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A point mutation in the chloroplast 16 S rRNA gene of a streptomycin resistant *Nicotiana tabacum*

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We sequenced a 82 bp fragment in the chloroplast 16 S rRNA gene of the streptomycin resistant mutant SR1 of *Nicotiana tabacum*. The sequence contains a transition from cytosine to adenine in position 860 of the 16 S rRNA. We suggest that this mutation is sufficient for the streptomycin resistance phenotype in the SR1 mutant.

Chloroplast; 16 S rRNA; 70 S ribosome; Antibiotic resistance; Direct sequencing; (SR1)

1. INTRODUCTION

Among mutant lines of higher plants resistant to the antibiotic streptomycin, *Nicotiana tabacum* SR1 [1,2] has been investigated most intensively. The location of the mutation in SR1 is assigned to the chloroplast genome [3,4], although direct proof is still lacking.

The 30 S ribosomal subunit isolated from SR1 chloroplasts has been shown to bind streptomycin 10-times less than its wild type counterpart [5]. Two-dimensional electrophoresis of chloroplast ribosomal proteins showed one [7] or possibly two [6] proteins of altered charge when SR1 and wild type tobacco were compared.

These findings indicated that one or two genes of the chloroplast genome encoding ribosomal proteins are mutated in the SR1 mutant. However Hildebrand and Bourque [21] suggested a mutation in the 16 S rRNA responsible for streptomycin resistance of SR1. Studying chloroplast DNA recombination in *Chlamydomonas reinhardtii*, Lemieux et al. [8] showed linkage between a nonmendelian locus for streptomycin resistance and a chloroplast DNA restriction fragment coding for 16 S rRNA. In *Euglena gracilis* a single point mutation in the chloroplast 16 S rRNA gene might be sufficient to give a streptomycin resistance phenotype [10]. In this particular case two independently generated streptomycin resistant mutants [9] show the same transition from cytosine to thymidine in position 876 in their single intact 16 S rRNA gene. No further mutations were detected in the other parts of the 16 S rRNA genes or in genes coding for the ribosomal S 12 proteins.

In an attempt to determine the mutation responsible for the streptomycin resistance phenotype in *N. tabacum* SR1, we have sequenced that part of the tobacco 16 S rRNA gene which corresponds to the region around position 876 in the *Euglena* 16 S rRNA and found a transversion from cytosine to adenine at the equivalent site.

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2. MATERIALS AND METHODS

2.1. *Isolation of chloroplast DNA*

Intact chloroplasts were isolated as described in [11] from 2-month-old SR1 plants grown in the greenhouse. The purified chloroplasts were lysed

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and the DNA was separated by CsCl gradient centrifugation.

2.2. *Primers*

Two 19mer oligonucleotides (A: 5′-GCTAACGCGTTAAGTATCC-3′; B: 5′-GGTTCTTCGCTTTGCATCG-3′) were made on a 380B DNA synthesizer from Applied Biosystems. The sequence was derived from the published wild type 16 S rRNA gene from tobacco [15].

2.3. *Amplification*

Oligonucleotide primers which are complementary to opposite strands and flanking the region of interest in the 16 S rRNA gene of tobacco (fig.1) were used in a polymerase chain reaction as described by Saiki et al. and Wrischnik et al. [12,13].

The DNA fragment between the 3′-hydroxyl ends of these primers was amplified by successive denaturation, polymerisation and renaturation with an excess of free primers.

The reaction volume was initially 90 μl containing 30 mM Tris-acetate (pH 7.9), 60 mM Na-acetate, 10 mM Mg-acetate, 0.25 mM DTT, 1.5 mM of each dNTP, 0.8 μM primer A, 1 μM primer B and about 200 ng of chloroplast DNA. The mixture was heated for 10 min at 95°C and cooled for 2 min at room temperature. After adding 1 unit Klenow (New England Biolabs – diluted to 1 U/μl in reaction buffer), the mixture was incubated at 37°C and then heated for 2 min at 95°C. The procedure from heating to the incubation at 37°C was repeated 20 times. In order to label the amplified fragment radioactively, 20 pmol of primer A were incubated with 3 μCi [γ-^{32}P]ATP and the amplification cycle was repeated another 5-times more.

The DNA was precipitated with ethanol and subjected to gel electrophoresis on a 6% non-denaturing polyacrylamide gel. The amplified DNA was visualized as a 120 bp fragment by autoradiography, eluted from the polyacrylamide and precipitated with ethanol.

2.4. *Sequence determination*

In contrast to the above mentioned publication [13] we have used the same primers A and B to determine the sequence of the amplified double-stranded DNA fragment by the dideoxy chain termination method [14].

A standard sequencing protocol was used and the DNA was labelled with [α-^{35}S]dATP. For a complete set of sequence reactions on both strands with primers A and B one-third of the DNA obtained by the amplification and 10 pmol of each primer was used.

3. RESULTS AND DISCUSSION

We have determined the 82 bp sequence between the 3′-hydroxyl ends of primers A and B from the amplified fragment of 120 bp on both strands (figs 1,2). The only nucleotide exchange with respect to the wild type sequence was an adenine instead of a cytosine in position 860.

Since the 16 S rRNA gene of *N. tabacum* resides in the 25339 bp inverted repeat of the chloroplast genome and both repeats contain identical sequences [15], we assumed that the primers A and B would both hybridize to the two 16 S rRNA genes of the mutant SR1 chloroplast DNA. We determined unambiguously an adenine and a thymine, on the complementary strand, indicating

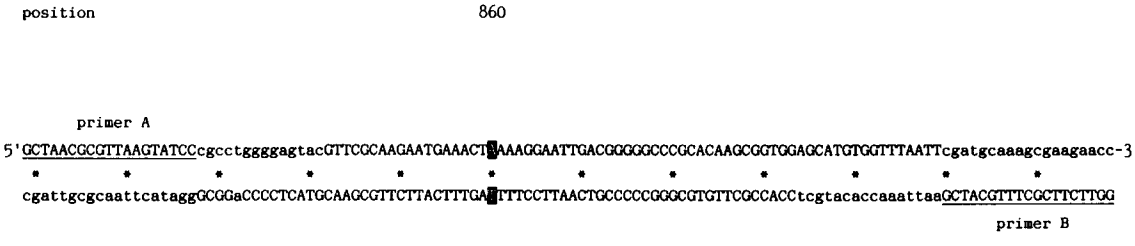

Fig.1. Amplification and sequencing of a 82 bp fragment of the SR1 16 S rRNA gene. The primers A and B which were used in the amplification and sequencing reactions are underlined and in capital letters. The nucleotides that were determined are shown in capital letters. The mutation in position 860 is highlighted in black boxes. The numbering of bases is given according to Shinozaki et al. [15]. The asterisks indicate distances of ten bases.

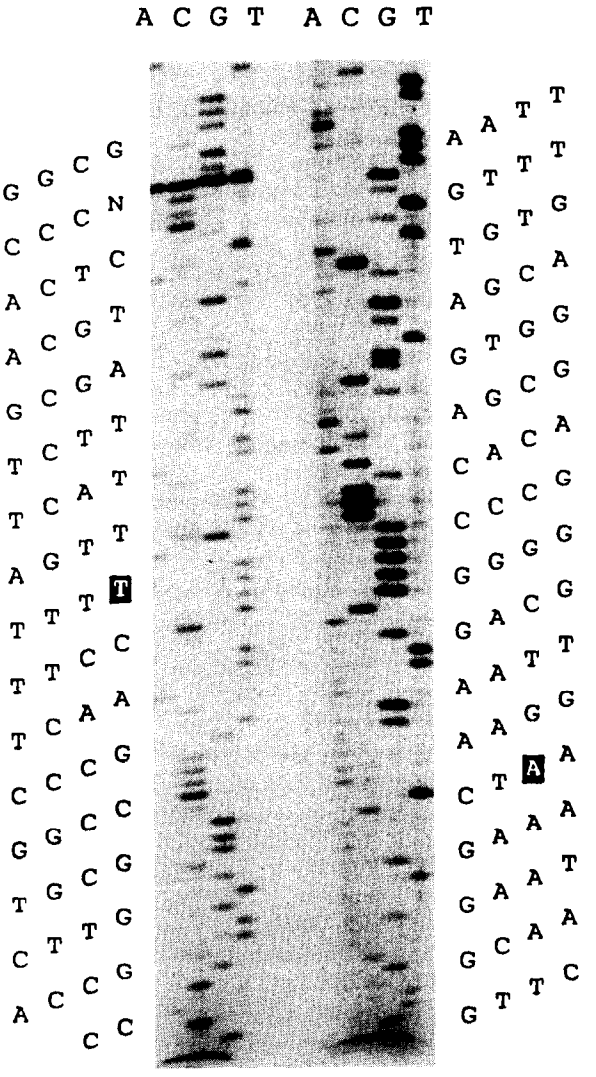

Fig.2. DNA sequencing gel showing the nucleotides on both strands of the amplified fragment of the SR1 16 S rRNA gene. The sequence on the right side was determined with primer A and represents the plus strand. Single bases are shown next to the bands in the gel. The first readable base is the guanine in position 842 shown as a G at the bottom of the gel. The following nucleotides are shown in packets of four from the left to the right. The sequence on the left side was determined with primer B and shows the complementary strand starting with the cytosine in position 893 and is read as mentioned before. The mutations are highlighted in both strands by black boxes. The A, C, G and T at the top of the two sequencing gels indicate the reactions containing the equivalent dideoxy nucleotides.

that indeed the mutation is present in both 16 S rRNA genes of the SR1 chloroplast genome.

The secondary structure of the small subunit ribosomal RNAs, 16 S rRNA from bacteria and chloroplasts, 18 S cytosolic rRNA and 12 S and 15 S rRNA of mitochondria, has essentially been solved by comparative sequence analysis [16,17]. The conserved secondary structure elements of all small subunit rRNAs are shown in a review edited by Gutell et al. [17]. Among these is a hairpin formed by nucleotides 836–860 in the *N. tabacum* 16 S rRNA (fig.3a) and by nucleotides 852–876 in the *E. gracilis* 16 S rRNA (fig.3b).

In the mutant SR1 and the two streptomycin resistant mutants of *Euglena* described by Montadon et al. [10], the first base pair of this hairpin is destabilized by a point mutation in the same position.

The stability of the hairpin structures shown in fig.3 was calculated using values for stacking energies given by Salser [18]. In tobacco the free energy of stem formation increases by 36% from −5.9 kcal/mol (wild type) to −3.8 kcal/mol (SR1) and in *Euglena* by 35% from −4.9 kcal/mol (wild type) to −3.1 kcal/mol (mutant). This indicates that the two different point mutations have the same effect on the stability of that stem in both cases.

The cytosolic small subunit rRNAs from maize, *Xenopus*, rat and yeast [10] show a sequence of the upper stem identical to that of the *Euglena* mutant

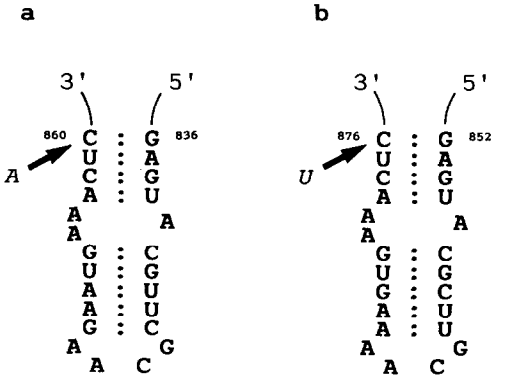

Fig.3. The sequence and the deduced secondary structure of the region between nucleotides 836–860 (a) and 852–876 (b) from wild type tobacco (a) and *Euglena* (b) 16 S rRNA. The nucleotide exchange in the streptomycin resistant mutants is indicated by an arrow.

(fig.3b). Interestingly eucaryotic cytoplasmatic ribosomes are not known to bind streptomycin or to be affected by it.

All this could mean that the above mentioned mismatch prevents formation of the binding site in the small subunit rRNA for streptomycin and that as a consequence of this the ribosome becomes resistant to streptomycin.

A point mutation in the small subunit rRNA is also linked to resistance against spectinomycin in *E. coli* [17] and in *Chlamydomonas reinhardtii* chloroplasts [19], hygromycin B in *Tetrahymena thermophila* [17] and paromomycin in various other organisms [17]. In all these cases a mispair in the secondary structure of the respective rRNA is introduced by a point mutation.

We present circumstantial evidence that the point mutation in position 860 of the SR1 chloroplast 16 S RNA is sufficient for the streptomycin resistant phenotype. However there are reports by Yurina et al. [7] and Capel et al. [6] suggesting that one or two mutations in ribosomal protein genes are responsible for that phenotype. Our result raises the number of mutations in SR1 to two or three. The reported frequency of spontaneous streptomycin resistance of 10^{-6} per cell [1] indicates that probably only one mutation event is necessary for that phenotype. We believe therefore that the mutations found in the chloroplast ribosomal proteins of SR1 are not responsible for its streptomycin resistance phenotype. Chloroplast transformation [20] and marker exchange [4] could be used to decide whether one or more mutations are necessary and sufficient to establish streptomycin resistance.

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