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Evolution of Chloroplast 16S Ribosome RNA Dependent Spectinomycin Resistance and Implications for Chloroplast Transformation

Shi-Hua Wang[1,2], Shu-Min Zhang[1], Hong-Yun Liu[1], Ning Sun[3], Shuang-Cheng Gao[2], Xiao-Qin Wang[4] & Zheng Liu[1]

[1] Key Laboratory of Microbial Diversity Research and Application of Hebei Province, College of Life Sciences, Hebei University, Baoding, China

[2] College of Agriculture, Henan University of Science and Technology, Luoyang, China

[3] The Affiliated School of Hebei Baoding Normal, Baoding, China

[4] Key Laboratory of Urban Agriculture (North) Ministry of Agriculture, Beijing University of Agriculture, Beijing, China

Correspondence: Zheng Liu, College of Life Sciences, Hebei University, Baoding, China. Tel: 86-0312-507-9364. E-mail: liuzhengxp@yeah.net; Xiao-Qin Wang, Key Laboratory of Urban Agriculture (North) Ministry of Agriculture, Beijing University of Agriculture, Beijing, China. Tel: 86-010-8079-7308. E-mail: wxqtycn@gmail.com

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Abstract

Spectinomycin is an aminoglycoside antibiotic that inhibits prokaryotic-like protein synthesis by blocking translocation of messenger RNA and transfer RNAs on the ribosome. Mutants resistant to this antibiotic have been isolated from a wide range of bacteria, the alga *Chlamydomonas* and from chloroplasts of different species of higher plants. Usually a single nucleotide substitution occurred on chloroplast 16S ribosome RNA gene is sufficient to resist spectinomycin. Mutation rates of 16S rRNA gene of *Nicotiana tabacum* L treated with different concentrations of spectinomycin were studied. The results showed a sharp decrease of green resistant shoots from 30 to 300 mg l^{-1} spectinomycin, then an increase from 300 to 500 mg l^{-1} and finally a gradual decrease from 500 to 1000 mg l^{-1}. The increasing number of green resistant shoots from 300 to 500 mg l^{-1} spectinomycin indicates that the higher selective pressure applied by the antibiotic promoted a faster evolutionary rate of 16S rRNA gene. Furthermore, a new substitution site of 16S rDNA which conferred resistance to spectinomycin was isolated and confirmed by sequencing, endorsing that mutation is due to selection through high concentration of the antibiotic.

Keywords: 16S rRNA gene, mutational evolution, *Nicotiana tabacum*, spectinomycin, tissue culture

Abbreviations: 6-BAP, 6-Benzylaminopurine; 16S rDNA, 16S ribosome DNA; 16S rRNA, 16S ribosome RNA; *aadA*, aminoglycoside 3'-adenyltransferase; NAA, α-Naphtaleneacetic acid

1. Introduction

Spectinomycin is an aminocyclitol antibiotic produced by *Streptomyces spectabilis,* being active against many gram-negative bacterial species. It has been used in the treatment of gonorrhea, where it inhibits microbial protein synthesis by binding to the 30S ribosomal subunit (Borovinskaya et al., 2007). Spectinomycin resistance can arise due to several RNA and protein mutations. In one type of mutant, ribosomal protein S5 of the 30S subunit of the ribosome has a single amino acid substitutions within a restricted region. In another type of mutant, there is a transition from C to T at position 1192 of a 16S ribosomal RNA gene (Fromm et al., 1987), suggesting a probable binding site in helix 34 of 16S rRNA. Binding of spectinomycin at this location is thought to stabilize helix 34, inhibiting the binding of elongation factor G, thereby blocking translocation of peptidyl-tRNAs from the ribosomal A site to the P site (Zimmerman & Maher, 2002). However, such a single nucleotide change of 16S ribosome DNA is sufficient to confer spectinomycin resistance.

Chloroplast ribosomes resemble their bacterial counterparts in monomer and subunit sedimentation coefficients, RNA primary- and secondary-structural characteristics, amino acid sequence of several ribosomal proteins and sensitivity to several antibiotics (Fromm et al., 1987). More specifically, a sequence of 21 nucleotides (nucleotides 1186-1206) surrounding the rRNA mutation site conferring spectinomycin resistance in *Escherichia coli* (Brosius et al., 1981; Sigmund et al., 1984) is also present in the chloroplast 16S rRNA genes of *Nicotiana* chloroplasts (nucleotides 1133-1153). Two G to A base transitions were found at positions 1140 and 1012 of the tobacco-chloroplast 16S rRNA gene that conferred spectinomycin resistance. Although the mutations 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. However, one mutation site was also found at position 1333 in tobacco which is quite far away from this conserved mutation region (Svab & Maliga, 1991).

Chloroplast transformation technology is a promising tool in biotechnology and has the potential to solve some of the problems associated with traditional plant genetic engineering, the major concern of which is the escape of foreign genes through pollen transmission to wild relatives. Genetic engineering of chloroplasts is a method for containment of such potential gene pollution. The commonly used method for chloroplast transformation is to use biolistics to bombard plasmid-coated gold particles into chloroplasts and to use the bacterial *aadA* gene as a selectable marker to select the tissue on spectinomycin medium (Svab & Maliga, 1993). The *aadA* gene encodes aminoglycoside 3'-adenyltransferase conferring resistance to aminoglycoside type antibiotics such as spectinomycin and streptomycin (Goldschmidt-Clermont, 1991). Spectinomycin is mainly used for higher plant plastid transformation because it does not have much effect on plant cells. It was claimed that spectinomycin selection on the concentration of 500 mg l^{-1}, on average, yields one transplastomic line per bombarded leaf sample (Maliga, 2003), while some other laboratories have reported as high as 90% mutants among spectinomycin resistant clones (Eibl et al., 1999; Sidorov et al., 1999). Transplastomic clones are identified as green shoots on spectinomycin medium, which suppresses greening and shoot formation of wild-type tobacco cells as higher plant cells in photoheterotrophic culture respond to spectinomycin by bleaching and retarded growth. Resistant mutants are also identified by their green color in the selective medium which makes it hard to identify them from true transformants by phenotype. Mutants resistant to spectinomycin can be isolated from 500 mg l^{-1}, a routine concentration used for tobacco plastid transformation, which makes identification of true transformants complicated (Svab & Maliga, 1991).

It is widely believed that the rate of evolution may rise as selection pressure increases. When the concentrations of an antibiotic increase during leaf tissue culture (i.e. increasing selection pressure for the evolution of the antibiotic resistance), but within a non-lethal scope of the antibiotic application, does the evolutionary rate of the antibiotic resistance increase at the same time? In this paper we presented the direct evidence to answer this question by studying on evolution of spectinomycin resistance of the tobacco16S rRNA gene. We also found that 500 mg l^{-1} spectinomycin is not the optimal concentration for initial selection of plastid transformation. Finally, a new chloroplast 16S rRNA mutation consisting of a change from G to U at position 1012 was identified by sequencing one mutant plant grown on 500 mg l^{-1} spectinomycin.

2. Method

2.1 Culture Media

The MSR3 medium for tobacco (*Nicotiana tabacum, cv. Petite Havana*) growth consisted of 4.3 g l^{-1} MS macro- and microelements (Murashige & Skoog, 1962) (Duchefa, Haarlem, The Netherlands), 30 g l^{-1} sucrose, 1 mg l^{-1} R3 vitamins (R3 Vitamins: 1.0mg ml^{-1} Thiamine, 0.5 mg ml^{-1} Nicotinic Acid, 0.5 mg ml^{-1} Pyridoxine) and 7g l^{-1} agar. The pH was adjusted to 5.8 with NaOH before autoclaving at 120ºC and 103.5 kPa for 20 min. The RMOP medium (pH, 5.8) for leaf regeneration contained 4.3 g l^{-1} MS macro- and microelements, 30 g l^{-1} sucrose, 6.5 g l^{-1} agar, 0.1 g l^{-1} Myo-Inositol. Filter-sterilized 0.1 ml l^{-1} α-Naphtaleneacetic acid (NAA) (Sigma-Aldrich, Steinheim, Germany), 1ml l^{-1} 6-Benzylaminopurine (6-BAP) (Sigma-Aldrich, Steinheim, Germany), 1 ml l^{-1} Thiamine, 0.4 g l^{-1} Amoxycillin Na/K Clavulanate (Duchefa, Haarlem, The Netherlands) were added after autoclaving.

2.2 Plant Materials and Tissue Culture

Tobacco wild type seeds were sterilized in 10% NaOCl (Jeyes Limited, Norfolk, England) for 5 min and rinsed 3 times in sterile water. Sterile seeds were then germinated on MSR3 medium and young seedlings were cultivated in a growth room at 24 ºC and 16h photolight period of 45 µmol m^{-2} s^{-1} provided by cool-white fluorescent tubes. Every 5-6 cm fully expanded leaf was excised using a sterile scalpel and put on RMOP medium with the upper side down, pre-incubated in a petri dish (9 cm in diameter containing 20 ml medium) for 2 or 3 days at the same

environmental conditions. Then the leaves were cut into small pieces (5 mm × 5 mm) and placed on RMOP medium containing the selective antibiotic (i.e. 30, 50, 100, 300, 500, 600, 1000 mg l^{-1} spectinomycin respectively) (Duchefa, Suffolk, UK) and incubated at 24 °C with 16h photoperiod. Usually small segments of one leaf were put in 2 petri dishes and each petri dish contained 25 leaf segments. Ten leaves (approximately 500 leaf segments) were used for each antibiotic concentration. After 2 weeks of cultivation, the swollen leaf explants were cut into small pieces (5 mm × 5 mm) and transferred to fresh RMOP medium containing the same concentration of spectinomycin. Subsequently the medium was renewed every 3 weeks routinely to maintain selection pressure. After about 4 or 5 weeks the first green shoot or callus was normally regenerated from the bleached leaf segments. Green calli or shoots were then subcultured onto the same selective medium. Leaves from shoots were then recallused a second or third time and subsequent shoots rooted on MSR3 medium. The experiment was repeated three times.

2.3 Sequencing of One 16S rDNA Mutant Plant From the 500 mg l^{-1} Spectinomycin Medium

Total genomic DNA was extracted from young leaves of a 16S rDNA mutant plant using the GenElute™ Plant Genomic DNA Miniprep Kit (Sigma). Two tobacco-specific primers 16S102489f (5'-ACC TTG ACG TGG TGG AAG TC-3') and 16S104303r (5'-GGG TGT GAA GCA GTG TCA AA-3') were used for cloning of mutant 16S rDNA (GenBank Z00044.2). PCR amplification was carried out using *pfu* DNA polymerase purchased from Promega, UK by following the usage information (Part# 9PIM774). The 120ng of tobacco total DNA was used for the PCR reaction in 20µl. PCR cycles were set as follows: 94ºC 3 min for denaturing; thirty cycles for 94 ºC 1 min, 60 ºC 1 min and 72 ºC 3.5 min; 72 ºC 10 min for extension. Eppendorf mastercycler was used for PCR amplification. After purification, the PCR product was then directly used for sequencing by using the primers for PCR amplication.

3. Results and Discussion

3.1 Evolution of Chloroplast 16S Ribosome RNA Dependent Spectinomycin Resistance

An interesting phenomenon was observed in our tobacco tissue culture for chloroplast transformation. As shown in Figure 1 and 2, there was a trend of reduced number of green shoots and calli when spectinomycin was increased from 30 mg l^{-1} to 100 mg l^{-1}. When spectinomycin concentration reached 300 mg l^{-1}, there was no shoot or callus found even in 30 leaves. However, there suddenly appeared about 1 green shoot or callus per leaf at 500 mg l^{-1} of the antibiotic. Continuing to increase the concentration of spectinomycin to 600 and 1000 mg l^{-1}, led to reduced number of green shoots or calli again.

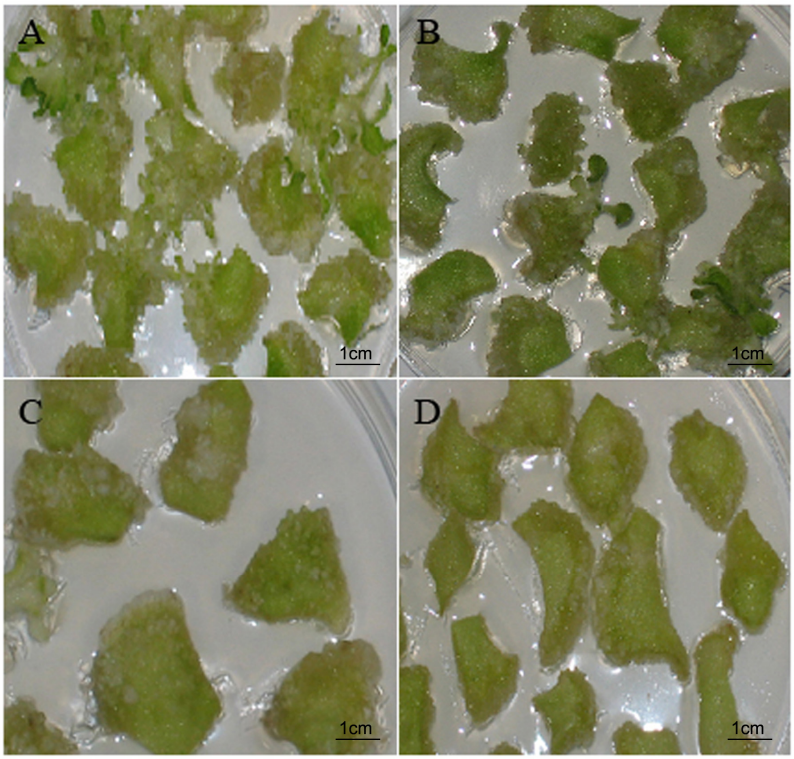

Figure 1. Green calli or shoots on different concentrations of spectinomycin

A) 30 mg l^{-1} spectinomycin. B) 50 mg l^{-1} spectinomycin.

C) 100 mg l^{-1} spectinomycin. D) 300mg l^{-1} spectinomycin.

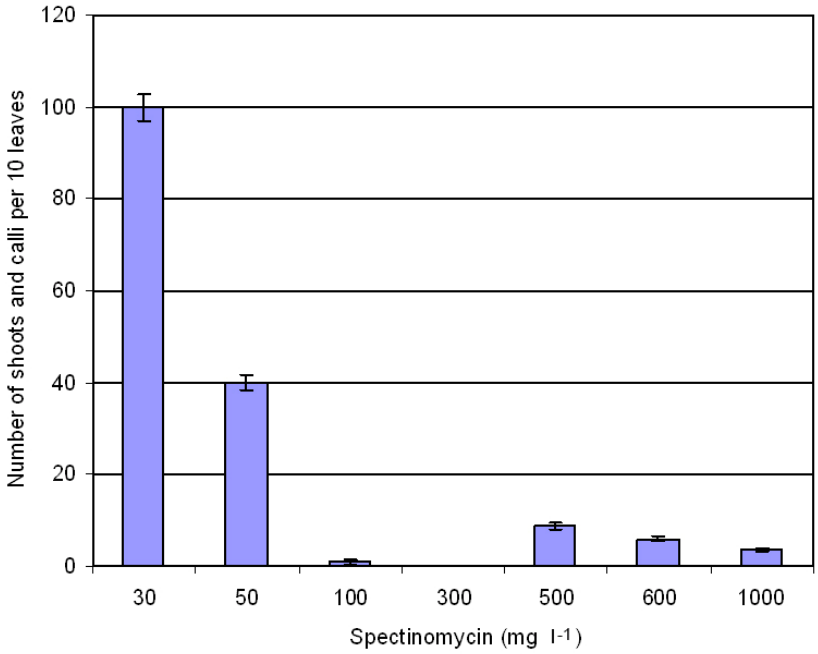

Figure 2. Mean ± SE of mutant shoots on different concentrations of spectinomycin (number of shoots or calli per 10 leaves)

In bacteria and plant chloroplasts, spectinomycin-resistant mutants have alterations in the 30S subunit of the ribosome. Some single sites of 16S rDNA mutation have been identified in *Nicotiana tabacum* which were sufficient to confer spectinomycin resistance (Fromm et al., 1987; Svab & Maliga, 1991). At very low concentrations of spectinomycin (e.g. 30 and 50 mg l^{-1}), there was a large number of green calli or shoots appeared. It seemed that low dosage of the antibiotic cannot inhibit the function of plastid ribosomes, thus wildtype escape would occur. When spectinomycin concentrations increase from 30-300 mg l^{-1}, the number of green shoots or calli reduced correspondingly. However, by comparing the number of green shoots and calli on 300 and 500 mg l^{-1} spectinomycin medium, we found that no green shoot or calus appeared on 300 mg l^{-1} spectinomycin, whereas the total number of 10 green shoots or calli in 10 leaves appeared on 500 mg l^{-1} spectinomycin. This reflects that mutation rate of 16S rDNA is higher on 500 mg l^{-1} than that on 300 mg l^{-1} spectinomycin. In order to prove that mutation happened on high concentration of spectinomycin, the 16S rDNA of a green shoot which resists 500 mg l^{-1} spectinomycin was sequenced, resulting in a novel mutation site was found as shown in Figure 3.

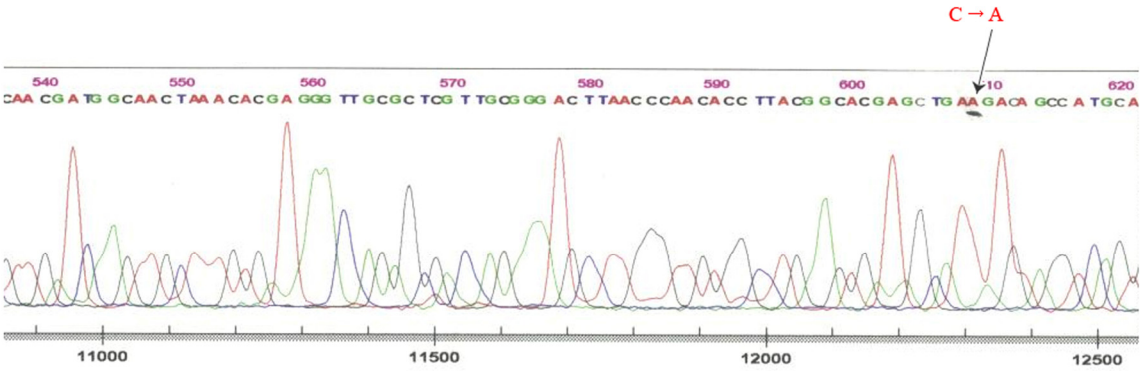

Figure 3. Sequencing of 16S rDNA mutant plant

It is widely accepted on human diseases study that there is a strong correlation between selective pressure and the nucleotide substitution rate for distinct virus genes (Shackelton & Holmes, 2006; Shackelton et al., 2005). For example, capsid proteins of porcine parvovirus (PPV) interact with the host immune system. Substitution of a amino acid in a specific site of the protein is related to a possible decrease in the affinity of neutralizing antibodies and provides some adaptive advantage to the virus (Zeeuw et al., 2007). Therefore, a higher evolutionary rate was found for PPV capsid genes (~3×10^{-4} – 4×10^{-4} substitutions per site per year) than that for PPV non-structural genes (~5×10^{-5} substitutions per site per year) during recent years by frequent use of vaccines resulting in high selective pressure (Streck et al., 2011). Thus, it is rational to understand that under high selective pressure (500 mg l^{-1} spectinomycin), tobacco calli showed higher evolutionary rate of the antibiotic resistance compared to that under 300 mg l^{-1} spectinomycin. However, when the concentration of spectinomycin increased to 600 mg l^{-1} or higher, the damaging effects to the cells were overwhelming as the result that mutation rates reduced. It should be noted that within the range from 300 to 500 mg l^{-1} spectinomycin the number of green resistant shoots or calli increases to 1 per leaf from zero when the medium was regularly changed every three weeks. However, a more interesting finding was that when the medium containing 500 mg l^{-1} spectinomycin was changed every two weeks, more green shoots appeared than that when the medium was changed every three weeks. With passing time, the antibiotic was gradually degraded. Thus the more frequent changes of medium, the higher spectinomycin concentration was maintained. The mutation rate is therefore related to the pressure of selection (concentration of the antibiotic). Our results gave the direct evidence in plants that within a certain scope of selective pressures, higher selective pressure applied by the antibiotic confers a faster rate of evolution caused by the antibiotic resistance of the cells.

3.2 Implications for Chloroplast Transformation

All reports on chloroplast transformation of tobacco claimed to use 500 mg l^{-1} spectinomycin (Bock, 1998; Iamtham & Day, 2000; Reed et al., 2001; Svab & Maliga, 1993) or even higher (Kavanagh et al., 1999). The great problem when using the antibiotic for selection is that the mutation rate of the chloroplast 16S rRNA gene is high. Although it was reported that one bombarded leaf gave one plastid transformant (Svab & Maliga, 1993),

other laboratories have reported as high as 90% mutants among spectinomycin resistant clones (Eibl et al., 1999; Sidorov et al., 1999). Our preliminary work showed that the background mutation rate of wild type tobacco leaves is that one leaf gave one green mutant shoot or callus on 500 mg l^{-1} of spectinomycin. It seems that 500 mg l^{-1} spectinomycin is not an optimal level for the initial step of chloroplast transformation as there is less than one green shoot or callus per 10 wild type leaves on 100 mg l^{-1} of spectinomycin. There is no logical reason to use very high amounts of antibiotic at the beginning stage of selection when only one or at most a few transgenes have been shot into a chloroplast and integrated into the chloroplast genomes (Bock, 2001). High concentration of spectinomycin, e.g. 500 mg l^{-1}, only produces more mutants which have the same phenotype as that of the true transgenics. This only produces extra work for screening of plastid transformants among the regenerants.

As 100 mg l^{-1} spectinomycin only produces less than one mutant green shoot or callus per 10 leaves, this concentration could be ideal for preliminary selection after bombardment with a plastid transformation vector. Furthermore, for putative chloroplast transformants, as only one or a few chloroplast genomes contain an integrated transgene after bombardment, a high concentration of the antibiotic would kill the cell. Homoplastomic plants with the *aadA* gene as a selectable marker were also tested for different concentrations of spectinomycin. We found that such plant leaves can even regenerate and keep green at more than 1500mg l^{-1} of spectinomycin. This is reasonable because aminoglycoside-3''-adenyltransferase can be encoded at the same time from about 10000 *aad A* containing plastid genome copies in one single leaf cell. There exists an extremely high accumulation of the enzyme in the leaf cells. So a logical method for selection of plastid homoplastome would be a dynamic and gradual increase of the antibiotic for each round of selection.

There is also another problem of transgene loss from transformed plants. For chloroplast transformation, primary transplastomic cell lines contain a mixed population of wild-type and transformed plastid genomes. Such cells, tissues or plants are also referred to as heteroplasmic (or, more specifically, "heteroplastomic") and are genetically unstable. More or less frequently, the cell lines resolve spontaneously into either of the two types of genome homogeneity ("homoplasmy") (Bock, 2001). We have to face such a dilemma in the selection of plastid transformants. On the one hand, a low concentration of the antibiotic cannot eliminate the wild type genomes. In cells there will always exist a large population of wild type genotype which interfers the homogeneity of the transgene. However, a high concentration of the antibiotic for selection in the early stage will kill transformed cells before they can be identified. On the other hand, long term exposure of plant cells to the antibiotic would cause secondary mutations. Once the heteroplastomic plant becomes mutant, selection would favor the amplification of chloroplast mutant genomes with gradual elimination of true transgene containing genomes. Therefore, an effective tissue culture system is the key factor for plastid transformation and correct application of the antibiotic to avoid mutation and dilution of true transgene is critical for success of plastid transformation. In all, it is concluded that 500 mg l^{-1} spectinomycin is too high for initial selection of tobacco chloroplast transformation as the high background mutation rate and damaging effects on cells containing few copies of true transformants.

3.3 A Novel 16S rDNA Mutant Which Confers Resistant to 500 mg l^{-1} Spectinomycin

The sequencing data showed that at position 1012, contrary to the reported mutation G→A in 16S rRNA (Fromm et al., 1987), there is a new mutation of 16S chloroplast rRNA of *N. tabacum* (G→U) (16S cDNA of 'A' in position of 609 as shown in Figure 3 which was transcribed to 'U' in 16S rRNA). This mutant plant was obtained by selection on 500 mg l^{-1} spectinomycin and it showed green color of leaves as normal plant, indicating a point mutation from G to U in 16S rRNA is sufficient for the plant to resist 500 mg l^{-1} spectinomycin. Previous reports already found some single nucleotide acid substitution sites of the 16S rDNA that can confer resistance to different concentrations of spectinomycin (Svab & Maliga, 1991). For example, a transition from C to T at position 1192 of a 16S ribosomal RNA gene was detected; mutation at Spe^R40 site, a G to A transition at position 1012 of the 16S rRNA confers resistant to 80 mg l^{-1} of spectinomycin (Fromm et al., 1987); in spectinomycin resistant line SPC23, a G to A change at position 1333, only confers resistance to low levels (50 mg l^{-1}) of spectinomycin, however, in SPC1 line, an A to C change at position 1138 gives resistance to 500 mg l^{-1} spectinomycin. In the SPC2 line, a C to U change at position 1139 was found on a medium containing 1000 mg l^{-1} spectinomycin (Svab & Maliga, 1991). Our finding further revealed that position 1012 is a hot spot for substitution under spectinomycin. Although G to A transition at this site only confers resistance to 80 mg l^{-1} of spectinomycin, our results showed that G to U transition at the same site is enough to resist 500 mg l^{-1} of spectinomycin.

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Conflict of interset

The authors declare no conflict of interest.

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