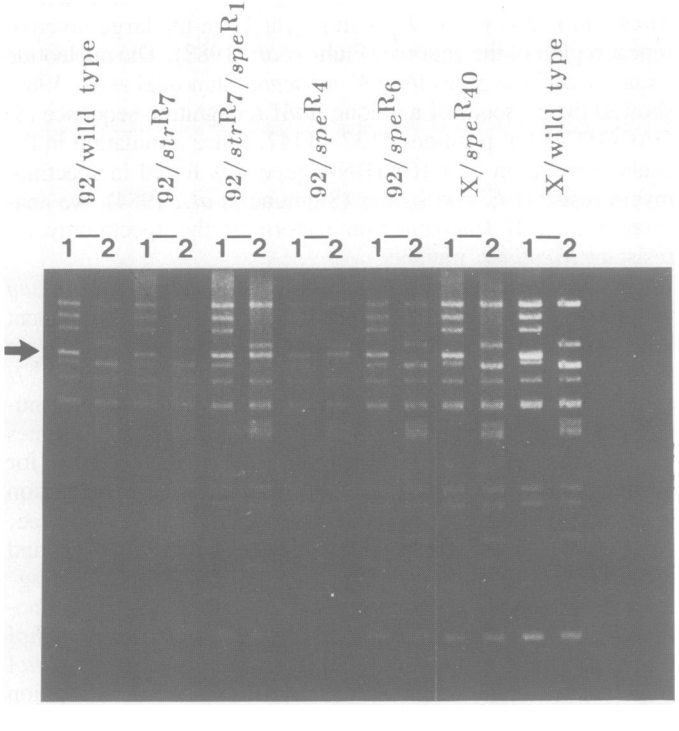

Fig. 1. Endonuclease restriction pattern of chloroplast DNA from wild-type and mutant *Nicotiana* lines. Chloroplast DNA from each line was digested with *Xho*I (**lanes 1**). Half of the *Xho*I digested fraction was then further digested with *Aat*II (**lanes 2**). Samples (~1 μg) were run on a 0.8% agarose gel in Tris-acetate buffer and stained with ethidium bromide. The arrow points to fragment *Xho*I-11 containing the 16S rRNA gene (Fluhr *et al.*, 1983).

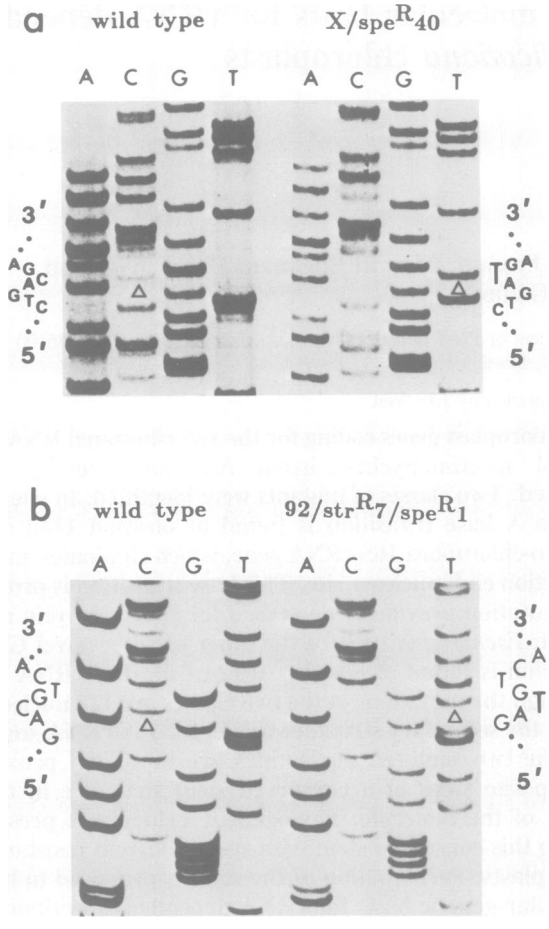

Fig. 2. Base changes in the 16S rRNA genes in spectinomycin-resistant lines. Nucleotide sequencing was carried out as described in Materials and methods. The sequence of the strand complementary to 16S rRNA is shown. Triangles indicate the base changes. (**a**) The nucleotide sequence shown, 5′ CTGACGA 3′ (wild-type), is complementary to nucleotides 1010–1016 (Shinozaki *et al.*, 1986) in the RNA-like strand. The C to T base change found in line X/*spe*^{R}40 predicts a G to A base change at position 1012 of the 16S rRNA. (**b**) The nucleotide sequence shown, 5′ GACGTCA 3′ (wild-type), is complementary to nucleotides 1136–1142 in the RNA-like strand. The C to T base change found in line 92/*str*^{R}7/*spe*^{R}1 (and line 92/*spe*^{R}4, not shown) predicts a G to A base change at position 1140 of the 16S rRNA.

Table I. Characterization of *Nicotiana* lines sensitive (S) or resistant (R) to 80 μg/ml spectinomycin

Line	Phenotype	*Aat*II site	Position 1012	Position 1140
92/wild-type	S	+	G	G
92/*str*^{R}7	S	+	G	G
92/*str*^{R}7/*spe*^{R}1	R	–	G	A
92/*spe*^{R}4	R	–	G	A
92/*spe*^{R}8	R	–	n.d.[a]	n.d.
92/*spe*^{R}6	R	+	n.d.	n.d.
X/*spe*^{R}40	R	+	A	G
X/wild-type	S	+	G	G

Chloroplast DNA of the *Nicotiana* lines indicated was analyzed by *Aat*II restriction endonuclease fragmentation and by nucleotide sequencing of the genes coding for 16S rRNA. The presence (+) or absence (–) of the unique *Aat*II site within the 16S rRNA gene is indicated, as is the rRNA nucleotide composition at the indicated position.
[a]Not determined.

resistance mutations found in *Nicotiana*, form two sides of a conserved stem structure in 16S rRNA (Noller, 1984). We noted that the nucleotide sequence of the 16S rRNA gene reported for maize chloroplasts (Schwarz and Kossel, 1980) predicts an imperfect secondary structure in these same regions. If instability of this conserved stem structure is the cause of spectinomycin resistance, it would suggest that maize chloroplasts are naturally resistant to this aminoglycoside. We tested this, preliminarily, by germinating maize seeds on media containing spectinomycin at the same concentration used for testing tobacco seeds (100 μg/ml). Under these conditions, maize was resistant to spectinomycin while tobacco was sensitive (not shown). The matter was approached more definitively by measuring protein synthesis in the presence of spectinomycin in isolated chloroplasts of maize and tobacco. As seen in Figure 3, 80 μg/ml of spectinomycin inhibited protein synthesis by 60% in chloroplasts of *Nicotiana* line 92/wild-type, but only 30% in chloroplasts of mutant line 92/*spe*^{R}4. Similarly, an inhibition of 50% was found for *Nicotiana* X/wild-type, in contrast to only 5% inhibition for mutant line X/*spe*^{R}40. However, when wild-type maize chloroplasts were tested in the presence of 80 μg/ml spectinomycin, they fully retained their ability to synthesize protein. In control experiments (not shown), we determined that the isolated chloroplasts from these *Nicotiana* spectinomycin-resistant lines, as well as from wild-type *Nicotiana* and maize, were all sensitive to streptomycin.

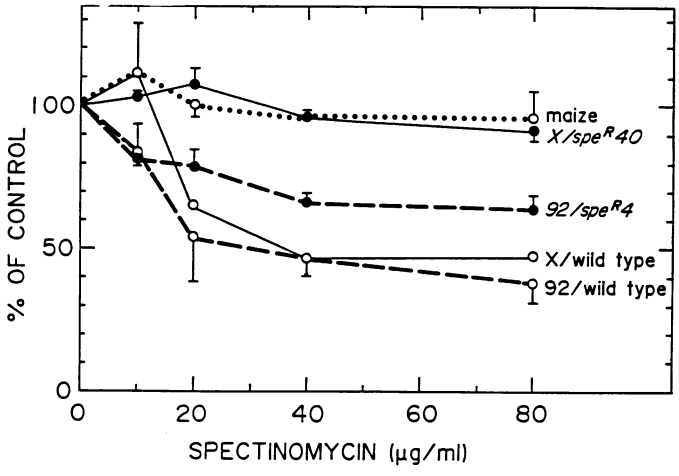

Fig. 3. Protein synthesis in isolated chloroplasts. Isolation of chloroplasts and incorporation of [^{35}S]methionine into proteins is described in Materials and methods. The values obtained for control treatments (0 μg/ml spectinomycin) were set at 100% on the abscissa, and represent about 1×10^4 c.p.m. per μg protein. The bars indicate the standard error calculated from two to five repeats of the experiments. ○, wild-types; ●, mutant lines.

Molecular-genetic correlation between 16S rRNA stem structure and spectinomycin resistance

The phylogenetic relationship between spectinomycin resistance and the 16S rRNA conserved stem structure is summarized in Figure 4. The sequence shown is that of wild-type *Nicotiana* and *Chlamydomonas* chloroplasts, and of *E. coli*. The known mutational divergence from this sequence in these three genomes is indicated above and below the stem structure. The consensus that emerges from the molecular-genetic analysis is that mutations which disrupt the right arm of the stem will confer resistance to spectinomycin on 70S ribosomes. Furthermore, a correspon-

GENOME	LINE	RESISTANCE	CHANGES	POSITION
Nicotiana chloroplast	X/*spe*^{R}40	high	- A - - -	1012
Nicotiana chloroplast	92/*spe*^{R}4	low	A - - - -	1140
Chlamydomonas chloroplast	spr-u-1-27-3	low	A - - - -	1137
Chlamydomonas chloroplast	spr-u-1-6-2	high	- - G - -	1135
Chlamydomonas chloroplast	spr-u-sp-23	high	- - C - -	1135
E. coli	spc-1	R	- U - - -	1192
Maize chloroplast	wild type	R	C - G - - -	1140, 1138
Euglena chloroplast	wild type	R	- - - - C	1141
Anacystis	wild type	S	- - - - C	1132

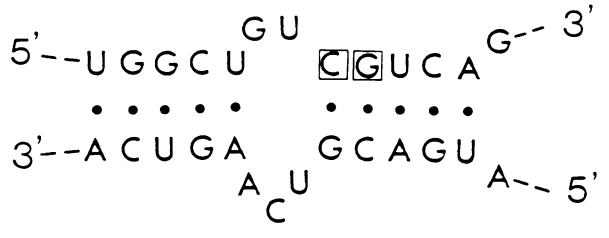

Fig. 4. Relationship between 16S rRNA stem structure and spectinomycin resistance. The secondary structure model for the depicted stem is according to Noller (1984). It holds for wild-type (i.e. spectinomycin sensitive) *E. coli* 16S rRNA, and *Nicotiana* and *Chlamydomonas* chloroplast 16S rRNAs, which are identical in sequence over these regions. The relative level of resistance to spectinomycin is indicated for the two *Nicotiana* (this work) and three *Chlamydomonas* (Harris *et al.*, 1987) mutant lines. Resistance (R) or sensitivity (S) is indicated for the single *E. coli* mutant (Sigmund *et al.*, 1984) and the three wild-type species analyzed by us. Base changes affecting the secondary structure of the conserved stem are localized in the right arm and are shown aligned above and below the sequence. The positions of the changed base(s) in the 16S rRNA genes are listed. Numbering for *Nicotiana*, *Chlamydomonas*, *E. coli*, maize, *Euglena* and *Anacystis* are according to Shinozaki *et al.* (1986), Dron *et al.* (1982), Brosius *et al.* (1981), Schwarz and Kossel (1980), Graf *et al.* (1982) and Tomioka and Sugiura (1983) respectively. Bases in the conserved stem structure which are proposed to interact with spectinomycin (Moazed and Noller, 1987) are boxed.

dence may exist between the level of resistance and the position of the altered nucleotide within the arm. Higher resistances appear when mutations are deeper in the arm (e.g. X/*spe*^{R}40; spr-u-1-6-2; spr-u-sp-23) as compared with the edge of the arm (e.g. 92/*spe*^{R}4; spr-u-1-27-3).

Wild-type 16S rRNA sequences from *Anacystis nidulans*, and from maize and *Euglena gracilis* chloroplasts, are also shown in Figure 4. These sequences differ within the arm from the sequence depicted. Since mutant sequences are not available for comparison in these cases, relative resistances cannot be defined. We find wild-type *Euglena*, like maize, to be naturally resistant to 100 μg/ml spectinomycin, however, *Anacystis* is sensitive to 5 μg/ml. The discrepancy between *Anacystis* and *Euglena*, which have the same nucleotide change but exhibit different phenotypes, cannot be explained unambiguously. Differential uptake of spectinomycin has not been ruled out. However, as the altered nucleotide is at an edge of the arm, perhaps species-specific stabilization by other regions of *Anacystis* 16S rRNA, or by adjacent proteins, is sufficient to maintain integrity of the *Anacystis* stem structure but not the *Euglena* structure.

It should be pointed out that any given alteration in nucleotide sequence found in a mutant is not necessarily related to the novel mutated phenotype. However, the numerous correlations summarized in Figure 4 amount to *prima facie* evidence that the alterations in the right arm of the conserved stem structure indeed are the cause for spectinomycin resistance in the *Nicotiana* chloroplast mutants. A similar situation, where mutations conferring antibiotic resistance (to paromomycin) appear on both sides of an evolutionary conserved stem structure of ribosomal RNA, was described for the 15S rRNA mitochondrial gene of yeast and the 17S rRNA nuclear gene of *Tetrahymena* (Spangler and Blackburn, 1985).

Genetic versus chemical analyses

Moazed and Noller (1987) have recently reported that several classes of antibiotics protect specific nucleotides in *E. coli* 16S rRNA from chemical reaction when bound to ribosomes. Spectinomycin was found to strongly shield nucleotide G 1064, and possibly also C 1063, from attack by dimethyl sulphate. Implicit in these findings is that spectinomycin interacts with these two particular nucleotides. Satisfyingly, G 1064 and C 1063 are components of the right arm of the conserved stem structure shown in Figure 4 (see boxed nucleotides in Figure 4). Thus, the chemical and genetic findings, arrived at independently, fully complement each other, and present a molecular scenario for rRNA-dependent spectinomycin resistance as follows. Spectinomycin specifically interacts with a nucleotide(s) which is part of a conserved stem structure. Alterations in either strand of the stem, and in the precise vicinity of the liganded nucleotide(s), which are induced by mutations or introduced by evolution lead to (strong or weak) structural perturbations. These perturbations adversely affect the interaction of spectinomycin with its nucleotide ligand, resulting in the resistant phenotype.

Materials and methods

Plant material

Nicotiana line 92, an alloplasmic line containing *N. undulata* chloroplast DNA (Fluhr *et al.*, 1984), was the wild-type source of all spectinomycin-resistant mutants except X/*spe*^{R}40, which was derived from *N. tabacum* var. Xanthi. Line 92/*str*^{R}7 is a streptomycin-resistant chloroplast mutant (Fluhr *et al.*, 1985) which served as the source for mutagenesis and selection of the double mutant 92/*str*^{R}7/*spe*^{R}1. The spectinomycin-resistant mutants derived from line 92 were propagated by cross-pollination with *N. tabacum* var. Xanthi and the resistance was maternally transmitted. *Zea mays* var. Jubilee (maize) was grown as described by Bogorad *et al.* (1983).

Chloroplast DNA

Chloroplast DNA was isolated and fragmented according to Fluhr and Edelman (1981). Restriction endonucleases were from New England Biolabs.

Protein synthesis in isolated chloroplasts

Nicotiana and maize chloroplasts were isolated according to Orozco *et al.* (1986) from 10 g of fresh leaves. The lower band of chloroplasts from a Percol gradient was washed with 10 vol grinding buffer, precipitated and resuspended in about 1 ml of reaction buffer (330 mM sorbitol, 50 mM Tricine–KOH pH 8.3, Fluhr *et al.*, 1985). 24 μl [^{35}S]methionine (1300 Ci/mmol, Amersham) were added, and 50 μl chloroplast suspension (about 5 μg protein) were removed to ice cold tubes containing spectinomycin (Sigma) dissolved in water. Tubes were incubated at 23°C under fluorescent light (25 μmol/m^2/s) for 30 min. Reactions were stopped by immersing the tubes in ice, and adding 0.5 vol of 3 × sample buffer (9% SDS, 2 M β-mercaptoethanol, 0.18 M Tris–HCl pH 6.8, and a trace of bromophenol blue). Incorporation of radiolabeled amino acids into proteins was assayed as in Roberts and Patterson (1973).

Cloning of the 16S rRNA gene

A 3.3 kbp *Bam*HI DNA fragment from within the inverted repeat region of *Nicotiana* chloroplast DNA, which contains the entire 16S rRNA gene, was cloned into the *Bam*HI site of M13mp19 vectors. Unexpectedly, only the rRNA-like orientation could be cloned under these conditions. The orientation complementary to 16S rRNA was obtained by cloning DNA fragments into the double strand vector pEMBL18+. Upon transfection with wild-type helper phage, single strand phage DNA was produced from clones of both orientations as described by Dente *et al.* (1983). *E. coli* strain BMH-71/18 (Kramer *et al.*, 1982) was used as host for all vectors described above.

Sequencing of the 16S rRNA gene

Sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) using [^{35}S]dATP. 17-mer oligonucleotide primers, specifically complementary to each strand of the *Nicotiana* 16S rRNA gene, were kindly provided by A.Weissbach. The wild-type gene from *Nicotiana* line 92 was sequenced in both orientations. Genes from other lines were sequenced in one orientation and electrophoresed on 6% acrylamide, 7 M urea gels, next to the line 92/wild-type sequence of the same orientation. All sequences were verified in at least two independent experiments.

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