Spontaneous spectinomycin resistance mutations detected after biolistic transformation of *Daucus carota* L.

Elena A. Filipenko · Yuri V. Sidorchuk · Igor I. Titov · Valery P. Maltsev · Elena V. Deineko

Published online: 6 February 2011

© Prof. H.S. Srivastava Foundation for Science and Society 2011

Abstract Spectinomycin resistant mutant carrot (*Daucus carota* L.) callus lines detected in the experiments on biolistic transformation of plastome were analyzed. It has been found that this antibiotic resistance is determined by point nucleotide substitutions at two distinct sites of the chloroplast gene *rrn16*, coding for 16S rRNA, namely, G1012T, G1012C, and A1138G. The detected mutations are localized to the 16S rRNA region forming helix h34, which contains spectinomycin binding site, and lead to its destabilization by several kilocalories per mole. Comparative analysis of *rrn16* gene sequences has demonstrated conservation of the positions of the nucleotide substitutions determining this antibiotic resistance in carrot (*D. carota* L.), tobacco (*Nicotiana tabacum* L.), and bladder pod (*Lesquerella fendleri* L.), as well as in *Escherichia coli*.

Keywords *Daucus carota* L. · Spectinomycin resistance · Spontaneous mutation · *rrn16* gene · Callus

Introduction

The technologies for construction of transgenic plants allowing foreign genes to be introduced into the chloroplast genome (plastome) are currently developed. Construction of transplastomic plants has several advantages as compared with nuclear transformation, including high expression of the transferred genes, absence of silencing effect, and ecological safety, since the extranuclear genes cannot be transferred with pollen (Verma and Daniell 2007).

The technology for constructing transplastomic plants comprises two main stages. The first stage is the transfer and integration of a target gene within a plasmid DNA into the chloroplast genome. At the second stage, the transformed cells are selected on the selective media containing antibiotics, and the full-fledge plants are restored.

A long-term cultivation of plant cells on selective medium can be accompanied by mutations in the chloroplast genome conferring antibiotic resistance (Svab and Maliga 1991). *In vitro* cultivation of the cells carrying mutations in their chloroplast can eventually bring out the plants that are not transgenic yet retain the resistance to selective agents. The plants resistant to antibiotic but lacking the transgene in their chloroplast genome were detected when producing transplastomic plants of tobacco (Svab and Maliga 1993), *Arabidopsis* (Sikdar et al. 1998), potato (Sidorov et al. 1999), bladder pod (Skarjinskaia et al. 2003), and poplar (Okumura et al. 2006). Note that the nucleotide mutations found in such plants are localized to a small region (position 1012–1140) in a gene *rrn16*, which is responsible for

E. A. Filipenko (✉) · Y. V. Sidorchuk · I. I. Titov · E. V. Deineko
Institute of Cytology and Genetics, Siberian Branch,
Russian Academy of Sciences,
pr. Lavrentieva 10,
Novosibirsk 630090, Russia
e-mail: filipenko@bionet.nsc.ru

Y. V. Sidorchuk
e-mail: sidorch@bionet.nsc.ru

I. I. Titov
e-mail: titov@bionet.nsc.ru

E. V. Deineko
e-mail: deineko@bionet.nsc.ru

V. P. Maltsev
Institute of Chemical Kinetics and Combustion, Siberian Branch,
Russian Academy of Sciences,
Institutskaya 3,
Novosibirsk 630090, Russia
e-mail: maltsev@ns.kinetics.nsc.ru

formation of helix h34 at the 3'-end of 16S rRNA (Fromm et al. 1987; Kavanagh et al. 1994; Svab and Maliga 1991). Spectinomycin-resistant mutants of various microorganisms are also known (Binet and Maurelli 2005; Galimand et al. 2000), including *Escherichia coli* (Mark et al. 1983); moreover, their antibiotic resistance was determined by nucleotide substitutions in the analogous region of the *rrn16* gene (Johanson and Hughes 1995).

When using a biolistic transformation of carrot callus cells, we have earlier obtained callus lines displaying resistance to the antibiotic spectinomycin. PCR-based molecular analysis did not detect any exogenous DNA in the genomes of spectinomycin-resistant calluses. These results suggest that the antibiotic resistance in carrot callus lines can be determined by spontaneous mutations that emerged in both the plastid and nuclear genomes.

Since the earlier detected mutations conferring spectinomycin resistance were localized to the region within the *rrn16* gene, encoding 16S rRNA (Fromm et al. 1987; Skarjinskaia et al. 2003; Svab and Maliga 1991), it was of interest to study the possibility that such mutations could arise in the carrot callus lines that we produced. The goal of this work was to determine the types of mutations and their localization in the genome and to deduce the secondary structure of the 16S rRNA region containing mutations by computer analysis.

Materials and methods

Plant material

The material for this study was ten spectinomycin-resistant callus lines detected in the experiments on biolistic transformation of plastome. Vector PRB94 for plastome transformation, carrying the spectinomycin resistance gene, *aadA*, under the control of Prn promoter of tobacco *rrn16* gene, and terminator of *psbA* gene, was constructed by the team headed by Ruf S. (University of Freiburg, Germany) and kindly provided by Hammond R. (Agricultural Research Service, Beltsville, Maryland, United States). The carrot calluses that were not subjected to biolistic treatment were used as control.

Molecular analyses

Total carrot genomic DNA was prepared using a GenElute Plant Genomic DNA Miniprep Kit (Sigma, United States) according to the manufacturer's protocol. DNA extracted from all spectinomycin-resistant callus lines and control callus line (without spectinomycin resistance) were used for PCR amplification.

PCR amplification was conducted in 20 µl of the buffer containing 67 mM Tris-HCl (pH 8.9), 19 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 0.01 % Tween 20, 100 µM dNTP (Biosan, Russia), and 1 AU of *Taq* polymerase (SibEnzim, Russia). The amplification with primers named **rr-car-1** (5'-ACAGGTGGTGCATGGCTGACG-3') and **rr-car-2** (5'-GAGGACGGGTTTTGGAGTTAGC-3') was conducted in the following mode: initial denaturation for 3 min at 95 °C and 34 cycles of denaturation for 10 s at 94 °C, primer annealing for 10 s at 62 °C, and elongation for 15 s at 72 °C. For quality control of *AatII* digestion of the amplified fragment with a length of 254 bp, the nucleotide T was replaced with A (at position 1009 of *rrn16* gene) in the sequence of primer **rr-car-1** (5'-ACAGGTGGTGCATGGCTGACG-3'); this substitution created additional *AatII* site. The amplification with the primers named **cr-m1** (5'-AACACCTTACGGCACGAGTCGA-3') and **cr-m2** (5'-GAAGAACCTTACCAGGGGTCGACA-3') was conducted in the following mode: initial denaturation for 3 min at 95 °C and 34 cycles of denaturation for 10 s at 94 °C, primer annealing for 20 s at 66 °C, and elongation for 20 s at 72 °C. To create the digestion site for the restriction enzyme *SalGI* into the amplified fragment of *rrn16* gene with a length of 114 bp, two nucleotide substitutions were made when constructing the primer **cr-m1**, namely, T and C for C and T (at positions 1015 and 1016 of the gene *rrn16*) respectively: 5' AACACCTTACGGCACGAGTCGA 3'. To provide for the quality control of *SalGI* digestion of the 114-bp amplified fragment, the nucleotide substitutions were also introduced into the primer **cr-m2**, namely G and C for C and T (at positions 930 and 932 of the gene *rrn16*) respectively: 5' GAAGAACCTTACCAGGGGTCGACA-3'. All amplification reactions were conducted using 20–50 ng of DNA template in a Tertsik (DNK-tehnologiya, Russia) minicycler. Amplified fragments (200–300 ng) were digested with the restriction enzyme *AatII* (the DNA fragments amplified using the primers rr-car-1 and rr-car-2) or *SalGI* (the DNA fragments amplified using the primers cr-m1 and cr-m2). The digestion was conducted in a volume of 20 µl overnight at 65 °C for *SalGI* and at 37 °C for *AatII*, using 10 U of enzyme per 1 µg DNA in their corresponding buffers as per the manufacturer's instructions. Both restriction enzymes were obtained from SibEnzim (Russia). The fragments were separated by electrophoresis on a 8 % polyacrylamide gel (8 % PAGE) in 0.5× TBE buffer together with a DNA of the plasmid pBluescript II SK(+) digested with the *MspI* (Medigen, Russia).

The amplified fragments were purified for sequencing reaction using Extract-N-Amp Plant PCR Kit and Extract-N-Amp™ Reagent Kit (Sigma, United States) according to the manufacturer's protocol. The nucleotide sequences of

DNA fragments were determined using an ABI PRISM Big Dye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Amersham, United Kingdom) at the interinstitutional sequencing center (Institute of Chemical Biology and Fundamental Medicine, Siberian Branch, Russian Academy of Sciences, Novosibirsk, Russia). The primary sequences were analyzed using the BLAST software and GenBank database.

Computer analysis

The secondary structure model of tobacco 16S rRNA (Cannone et al. 2002) was used for deducing the secondary structure of carrot 16S rRNA. The changes in the secondary structure energy were calculated using the GArna program (Titov et al. 2002) based on thermodynamic rules (Jaeger et al. 1989).

Results

Detection of the *rrn16* gene regions with the mutations conferring spectinomycin resistance

It is known that the cause of spectinomycin resistance in plants is mutations in two regions of the *rrn16* gene, coding for 16S rRNA; these mutations result in a loss of the *AatII* site at positions 1138–1140 of the gene and a substitution of G at position 1012 with any other base (Fromm et al. 1987; Svab and Maliga 1991).

The PCR products of carrot spectinomycin-resistant callus lines amplified with the primers specific to two different regions of the gene were analyzed by RFLP using *AatII* or *SalGI* restriction endonucleases. It should be noted that amplification of the DNA fragments containing position 1012 of the gene was accompanied by base substitutions at positions 1015 and 1016 provided by structure of primers specific to this gene region. This created a *SalGI* site, so that the enzyme digested DNA between G at position 1012 and T at position 1013. This allowed us to perform RFLP analysis for detecting the mutation at position 1012 in spectinomycin-resistant callus lines. The loss of the target *SalGI* site, i.e., the mutation at position 1012, was shown in six of the ten analyzed lines (nos. 2, 3, 5–7, and 10) (Fig. 1). The remaining lines (nos. 1, 8, and 9) did not undergo mutation at position 1012; therefore, they were assayed by the same method for detecting mutation at the *AatII* site. In our experiment, three spectinomycin-resistant lines (nos. 1, 8, and 9) were altered at the *AatII* site (Fig. 2). No mutations in either analyzed gene region were detectable in line 4 (Figs. 1 and 2).

Fig. 1 PAGE (8 %) pattern of the amplified DNA fragments digested with *SalGI* using the following sequences as PCR templates: (1–10) DNA of spectinomycin-resistant callus lines; (C2) DNA of the control callus, not subjected to biolistic treatment. (C1) amplified DNA fragment not digested with *SalGI*. Short fragments with lengths of 20 and 18 bp are beyond the figure field

Determination of base substitutions at the *AatII* site

The PCR products from three spectinomycin-resistant callus lines containing mutations at the *AatII* site were sequenced in the region encompassing the target site to determine the exact base changes that had occurred. The PCR product from the control callus line, susceptible to the antibiotic, was also sequenced. It was shown that the cause of loss the *AatII* site in all the three mutant callus lines was the substitution of base A at position 1138, typical of the control line, with base G.

Determination of base substitutions at position 1012

The PCR products from six spectinomycin-resistant callus lines containing mutations at position 1012 of the gene *rrn16* were sequenced in the region encompassing the target site to determine the exact base changes that had occurred. The PCR product from the control callus line, susceptible to spectinomycin, was also sequenced. Comparison of the sequences of the mutant lines and the sequence of the control line demonstrated that three lines (nos. 1, 8, and 9) carried G→T substitution, whereas the other three lines (nos. 5–7) displayed G→C substitution.

Fig. 2 PAGE (8 %) pattern of the amplified DNA fragments digested with *AatII* using the following sequences as PCR templates: (1, 4, 8, and 9) DNA of spectinomycin-resistant callus lines; (C2) DNA of the control callus, not subjected to biolistic treatment. (C1) amplified DNA fragment not digested with *AatII*; (M) DNA of the plasmid pBluescript II SK(+) digested with the *MspI*. Short fragment with a length of 22 bp is beyond the figure field

Construction of the map of the mutations conferring spectinomycin resistance in the context of 16S rRNA secondary structure

The mutations conferring spectinomycin resistance are localized to the 3'-end of 16S rRNA molecule, the secondary structure of which is represented by helix, h34 (Carter et al. 2000). The gene *rrn16*, contained in the *rrn* operon of the chloroplast genome, is highly conserved, which is reflected in its nearly 100 % homology of both the nucleotide sequence and secondary structure within the higher plants (McNutt et al. 2007).

Using the tobacco 16S rRNA secondary structure (Cannone et al. 2002) as a reference as well as the nucleotide sequences of *N. tabacum*, *L. fendleri*, and *D. carota* gene *rrn16* available in gene banks, we constructed the map of mutations conferring spectinomycin resistance to these plants for the upper part of helix h34 (Fig. 3). The changes in the secondary structure energy caused by the mutations were calculated. It was shown that all the mentioned mutations destabilized helix h34 by 1.3 to 6.0 kcal/mol (Table 1).

Comparative analysis of mutations in the *rrn16* gene in higher plants

Comparative analysis of the mutations and their positions in the earlier studied spectinomycin-resistant *Nicotiana* and *Lesquerella* plants and the mutations found in carrot callus lines in this work was performed (Table 1 and Fig. 3). It was shown that the base substitutions in all plants took place in the same *rrn16* gene regions and at the same

positions, which is reflected in identical localization of the mutations on h34.

Discussion

The strategy in searching for mutations in spectinomycin-resistant carrot callus lines obtained by biolistic transformation

When transforming plant cells to create the plants able to produce foreign proteins with given properties, researchers encounter appearance of the regenerants resistant to selective agent that are phenotypically indistinguishable from the transplastomic plants but lack the foreign genes in their plastomes. It has been demonstrated that the resistance to selective agent (as a rule, to any antibiotic) is determined by mutations in the chloroplast genome. The earlier studies of spectinomycin-resistant plants have demonstrated that their antibiotic resistance is conferred by the mutations at two distinct sites within the chloroplast gene *rrn16*, encoding 16SrRNA, namely, the G→A substitution at position 1012 and the mutations at positions 1138–1140 of this gene leading to the loss of *AatII* endonuclease recognition site (Fromm et al. 1987; Skarjinskaia et al. 2003; Svab and Maliga 1991).

It is known that the gene coding for 16S rRNA is highly conserved and is present in the plastid genomes of all studied plants (Harris et al. 1994). This suggested that the analogous mutations in the plastid genome could be the cause of antibiotic resistance in the carrot callus lines isolated in our work.

So far the nucleotide sequences of plastomes have been determined for over 100 plant species (<http://chloroplast.cbio.psu.edu>), including *D. carota* (Ruhlman et al. 2006). Based on the primary nucleotide sequence of *D. carota* gene *rrn16*, the primers specific to two different regions of this gene were constructed; this allowed the amplified fragments to be analyzed by RFLP. RFLP analysis allows for a quick determination of whether the mutation took place in the target site. The possibility to use RFLP in our research was provided by an *AatII* site in the *rrn16* gene. In the region of the gene including position 1012, a *SalGI* site was created by PCR as a result of base substitutions at positions 1015 and 1016 provided by the structure of the primers specific to this region of the gene. In addition, extra *SalGI* and *AatII* sites were created by PCR due to base substitutions provided by the structure of the primers so that the resulting restriction endonuclease digestion formed small fragments (18 bp for *SalGI* and 22 bp for *AatII*). The analysis allowed us to explain why there was only one large fragment, namely, whether it was caused by the change of the restriction site or an incomplete enzyme

Fig. 3 A fragment of helix h34 in 16S rRNA in the region of the spectinomycin binding site of higher plants. The nucleotides interacting with spectinomycin (Brink et al. 1994; Carter et al. 2000) are boldfaced. Arrows denote the nucleotide mutations responsible for the antibiotic resistance; the corresponding positions in the *rrn16* gene are indicated. The indices of nucleotides indicate the corresponding reference, namely, (1) this work; (2) Fromm et al. 1987; (3) Skarjinskaia et al. 2003; and (4) Svab and Maliga 1991

Table 1 Nucleotide mutations in two sites of *rrn16* gene detected in higher plants

Species	Position	Mutation	Change in secondary structure energy, kcal/mol	Reference
<i>D. carota</i>	1012	G→C	+6.0	This work
<i>D. carota</i>	1012	G→T	+6.0	This work
<i>N. tabacum</i>	1012	G→A	+6.0	Fromm et al. 1987
<i>D. carota</i>	1138	A→G	+2.0	This work
<i>N. tabacum</i>	1138	A→C	+4.9	Svab and Maliga 1991
<i>N. tabacum</i>	1139	C→T	+1.3	Svab and Maliga 1991
<i>N. undulata</i>	1140	G→A	+2.9	Fromm et al. 1987
<i>L. fendleri</i>				Svab and Maliga 1991
				Skarjinskaia et al. 2003
<i>L. fendleri</i>	1140	G→C	+2.9	Skarjinskaia et al. 2003

digestion (Figs. 1 and 2). The absence of mutation could be easily determined by analyzing the number and length of fragments in the electrophoretic pattern (Figs. 1 and 2).

The performed RFLP analysis allowed us to minimize the subsequent volume of work in order to determine the exact base changes in the gene *rrn16*.

Comparison of the mutations detected in carrot callus lines with earlier detected mutations in higher plant species

It is known that all the nucleotide mutations conferring resistance to spectinomycin found during plastome transformation are localized to the gene *rrn16* (Svab and Maliga 1991). The mutations obtained during generation of transplastomic tobacco and *Lesquerella* plants were studied in detail. Comparison of the results obtained in this work for *D. carota* with the previously obtained data for *L. fendleri* (Skarjinskaia et al. 2003) and *Nicotiana* (Fromm et al. 1987; Svab and Maliga 1991) demonstrates that all the antibiotic-resistant plants carry mutations at the same sites within the *rrn16* gene.

Table 1 summarizes the already found base substitutions in the gene coding for 16S rRNA in the studied callus lines. Our study has demonstrated G→C or G→T transversions at position 1012 of *rrn16* gene and A→G transition at position 1138 of *AatII* site. In *Nicotiana* and *Lesquerella*, the base substitutions were also transitions and transversions. Note that we detected mutations at only one position (1138) within site 1138–1140, whereas mutations at positions 1139 and 1140 of this gene sequence have been found for *Nicotiana* (Fromm et al. 1987; Svab and Maliga 1991) (Table 1).

The possible causes for spectinomycin resistance in line 4

No mutations in either of the analyzed gene regions were detectable in line no. 4. It is known that the antibiotic

resistance is determined by changes in the structure of prokaryotic ribosomal complex, which can result from mutations in the genes encoding not only the RNA components, but also the ribosomal proteins (Davies et al. 1998). It has been shown that amino acid substitutions in the ribosomal protein S5 resulting from nucleotide mutations in the *rpsE* gene interfere with the binding of spectinomycin molecule to ribosome (Ramakrishnan and White 1992). Presumably, the cause of spectinomycin resistance displayed by line no. 4 is also mutations in the *rpsE* gene. However, the primary nucleotide sequence of this gene, localized to the *D. carota* nuclear genome, is yet unknown. This considerably complicates the search for the region that could potentially carry the target mutation and the corresponding molecular analysis.

The frequency of mutations conferring antibiotic resistance

Lines (2,3,10) and lines (5, 6, 7) were detected during passaging of calluses on selective medium after biolistic treatments in two independent experiments. Many cells (within one callus) were bombarded in each biolistic treatment. Consequently, it is impossible to trace the proliferation of each cell individually. Thus, we cannot assume whether lines (2,3,10) and lines (5, 6, 7) were the progenies of the same mutant cell or have been derived from different cells with independently emerged mutations. Line no. 4 was isolated in the same experiment as lines (2,3,10); however, the absence of mutation at position 1012 definitely suggests that this line can be regarded as independent. Callus lines (1, 8, and 9) were obtained in three different experiments; consequently, the mutations in these lines are independent. Thus, it is not possible to determine the mutation frequency in the chloroplast gene *rrn16* in the studied lines. No mutant forms resistant to spectinomycin were detectable in the control calluses (not subjected to biolistic treatment).

Conservation of the structure of prokaryotic and chloroplast 16S rRNA

The inhibition of protein translation in the cell by spectinomycin (and several other aminoglycoside antibiotics) is characteristic of the prokaryotic genome organization, which is also typical of the plastome. At molecular level, this appears as a structural similarity between the genes encoding the translation machinery of prokaryotic cell and the genes encoding the protein and RNA components of the ribosomes involved in the synthesis of chloroplast proteins. It is known that the genes contained in the *rrn* operon of chloroplast genome display a high degree of homology to the corresponding *E. coli* genes (Harris et al. 1994), which reaches 75 % for *rrn16* gene (McNutt et al. 2007). The performed phylogenetic analysis has detected a similarly pronounced conservation of the gene encoding 16S rRNA within the angiosperms (McNutt et al. 2007). A high homology of this gene in various taxa is explainable by the role of 16S rRNA in translation. Note that not only the primary sequence of 16S rRNA is important for successful protein synthesis, but also its secondary structure, characteristic of which is the presence of double-stranded regions, helices and loops. In particular, the conformation of 30S ribosomal subunit is changed with involvement of h34 helix, which is necessary at the stage of peptidyl-tRNA translocation from the A- to the P-site of ribosome. It has been shown that the secondary structure 16S rRNA is highly conserved in prokaryotes and eukaryotes as well as in lower and higher plants (Harris et al. 1994).

Comparative analysis of the nucleotide sequences of two regions within helix h34, 995–1015 and 1136–1157, has demonstrated their complete conservation in the higher plants *Nicotiana* and *D. carota*; as for their difference from analogous regions in the *E. coli* gene *rrn16*, it consists in substitution of only one complementary pair of nucleotides, G₉₉₉–C₁₁₅₄ → C₉₉₉–G₁₁₅₄ (positions numbered according to *E. coli*).

The effect of base substitutions in helix h34 on the binding with spectinomycin

It is known that the upper part of h34 contains a spectinomycin binding site (Carter et al. 2000); however, the roles of positions 1012 and 1138 are different (Fig. 3). Nucleotides G₁₀₁₂ and C₁₁₃₉ (corresponding to *E. coli* positions 1064 and 1192) directly interact with spectinomycin and are necessary for its binding. The mutations found in this work are located at this particular position, 1012. As for the mutations in neighboring positions, including position 1038, where we also have detected nucleotide substitutions, they destabilize helix h34 (Table 1). Destabilizing mutations conferring

antibiotic resistance have been also discovered in several bacteria (Binet and Maurelli 2005; Galimand et al. 2000), including *E. coli* (Mark et al. 1983). A normal translation of ribosomal proteins requires melting of helix h34 in 16S rRNA, whereas antibiotics stabilize this helix, entailing a frameshift, inhibition of peptidyl-tRNA translocation, and blocking of elongation stage (Kubarenko et al. 2006). The mutations in the upper part of h34, whereto the mutations found in this work are localized, neutralize a spectinomycin-induced stabilization of this helix and restore the conformational lability of ribosome, which leads to an unhindered synthesis of chloroplast proteins.

Calculation of the changes in the secondary structure energy demonstrates that all the mentioned mutations destabilize helix h34 by 1.3 to 6.0 kcal/mol (Table 1). Presumably, the value of free energy for spectinomycin binding to h34 falls within the same range yet with an opposite sign.

The possible ways to solve the problem of spontaneous spectinomycin resistance mutations

Researchers encounter a set of problems when constructing transplastomic plants. One of them is the need of a long-term *in vitro* cultivation of cells and plants on selective media and the corresponding emergence of spontaneous mutations in genomes of individual cells. Spontaneous mutations in chloroplast genomes have been described when obtaining transplastomic plants of the families Solanaceae (Svab and Maliga 1993), Brassicaceae (Sidorov et al. 1999; Skarjinskaia et al. 2003), and Salicaceae (Okumura et al. 2006). Phenotypic identity of the plants that regenerated from mutant cells to the truly transplastomic plants requires additional and comprehensive analysis.

In this work, mutant carrot callus lines were produced via a long-term selection of plant cells on selective medium with a high spectinomycin concentration (300–500 mg/l), which is a necessary condition for creation of transgenic plants. Note that the fact of emergence of nontransgenic plants resistant to a selective agent even at high concentrations is known (Okumura et al. 2006; Sidorov et al. 1999; Sikdar et al. 1998). The probability of green calluses to appear due to mutations can be reduced by additional selection using a second antibiotic. When selecting the cells transformed with the plasmid DNA carrying the gene *aadA*, the cultivation medium containing spectinomycin is supplemented with streptomycin. It is known that the enzyme aminoglycoside-3'-adenyltransferase inactivates both antibiotics (Shaw et al. 1993); therefore, only the plants with the selective gene integrated into their plastome will be viable. In our experiment, the calluses with phenotypic traits of spectinomycin resistance (green coloration) were also subjected to

further selection on the streptomycin-containing medium (200 mg/l). All green calluses in all lines acquired yellow color, characteristic of the control calluses, which confirmed their nontransgenic status.

Conclusions

The first plants with foreign genes successfully integrated into their plastomes were *N. tabacum* and *Arabidopsis thaliana* (Sikdar et al. 1998; Svab and Maliga 1993), which have become classical objects for plant gene engineering. The range of species diversity of the transplastomic plants obtained so far is rather wide. A new stage in the development of plant biotechnology is production of transplastomic plants with an agricultural and nutrition value (De Marchis et al. 2009; Dufourmantel et al. 2004; Kanamoto et al. 2006; Kumar et al. 2004a; Lelivelt et al. 2005; Liu et al. 2007; Singh et al. 2010). So far, the data on transformation of carrot plastome, described in this work, are sparse. The only work on production of transplastomic carrot does not mention any mutant plants displaying an antibiotic resistance (Kumar et al. 2004b). This work is the first to perform a detailed analysis of mutations conferring resistance to spectinomycin in a plant belonging to the family Apiaceae. It has been demonstrated that this antibiotic resistance is determined by mutations in two sites of the chloroplast gene *rrn16*, encoding 16S rRNA. The type of mutations (nucleotide substitutions) and their localization match the data obtained earlier for higher plants, which is explainable by a high degree of conservation of the gene coding for a significant component of the plastid translational machinery.

Acknowledgements The work was supported by the Russian Academy of Sciences under the programs Molecular and Cell Biology and Origin and Evolution of Biosphere, the President of the Russian Federation under the program for support of leading scientific schools (grant no. NSh-2447.2008.4) and by the Siberian Branch of the Russian Academy of Sciences (integration project no. 7).

References

- Binet R, Maurelli AT (2005) Fitness cost due to mutations in the 16S rRNA associated with spectinomycin resistance in *Chlamydia psittaci* 6BC. Antimicrob Agents Chemother 49:4455–4464
- Brink MF, Brink G, Verbeet M, de Boer HA (1994) Spectinomycin interacts specifically with the residues G₁₀₆₄ and C₁₁₉₂ in 16S rRNA, thereby potentially freezing this molecule into an inactive conformation. Nucleic Acids Res 22:325–331
- Cannone JJ, Subramanian S, Schnare MN, Collett JR, D'Souza LM, Du Y, Feng B, Lin N, Madabusi LV, Muller KM, Pande N, Shang Z, Yu N, Gutell RR (2002) The comparative RNA web (CRW) site: an online database of comparative sequence and structure information for ribosomal, intron, and other RNAs. BMC Bioinform 3:2
- Carter AP, Clemons WM, Brodersen DE, Morgan-Warren RJ, Wimberly BT, Ramakrishnan V (2000) Functional insights from the structure of the 30S ribosomal subunit and its interactions with antibiotics. Nature 407:340–348
- Davies C, Bussiére DE, Golden DE, Porter SJ, Ramakrishnan V, White SW (1998) Ribosomal proteins S5 and L6: high-resolution crystal structures and roles in protein synthesis and antibiotic resistance. J Mol Biol 279:873–888
- De Marchis F, Wang Y, Stevanato P, Arcioni S, Bellucci M (2009) Genetic transformation of the sugar beet plastome. Transgenic Res 1:17–30
- Dufourmantel N, Pelissier B, Garçon F, Peltier G, Ferullo JM, Tissot G (2004) Generation of fertile transplastomic soybean. Plant Mol Biol 55:479–489
- Fromm H, Edelman M, Aviv D, Galun E (1987) The molecular basis for rDNA-dependent spectinomycin resistance in *Nicotiana chloroplasts*. EMBO J 6:3233–3237
- Galimand M, Gerbaud G, Courvalin P (2000) Spectinomycin resistance in *Neisseria spp.* due to mutations in 16S rRNA. Antimicrob Agents Chemother 44:1365–1366
- Harris EH, Boynton JE, Gillham NW (1994) Chloroplast ribosomes and protein synthesis. Microbiol Rev 58:700–754
- Jaeger JA, Turner DH, Zuker M (1989) Improved predictions of secondary structures for RNA. Proc Natl Acad Sci USA 86:7706–7710
- Johanson U, Hughes D (1995) A new mutation in 16S rRNA of *Escherichia coli* conferring spectinomycin resistance. Nucleic Acids Res 23:464–466
- Kanamoto H, Yamashita A, Asao H, Okumura S, Takase H, Hattori M, Yokota A, Tomizawa K (2006) Efficient and stable transformation of *Lactuca sativa* L. cv. Cisco (lettuce) plastids. Transgenic Res 15:205–217
- Kavanagh TA, O'Driscoll KM, McCabe PF, Dix PJ (1994) Mutations conferring lincomycin, spectinomycin, and streptomycin resistance in *Solanum nigrum* are located in three different chloroplast genes. Mol Gen Genet 242:675–680
- Kubarenko A, Sergiev P, Wintermeyer W, Dontsova O, Rodnina MV (2006) Involvement of helix 34 of 16S rRNA in decoding and translocation on the ribosome. J Biol Chem 281:35235–35244
- Kumar S, Dhingra A, Daniell H (2004a) Stable transformation of the cotton plastid genome and maternal inheritance of transgenes. Plant Mol Biol 56:203–216
- Kumar S, Dhingra A, Daniell H (2004b) Plastid-expressed betaine aldehyde dehydrogenase gene in carrot cultured cells, roots, and leaves confers enhanced salt tolerance. Plant Physiol 136:2843–2854
- Lelivelt CL, McCabe MS, Newell CA, Desnoo CB, van Dun KM, Birch-Machin I, Gray JC, Mills KH, Nugent JM (2005) Stable plastid transformation in lettuce (*Lactuca sativa* L.). Plant Mol Biol 6:763–774
- Liu CW, Lin CC, Chen JJ, Tseng MJ (2007) Stable chloroplast transformation in cabbage (*Brassica oleracea* L. var. capitata L.) by particle bombardment. Plant Cell Rep 10:1733–1744
- Mark LG, Sigmund CD, Morgan EA (1983) Spectinomycin resistance due to a mutation in an rRNA operon of *Escherichia coli*. J Bacteriol 155:989–994
- McNutt PM, Dehart MJ, Matej LA (2007) Characterization of the 16S–23S internal transcribed spacer among 34 higher plants: suitability for interspecific plastid transformation. Plant Cell Rep 26:47–60
- Okumura S, Sawada M, Park YW, Hayashi T, Shimamura M, Takase H, Tomizawa K (2006) Transformation of poplar (*Populus alba*) plastids and expression of foreign proteins in tree chloroplasts. Transgenic Res 15:637–646
- Ramakrishnan V, White SW (1992) The structure of ribosomal protein S5 reveals sites of interaction with 16S rRNA. Nature 358:768–771

- Ruhlman T, Lee SB, Jansen RK, Hostetler JB, Tallon LJ, Town CD, Daniell H (2006) Complete plastid genome sequence of *Daucus carota*: implications for biotechnology and phylogeny of angiosperms. BMC Genomics 7:222. doi:10.1186/1471-2164-7-222
- Shaw KJ, Rather PN, Hare RS, Miller GH (1993) Molecular genetics of aminoglycoside resistance genes and familial relationships of the amino glycoside-modifying enzymes. Microbiol Res 57:138–163
- Sidorov VA, Kasten D, Pang SZ, Hajdukiewicz PT, Staub JM, Nehra NS (1999) Technical advance: stable chloroplast transformation in potato: use of green fluorescent protein as a plastid marker. Plant J 9:209–216
- Sikdar SR, Serino G, Chaudhuri S, Maliga P (1998) Plastid transformation in *Arabidopsis thaliana*. Plant Cell Rep 18:20–24
- Singh AK, Verma SS, Bansal KC (2010) Plastid transformation in eggplant (*Solanum melongena* L.). Transgenic Res 1:113–119
- Skarjinskaia M, Svab Z, Maliga P (2003) Plastid transformation in *Lesquerella fendleri*, an oilseed Brassicaceae. Transgenic Res 12:115–122
- Svab Z, Maliga P (1991) Mutation proximal to the tRNA binding region of the *Nicotiana* plastid 16S rRNA confers resistance to spectinomycin. Mol Gen Genet 228:316–319
- Svab Z, Maliga P (1993) High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. Proc Natl Acad Sci USA 90:913–917
- Titov II, Vorobiev DG, Ivanisenko VA, Kolchanov NA (2002) A fast genetic algorithm for RNA secondary structure analysis. Russ Chem Bull 51:1135–1144
- Verma D, Daniell H (2007) Chloroplast vector systems for biotechnology applications. Plant Physiol 145:1129–1143