

# PRODUCTION OF A NITRATE REDUCTASE DEFICIENT STREPTOMYCIN RESISTANT MUTANT OF *NICOTIANA TABACUM* FOR SOMATIC HYBRIDIZATION STUDIES

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## 1. INTRODUCTION

We have sexually crossed an auxotrophic mutant of *Nicotiana tabacum* (*nia*-130), incapable of utilizing nitrate as the sole source of nitrogen, with a streptomycin resistant mutant of *N. tabacum* (SR1) to produce a double mutant. The *nia*-130 mutant lacks nitrate reductase apoenzyme activity and is a recessive nuclear mutant. As *N. tabacum* is an allotetraploid (Gray *et al.*, 1974), mutations at two separate loci are necessary to produce nitrate reductase deficiency and thus, chlorate resistance (Müller, in press). Mutant plants were regenerated from a cell line selected for potassium chlorate resistance by a procedure previously described (Müller and Grafe, 1978). *N. tabacum* SR1 is a cytoplasmic mutant (Maliga *et al.*, 1973, 1975). A nitrate reductase deficient (NR<sup>-</sup>), streptomycin resistant (SR<sup>+</sup>) mutant will be useful for somatic cell fusion studies as hybrid colonies can be selected in culture for streptomycin resistance and the ability to use nitrate as the sole source of nitrogen.

## 2. MATERIALS AND METHODS

*Nicotiana tabacum nia*-130 were grown in hydroponics. The nutrient solution has the following composition: KNO<sub>3</sub>, 200 mg l<sup>-1</sup>; CaCl<sub>2</sub>·2H<sub>2</sub>O, 175 mg l<sup>-1</sup>; MgSO<sub>4</sub>·7H<sub>2</sub>O, 140 mg l<sup>-1</sup>; KH<sub>2</sub>PO<sub>4</sub>, 130 mg l<sup>-1</sup>; FeEDTA (FeSO<sub>4</sub>·7H<sub>2</sub>O, 5 mg l<sup>-1</sup> and Na<sub>2</sub>EDTA, 7 mg l<sup>-1</sup>), NH<sub>4</sub>NO<sub>3</sub>, 350 mg l<sup>-1</sup>; MnSO<sub>4</sub>·4H<sub>2</sub>O, 2 mg l<sup>-1</sup>; CuSO<sub>4</sub>·5H<sub>2</sub>O, 250 µg l<sup>-1</sup>; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 300 µg l<sup>-1</sup>; H<sub>3</sub>BO<sub>3</sub>, 2 mg l<sup>-1</sup> and (NH<sub>4</sub>)<sub>6</sub>MoO<sub>24</sub>, 35 µg l<sup>-1</sup>. This nutrient solution is modified from Long Ashton nutrient solution (Hewitt, 1966). The above nutrient solution will be referred to as MN medium. *N. tabacum* SR1 plants were grown as for wild type as reported previously (Pental *et al.*, 1982). In sexual crosses the SR1 mutant was used as the female parent to ensure transmission of the streptomycin resistant chloroplast. Plants of the F1 were grown as for SR1. To allow segregation of nitrate reductase deficient genotypes, the F1 generation was either selfed or backcrossed to the *nia*-130 parent using the F1 as the female.

Seeds from the selfed and backcrossed F1 plants were surface sterilized with 20 per cent v/v Domestos (Lever Bros. U.K.) for 45 min followed by thorough rinsing in sterile water. Sterilized seeds were germinated on MN medium solidified with 8 g l<sup>-1</sup> agar (Sigma) in 9 cm petri dishes (Sterilin, Teddington) under continuous fluorescent light (3000 lux) at a temperature

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of  $22 \pm 2^\circ\text{C}$ . To select double mutant seedlings, MN medium was supplemented with  $1 \text{ g}^{-1}$  streptomycin sulphate (Sigma), and  $2.5 \text{ g}^{-1}$  potassium chlorate. Seeds germinated within 3–4 days and after another 6–8 days the difference between chlorate sensitive and resistant seedlings was apparent. The former were shrivelled and became necrotic, the latter possessed morphologically normal cotyledons.

Potassium chlorate, streptomycin resistant seedlings were aseptically removed and cultured on a shoot culture medium (Pental *et al.*, 1982) or were grown in hydroponics as for *nia*-130 mutants. Nitrate reductase activity of chlorate and streptomycin resistant plants was assayed following the general procedure of Mendel and Müller (1978). Protoplasts from chlorate and streptomycin resistant plants were isolated and cultured as previously described (Pental *et al.*, 1982). To check for mutant traits, protoplasts were cultured in an amino acid medium with chlorate and streptomycin or a medium with nitrate as the sole source of nitrogen.

### 3. RESULTS

#### (i) Crosses for producing double mutant

The progeny of crosses between SR1  $\times$  *nia*-130 were resistant to streptomycin and sensitive to chlorate. F1 hybrids were selfed or backcrossed to *nia*-130 and the progeny were scored for segregation for chlorate sensitivity: chlorate resistance. Results are given below in tabular form.

Cross	Expected ratios sensitive : resistant	Observed ratios sensitive : resistant	$\chi^2$ at $p = 0.05$
F1 selfed	15 : 1	1438 : 105	$0.5 > \chi^2 > 0.3$
F1 $\times$ <i>nia</i> -130	3 : 1	1113 : 347	$0.3 > \chi^2 > 0.2$

At  $p = 0.05$ ,  $\chi^2$  analysis shows that deviations of observed from expected are not significant. These results confirm that chlorate resistance is due to recessive mutations at two loci. All offspring were streptomycin resistant due to maternal inheritance of chloroplasts in *Nicotiana* (Kirk and Tilney-Bassett, 1978).

#### (ii) Characterisation of double mutants

Like their nitrate reductase deficient parent *nia*-130, seedlings of the streptomycin and chlorate resistant double mutants were unable to grow on modified MN medium containing  $2 \text{ g l}^{-1}$  potassium nitrate as sole source of nitrogen. Seedlings of the nitrate reductase proficient parent SR1 were capable of growing on the modified MN medium.

When grown in hydroponics, the nitrate reductase activity of leaf homogenates of wild type *N. tabacum* was 2–3 n moles  $\text{NO}_2^-$  produced  $\text{min}^{-1} \text{ mg}^{-1}$  protein at  $18^\circ\text{C}$ . No activity was detected in leaf homogenates of *nia*-130 parents and of double mutant plants.

Protoplasts isolated from the leaf mesophyll tissue of double mutant plants, divided with 50–60 per cent plating efficiency when cultured in a medium containing amino acids as a nitrogen source (Pental *et al.*, 1982). Protoplast-derived colonies were capable of growth as green colonies in amino acid medium containing  $1 \text{ g l}^{-1}$  streptomycin sulphate, and/or

2.5 g l<sup>-1</sup> potassium chlorate. However, double mutant protoplast colonies were incapable of growth in a medium containing nitrate as the sole source of nitrogen unlike protoplast-derived colonies of the wild type *N. tabacum* (Pental *et al.*, 1982).

#### 4. DISCUSSION

Both nitrate reductase deficiency and streptomycin resistance have been used as markers for selection of somatic hybrids in culture. For example, protoplasts from a nitrate reductase deficient cell line of *N. tabacum* were fused with protoplasts of a cytoplasmic albino mutant of *N. tabacum* and somatic hybrids were selected on the basis of chlorophyll synthesis and nitrate reductase proficiency (Glimelius *et al.*, 1981). Somatic hybrids were also selected on the basis of streptomycin resistance and hormone autotrophy by fusion between *N. tabacum* SR1 and a tumorous line of *N. tabacum* (Wullems *et al.*, 1980).

By sexual crosses we have combined streptomycin resistance and nitrate reductase deficiency, incorporating a positive and negative selection marker in the same *N. tabacum* plant. Theoretically, this *N. tabacum* NR<sup>-</sup>SR<sup>+</sup> mutant can be hybridized with any streptomycin sensitive wild type species by protoplast fusion. On selection medium containing nitrate as the sole source of nitrogen and also containing streptomycin, it is expected that only nuclear/cytoplasmic recombinant cells will be able to grow as green colonies (barring new mutations or revertants). In fusions between *N. tabacum* NR<sup>-</sup>SR<sup>+</sup> and other species, recombinant genomes may be expected to vary from true amphiploid somatic hybrids through a range of partial hybrids to a situation where only a chromosome carrying the nitrate reductase gene or the gene itself has been maintained in an otherwise *N. tabacum* genetic background.

The other expected recombinant cells are cybrids possessing the chloroplasts of *N. tabacum* and the nuclear genome of the chosen wild type parent. Thus the streptomycin resistant nitrate reductase deficient double mutant of *N. tabacum* will be valuable for assessing genomic compatibilities between tobacco and evolutionarily divergent species via protoplast fusion.

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