**Induction and characterization of streptomycin-resistant mutants in *Capsicum praetermissum***

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**Abstract.** Streptomycin-resistant mutants were isolated from mutagenised cotyledon explants of *Capsicum praetermissum* Heiser & Smith. The explants were mutagenised with N-ethyl-N-nitrosourea, which resulted in a high frequency of streptomycin-resistant mutants (18.0%) and a low frequency of chlorophyll-deficient (albino) mutants (8.0%). Complete streptomycin-resistant plantlets were obtained after rooting of the regenerated green shoots on rooting medium containing 1.0 mg L⁻¹ IAA and 500 mg L⁻¹ streptomycin sulphate. Leaf-segment assay of these plantlets revealed that they were resistant to streptomycin but sensitive to chloramphenicol, kanamycin, lincomycin, and spectinomycin. Reciprocal crosses between streptomycin-resistant and -sensitive plants showed a non-Mendelian transmission of resistance by female parents.

**Key words:** *Capsicum praetermissum*, cotyledon cultures, maternal inheritance, mutagenesis, streptomycin resistance.

**Introduction**

The genetic information that resides in mitochondria and chloroplasts codes for a number of significant agronomic characteristics. As the genomes of these organelles become better understood on the molecular and biochemical level, the ability to manipulate this information and to create unique genetic combinations will become of vital importance (Whitaker and Evans 1986). Inheritance of these organelles in most plant species is maternal (cytoplasmic), so that only maternal organelles are transmitted into the progeny (Connett 1987; Rose 1991). Thus the combining of cytoplasmic organelles with different genetic traits by sexual hybridization is not possible.

Somatic cell fusion can be useful to transfer agronomically important characters from one plant to another or to overcome the sexual barriers between two species. But improvement of certain agronomic traits is possible only when selectable and easily screened genetic markers are available. A prerequisite for such *in vitro* selection of biochemical markers is to have suitable explants and a well-established regeneration system. Genetic markers can be screened from extranuclear and/or intranuclear genomes. Several cytoplasmic markers, such as lincomycin, specinomycin and streptomycin resistance, have been successfully isolated in different systems by using efficient plastome-targeted mutagens (Cseplo and Maliga 1984; Fluhr et al. 1985; Dix et al. 1990; McCabe et al. 1989, 1990; Rao et al. 1997a,b).

Streptomycin resistance is the most extensively used marker in higher plants and has already been used in *Capsicum annuum* (Subhash et al. 1996; Rao et al. 1997a), *Onobrychis vicifolia* (Hamill et al. 1986), *Lycopersicon* species (Jansen et al. 1990; McCabe et al. 1990; Timmons and Dix 1993), *Nicotiana* species (Maliga et al. 1973; Fluhr et al. 1985; Svat and Maliga 1986; To et al. 1989; Toki et al. 1990); and *Solanum* species (Mc Cabe et al. 1989; Rao et al. 1993, 1997b).

The discovery that nitrosourea compounds are efficient plastome-targeted mutagens (Hagemann 1982) has resulted in strategies for producing anti-
biotic-resistant mutants. McCabe et al. (1989, 1990) reported a rapid and simple protocol for obtaining plastome-encoded streptomycin-resistant mutants of solanaceous plants. This was based on the use of nitrosomethyl urea and a highly regenerative explants system in which the antibiotic causes bleaching and suppresses adventitious shoot initiation. The appearance of resistant shoots depends on a complex process of sorting out of resistant and sensitive plastome types during a sustained period of cell division. Streptomycin binds to the 30S ribosomal subunit, inhibiting polypeptide synthesis and causing misreading of the genetic code (Edwards 1980; Moazed and Noller 1987; Dahlberg 1989; Harries et al. 1989). Resistance is caused by a single nucleotide change in the chloroplast 16S rRNA (Etzold et al. 1987; Fromm et al. 1989; Harris et al. 1989) or S12 ribosomal protein genes (Galili et al. 1989; Hsu et al. 1993).

Chloroplast mutation conferring streptomycin resistance has been successfully induced with mutagens like nitrosoethyl urea (NEU), nitrosomethyl urea (NMU), and ethyl methane sulphonate (EMS). A critical requirement to develop a plastid transformation technology is the availability of the selectable markers. Selectable markers have not yet been reported in C. praetermissum Heiser & Smith. In the present paper, we report a simple and efficient method for isolating streptomycin-resistant mutant plants in C. praetermissum by using NEU-mutagenised cotyledon explants.

Materials and methods

Plant material

Seeds of C. praetermissum PI 342947 were obtained from the Regional Plant Introduction Station, Griffin, Georgia, USA. The seeds were soaked for 24 h in sterile distilled water, surface sterilized with 0.1% HgCl₂ for 3–5 min, followed by three rinses with sterile distilled water, and germinated aseptically on MS (Murashige and Skoog 1962) basal medium. Cotyledons from 3-week-old seedlings were used as explants.

Mutagenesis and isolation of streptomycin-resistant plants

Cotyledon explants were incubated for 90 min in liquid MS medium, in which 5 mM N-ethyl-N-nitrosourea (NEU) (Sigma, USA) was dissolved, then placed on a rotatory shaker (100 rpm) at 25°C, and subsequently washed thrice with fresh sterile medium. Mutagenised explants were placed on MS medium supplemented with 1.0 mg L⁻¹ indole acetic acid (IAA) and 5.0 mg L⁻¹ N⁶-Benzylaminopurine (BA). The antibiotic streptomycin sulphate (500 mg L⁻¹) was added to shoot-regenerating medium. Regenerated shoot buds were separated and transferred to rooting medium containing 1.0 mg L⁻¹ IAA and 500 mg L⁻¹ streptomycin. The pH was adjusted to 5.8 and the medium was solidified with 0.8% Difco Bactoagar. Simultaneously, control explants (non-muta-genised) were also cultured. A single explant was placed in each tube and incubated at 25 ± 1°C under 16 h photoperiod. The data were scored after 6 weeks of culture of 100 replicates for each treatment.

Leaf-segment assay for antibiotic resistance

Leaf explants were collected from streptomycin-resistant (from all 10 resistant clones, Table 2) and streptomycin-sensitive plants, next surface sterilized for 1 min in 70% (v/v) ethanol, for 15 min in 10% (v/v) sodium hypochlorite, and rinsed thrice in sterile water for 3 min. The leaf explants from streptomycin-resistant clones and sensitive control plants were cut into segments (0.5–1.0 cm long) and placed on regeneration medium containing 5.0 mg L⁻¹ BA, 1.0 mg L⁻¹ IAA, 2.0% sucrose, 0.8% agar, and one of the following antibiotics: 500 mg L⁻¹ streptomycin sulphate, 100 mg L⁻¹ chloramphenicol, 100 mg L⁻¹ kanamycin monosulphate, 100 mg L⁻¹ lincomycin hydrochloride, or 100 mg L⁻¹ spectinomycin dihydrochloride. The cultures were incubated at 25 ± 1°C under 16 h photoperiod. Visual scores for greening response and formation of adventitious buds were also recorded. The complete study included three separate experiments, each having its own susceptible control. Formation of green calli and/or shoots from leaf-segments was scored as an indication of resistance.

Chromosome number determination

To determine the ploidy level of the regenerated plants, root tips were treated with 0.002 M 8-hydroxyquinoline at 18–20°C for 3 h, and fixed in ethanol-glacial acetic acid (3:1) for 24 h. They were placed in 1N HCl and 2% aceto-orcein (9:1) for 2 h and then squashed with a drop of 45% ace-
tic acid. Chromosomes were counted in 2 to 5 well-spread cells of each regenerated plant.

**Estimation of pollen fertility**

Anthers containing mature pollen grains were squashed in a drop of aceto-carmine. Stained pollen grains were scored as fertile, while non-stained pollen grains were classified as sterile. For each plant more than 2000 pollen grains from two separate flower buds were counted.

**Seedling assay for antibiotic resistance**

Seeds were collected from reciprocal crosses and selfing of streptomycin-resistant and -sensitive plants. The seeds were sterilized with 0.1% HgCl₂ for 5 min and rinsed thoroughly with sterile distilled water, then germinated on medium containing MS salts, 0.5% sucrose, 0.8% agar and 1 mg mL⁻¹ streptomycin sulphate. The cultures were incubated at 25 ± 1°C under 16 h photoperiod. The seedlings with green cotyledons were classified as resistant while those with white cotyledons were classified as sensitive.

**Results**

An initial experiment was conducted to determine if the concentration of streptomycin at 500 mg L⁻¹ could suppress shoot regeneration and cause efficient bleaching of the explants. In explants after NEU mutagenesis, streptomycin-resistant shoots appeared at a high frequency as green adventitious shoots. Streptomycin suppressed morphogenesis and caused bleaching of non-mutagenised controls. Data on normal and mutagenised explants and appearance of resistant shoots and albino shoots on medium containing 500 mg L⁻¹ streptomycin are given in Table 1. Some of the mutagenised explants produced a very low number of albino shoots. However, they were not able to turn green, as they were suppressed by the antibiotic present in the selection medium. The stability of the streptomycin resistance was increased when resistant explants (with green shoots) were cultured repeatedly on the same selection medium. After three passages of culturing the variegated shoots have disappeared and the maximum number of green shoots per explant was increased considerably (Table 1). Regenerated green and albino shoots were separated and placed on rooting medium containing 1.0 mg L⁻¹ IAA and 500 mg L⁻¹ streptomycin. Streptomycin-resistant green shoots rooted, whereas albino and some green shoots failed to root normally. Complete streptomycin-resistant plantlets were obtained after rooting of the regenerated green shoots on rooting medium.

The regenerated antibiotic-resistant plantlets were found to be diploid (2n = 24) and devoid of chromosomal aberrations. Resistance to streptomycin was verified by culturing of leaf-segments on a medium containing 500 mg L⁻¹ streptomycin. The stability of resistance to the antibiotic was scored basing on the formation of callus and/or differentiation of shoot buds on streptomycin medium from the explants. Leaf-segments from the 10 clones (Table 2) remained green and formed callus and/or shoot buds from cut ends of explants, whereas leaf-segments from control sensitive plants bleached rapidly. Formation of shoot buds from the cut ends of the explant confirmed their resistance to the antibiotic. The plants regenerated from mutagenised cotyledon explants retained streptomycin resistance, as indicated by the appearance of the shoots on leaf segments of these putative regenerants grown on selective medium containing selective levels of streptomycin, but they were sensitive to other antibiotics, such as chloramphenicol, kanamycin, lincomycin, and spectinomycin.

The plantlets were transferred to pots and raised to maturity. The resistant plants were normal with respect to morphology. The pollen fertility of sensitive (control) plants was 100%, while in resistant plants it ranged from 60% to

**Table 1. Production of albino (chlorophyll-deficient) and green (streptomycin-resistant) shoots from mutagenised cotyledon explants of Capsicum praetermissum on MS medium containing 500 mg L⁻¹ streptomycin sulphate**

<table>
<thead>
<tr>
<th>Explant type</th>
<th>Total number of explants</th>
<th>Explants bleached (%)</th>
<th>Explants producing albino shoots (%)</th>
<th>Explants producing green shoots (%)</th>
<th>Mean number ± S.E. of green shoots per explant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-mutagenised cotyledons (control)</td>
<td>100</td>
<td>100.0</td>
<td>2.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Cotyledons mutagenised with 5 mM NEU</td>
<td>120</td>
<td>74.0</td>
<td>8.0</td>
<td>18.0</td>
<td>6.5 ± 0.82</td>
</tr>
</tbody>
</table>

Data scored after three repeated passages on the same selection medium
85% (Table 2). All the resistant clones had normal flowers and were able to set seeds when self-fertilized and crossed reciprocally to sensitive plants.

Inheritance to streptomycin resistance of the clones was investigated by germinating self-fertilized and reciprocally crossed seeds on medium containing 1 mg mL\(^{-1}\) streptomycin sulphate. Reciprocal crosses between resistant and sensitive plants showed a non-Mendelian inheritance of the resistance. A cross between streptomycin-resistant and -sensitive plants produced all resistant phenotypes when the female parent was resistant. However, all the progeny were sensitive when the pollen of resistant plants was used in the cross. The results obtained confirm that the resistance is controlled by a maternally inherited mutation (Table 2).

### Table 2. The inheritance pattern and pollen fertility of streptomycin-resistant clones of Capsicum praetermissum

<table>
<thead>
<tr>
<th>Resistant clone</th>
<th>No. of resistant (SR) and sensitive (SS) seedlings in crosses*</th>
<th>Pollen fertility (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \text{SR } q \times \text{SS } q )</td>
<td>( \text{SR } q \times \text{SS } q )</td>
</tr>
<tr>
<td></td>
<td>resistant</td>
<td>sensitive</td>
</tr>
<tr>
<td>SR 1001</td>
<td>82</td>
<td>0</td>
</tr>
<tr>
<td>SR 1002</td>
<td>76</td>
<td>0</td>
</tr>
<tr>
<td>SR 1004</td>
<td>88</td>
<td>0</td>
</tr>
<tr>
<td>SR 1006</td>
<td>92</td>
<td>0</td>
</tr>
<tr>
<td>SR 1007</td>
<td>84</td>
<td>0</td>
</tr>
<tr>
<td>SR 1010</td>
<td>68</td>
<td>0</td>
</tr>
<tr>
<td>SR 1012</td>
<td>94</td>
<td>0</td>
</tr>
<tr>
<td>SR 1013</td>
<td>82</td>
<td>0</td>
</tr>
<tr>
<td>SR 1015</td>
<td>78</td>
<td>0</td>
</tr>
<tr>
<td>SR 1016</td>
<td>84</td>
<td>0</td>
</tr>
</tbody>
</table>

* Seeds germinated on medium containing 1 mg mL\(^{-1}\) streptomycin sulphate.

### Discussion

The present investigation was initiated to provide a protocol for \textit{in vitro} mutagenesis to induce antibiotic resistance encoded on chloroplast DNA in \textit{C. praetermissum}. The successful isolation of mutants depended upon establishing appropriate conditions, where bleaching was not accompanied by severe growth limitation. This certainly relates to the complex kiloploid nature of the plant chloroplast genome (Medgyesy 1990). Streptomycin resistance has also been shown to be a result of recessive mutations in the nucleus (Maliga 1981). One possible explanation for the failure to obtain nuclear mutations is that the concentration of streptomycin in the selective medium may not be suitable for selection of such mutations. Another possibility is that the samples used may not be large enough for screening nuclear mutations. Because of the polyploid nature of the chloroplast or mitochondrial genomes, it is expected that mutations could occur more frequently in the organelles than in the nucleus. Moreover, nuclear-encoded streptomycin resistance is always inherited as a recessive trait and has only been isolated when using haploid material (Maliga 1981). Thus, it is unlikely to expect a dominant, nuclear streptomycin-resistant mutant and even less likely to expect a homozygous recessive, nuclear mutation in diploid plant material.

The main observation from the current data is the greater yield of streptomycin-resistant shoots appearing from the NEU-treated cotyledon explants. Nitrosourea compounds create cytoplasmic variations when used for explant mutagenesis (Dix et al. 1990; Mc Cabe et al. 1990; Timmons and Dix 1993; Rao et al. 1997a,b) but fail to elicit any response when used for seed mutagenesis (Rao et al. 1997a). The recovery of healthy, green resistant shoots in mutagenised explants indicated the effectiveness of the mutagen and selection scheme. Our results confirm the general efficiency of the nitrosourea compounds (NEU or NMU) for inducing antibiotic resistance mutations, as has been reported by others (Cseplo and Maliga 1984; Svab and Maliga 1986; Dix et al. 1990; Mc Cabe et al. 1989, 1990; Rao et al. 1997a,b). EMS and nitrosoguanidine – other alkylating agents – have also been shown to be effective in inducing cytoplasmic mutations in higher plants (King 1984; Miller et al. 1986; To et al. 1989; Rao et al. 1993; Subhash et al. 1996, 1997).
The cross-resistance experiments show that the streptomycin-resistant mutants are sensitive to other antibiotics, like chloramphenicol, kanamycin, lincomycin, and spectinomycin. Similar results have also been obtained in streptomycin-resistant clones of *C. annuum* (Subhash et al. 1996), *N. plumbaginifolia* (To et al. 1989), and *S. melongena* (Rao et al. 1993), which were not resistant to other antibiotics. It has been found in eukaryotes that each of the antibiotics mentioned above has a specific binding site either in 16S rRNA or 23S rRNA gene (Harris et al. 1989). We speculate that the streptomycin-resistant mutants obtained in this study may result from a change either in chloroplast 16S rRNA gene or in S12 ribosomal protein gene. This speculation is consistent with the findings that streptomycin resistance is caused by a single point mutation either in the chloroplast 16S rRNA gene of *N. tabacum* (Etzold et al. 1987; Fromm et al. 1989) or S12 ribosomal protein gene of *N. tabacum* (Galili et al. 1989) and *N. plumbaginifolia* (Hsu et al. 1993).

In conclusion, we succeeded to produce streptomycin-resistant mutants by using nitrosoethyurea (NEU) in *C. praetemissum*. It is anticipated that these markers will be useful in studies on interspecific cytoplasmic genetics of *Capsicum* species, and in developing a plastid transformation technology. Plastid-encoded resistance to streptomycin and lincomycin in plant cell culture is used as a colour marker. Resistant cells are green, whereas sensitive cells are white on a selective medium. It has been shown that streptomycin and lincomycin resistance can be used to isolate cytoplasmic substitution lines and clones with recombinant plastids (Medgyesy et al. 1985; Medgyesy 1990; Moll et al. 1990).

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**REFERENCES**


