Co-segregation of nitrate-reductase activity and normal regeneration ability in selfed sibs of *Nicotiana plumbaginifolia* somatic hybrids, heterozygotes for nitrate-reductase deficiency

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Summary. The nitrate-reductase (NR) defective cell lines of *Nicotiana plumbaginifolia* isolated in our laboratory could not be regenerated into plants on the standard medium (Márton et al. 1982a). The normal regeneration potential, however, was restored in somatic hybrids obtained by fusing the NR" (green) lines with a pigment deficient (P"), but NR+ line, A28. Somatic hybrid plants were fertile in two combinations (A28+NA9 and A28+NX9). As expected, segregation for NR- and P- was found after selfing the somatic F1 (SF1) obtained by protoplast fusion, and in the F2. The variable segregation ratios are explained by chromosome abnormalities. Co-segregation of the NR" phenotype and the altered response to shoot induction on standard medium suggest the involvement of the nitrate-assimilatory pathway in determining shoot regeneration ability.

Key words: Nitrate reductase activity – Shoot induction – *N. plumbaginifolia* – Segregation

Introduction

Interest in nitrate metabolism and in generating auxotrophic mutations for plant cellular genetic studies has resulted in the isolation of nitrate-reductase deficient (NR") lines in a number of species.

Mutants isolated by screening seedlings were found to display residual NR activity (Oostindier-Braaksma and Feenstra 1973; Kleinhofs et al. 1980; Feenstra and Jacobsen 1980; Braaksma and Feenstra 1982). By tissue culture a number of cell lines were obtained which are fully defective in NR activity. Such mutants were obtained in *N. tabacum* (Müller and Grafe 1978), *Datura innoxia* (King and Khanna 1980), *Hyoscyamus muticus* (Straus et al. 1981), *N. plumbaginifolia* (Márton et al. 1982a; Negrutiu et al. 1983) and in *Petunia* (Steffen and Schieder, 1983).

In some of the NR+ cell lines isolated in *N. tabacum* (Müller 1983) and *N. plumbaginifolia* (Negrutiu et al. 1983) plants were regenerated and the inheritance of NR deficiency through meiosis has been studied. Plant regeneration, however, was not possible in some of the *N. tabacum* lines (Müller and Grafe 1978), in *Petunia* lines (Steffen and Schieder 1983), and in our *N. plumbaginifolia* cell lines which entirely lacked NR activity (Márton et al. 1982a).

Complemented somatic hybrids obtained by fusion regenerated normally in our lines (Márton et al. 1982b) and in *N. tabacum* (Glimelius et al. 1978). Restoration of shoot formation in complemented somatic hybrids suggested a causal relationship between the loss of NR activity and the loss of shoot regeneration ability. However, it could not be excluded that a mutation other than that responsible for the loss of NR activity was involved in the altered response to shoot induction due to unintentional selection in culture. As an example the work of Chaleff and Keil (1981) could be mentioned. They discovered that more than half of all tobacco cell lines isolated on the basis of resistance to the herbicide picloram were also resistant to hydroxyurea, traits which are genetically unlinked.

In this paper we provide evidence for the co-segregation of the NR" phenotype and the altered response to shoot induction in the seed progeny of somatic hybrids.

Materials and methods

Plant material

Isolation and properties of the NR" cell lines (Table 1) have been described earlier (Márton et al. 1982 a, b). The chromosome numbers were not determined before protoplast fusion. In shoot induction tests a wild-type *Nicotiana plumbaginifolia* callus (DPN) was used which was initiated at the time of the
isolation of the NR⁻ lines, and was maintained under identical conditions.

The pigment deficient line A28 was isolated in a haploid protoplast culture and was diploid at the time of protoplast fusion (Sidorov and Maliga 1982). Pigment deficiency in this line was shown to be recessive by fusion.

**Callus culture and plant regeneration**

The NR⁻ cell lines were maintained on the NH₄-S-RMOP-medium which is the Murashige and Skoog (MS) medium (1962) with the growth regulators naphthaleneacetic acid (0.1 mg/l) and benzyladenine (1 mg/l) (Sidorov et al. 1981), and was supplemented with 8.25 mM ammonium-succinate (Márton et al. 1982 a).

Shoots from somatic hybrids were obtained on RMOP medium (same medium but ammonium succinate omitted). Shoot induction was also obtained on RMB medium, which is the same as the RMOP medium, except that it contains 1 mg/l BA but no NAA. The shoots were rooted on P medium (Maliga et al. 1982).

**Determination of NR activity**

NR activity was assayed in vivo as previously described (Márton et al. 1982 a).

**Protoplast isolation, fusion and culture conditions**

Protoplasts were isolated and fused according to Menczel et al. (1981). Protoplasts were cultured in modified K3 medium containing 0.4 M glucose, 1 mg/l naphthaleneacetic acid and 1 mg/l benzyladenine. Details of protoplast culture have been described (Márton et al. 1982 a, b).

**Screening seedlings for NR⁻ and P⁻ phenotypes**

The seeds were surface sterilized, treated with gibberelic acid (GA₃) (0.5 mg/ml; 1 h) and germinated on a medium containing the salts of the Murashige-Skoog medium; 8.25 mM ammonium-succinate, 3% sucrose, 0.7% agar, pH 5.6. On this medium the P⁻ plants were white, whereas wild-type and NR⁻ seedlings were green. Details of protoplast culture have been described (Márton and Maliga 1975), supplemented with 8.25 mM ammonium-succinate. The in vivo NR activity was measured in ten-day-old calli. Nitrate utilization by seedling calli was tested by their ability to proliferate on the RMNO medium without ammonium-succinate, a medium which does not support the growth of cells lacking NR activity (Müller and Grafe 1978; Márton et al. 1982 a, b).

**Determination of chromosome numbers**

The chromosome numbers were determined in colchicine treated (0.5%; 3 h) root tips by the standard aceticarmine method.

**Results**

**Somatic hybrids by protoplast fusion**

Protoplasts obtained from calli of the NR⁻ cell lines (Table 1) were fused with diploid protoplasts from leaves of the recessive albino (P⁺) mutant A28. Complemented green and NR⁺ colonies were selected by culturing the fused products in ammonium-nitrate based K3 medium under light conditions. On this medium parental NR⁻ protoplasts did not divide whereas A28 protoplasts formed white calli. The frequency of somatic hybrid colonies (green colonies) was 3–10% in the fused population. No such green colonies were observed in mixed (non-fused) protoplast cultures or after the intra-line fusion of 10⁶ protoplasts. Somatic hybrids in each combination contained levels of NR activity close to that of the wild-type (50–100%, Table 1). Each of these somatic hybrid clones regenerated shoots, as did the wild-type control tissue.

In regenerated somatic hybrid plants chromosome numbers were tetraploid (4x = 40), or varied around the tetraploid level (Table 1).

**Marker segregation in the F2 and F3 generations**

Regenerated somatic hybrids, with respect to the nuclear genetic information, but not necessarily with respect to the cytoplasm are equivalent to an F1 (with increased ploidy) and will be termed below as somatic F1 (SF1). The seed progeny of SF1 obtained by selfing will be termed F2 since in this case inheritance is not expected to differ from that of plants descended from generative hybrids.

Somatic hybrid plants from most fusion combinations were self sterile except for some of those derived from fusions with NX9 and NA9. Marker segregation was studied in the seed progeny (F2) of self-fertile NX9 + A28 and NA9 + A28 somatic hybrid plants (SF1).

**Table 1. NR activity and chromosome numbers in the somatic hybrid (green) clones**

<table>
<thead>
<tr>
<th>Fusion combination</th>
<th>NR activity</th>
<th>No. of plants</th>
<th>Chromosome no.</th>
<th>No. of tetr(ploids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A28 + NA1</td>
<td>+</td>
<td>4 (3)</td>
<td>42–50</td>
<td>–</td>
</tr>
<tr>
<td>A28 + NA2</td>
<td>+</td>
<td>6 (5)</td>
<td>32–55</td>
<td>1</td>
</tr>
<tr>
<td>A28 + NA9</td>
<td>+</td>
<td>14 (6)</td>
<td>31–50</td>
<td>2</td>
</tr>
<tr>
<td>A28 + NA18</td>
<td>+</td>
<td>6 (3)</td>
<td>33–43</td>
<td>2</td>
</tr>
<tr>
<td>A28 + NA36</td>
<td>+</td>
<td>5 (1)</td>
<td>40–55</td>
<td>1</td>
</tr>
<tr>
<td>A28 + NX1</td>
<td>+</td>
<td>9 (5)</td>
<td>33–65</td>
<td>1</td>
</tr>
<tr>
<td>A28 + NX9</td>
<td>+</td>
<td>36 (13)</td>
<td>26–40</td>
<td>5</td>
</tr>
<tr>
<td>A28 + NX21</td>
<td>+</td>
<td>9 (2)</td>
<td>37–50</td>
<td>3</td>
</tr>
<tr>
<td>A28 + NX24</td>
<td>+</td>
<td>4 (4)</td>
<td>35–68</td>
<td>2</td>
</tr>
</tbody>
</table>

* In vivo NR activity was measured in four independent clones. Values varied between 50–100% of the wild-type level (260 nmoles NO₂⁻/100 mg callus/1 h)

b In brackets: the number of clones from which the plants were regenerated
c Chromosomes were counted in 5 root tip cells per plant
d Plants with 40 chromosomes