EFFECT OF RADIATION DOSAGE ON EFFICIENCY OF CHLOROPLAST TRANSFER BY PROTOPLAST FUSION IN NICOTIANA

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ABSTRACT

Chloroplasts of Nicotiana tabacum SR1 were transferred into Nicotiana plumbaginifolia by protoplast fusion. The protoplasts of the organelle donor were irradiated with different lethal doses using a 60Co source, to facilitate the elimination of their nuclei from the fusion products. After fusion induction, clones derived from fusion products and containing streptomycin-resistant N. tabacum SR1 chloroplasts were selected by their ability to green on a selective medium. When N. tabacum protoplasts were inactivated by iodoacetate instead of irradiation, the proportion of N. plumbaginifolia nuclear segregant clones was low (1-2%). Irradiation markedly increased this value: Using 50, 120, 210 and 300 J kg\(^{-1}\) doses, the frequency of segregant clones was 44, 57, 84 and 70 percent, respectively. Regeneration of resistant N. plumbaginifolia plants with SRI chloroplasts indicated that plastids can be rescued from the irradiated cells by fusion with untreated protoplasts. Resistant N. plumbaginifolia plants that were regenerated (43 clones studied) had diploid \((2n = 2X = 20)\) or tetraploid chromosome numbers and were identical morphologically to parental plants. The absence of aneuploids suggests that in these clones irradiation resulted in complete elimination of the irradiated N. tabacum nuclei. Resistance is inherited maternally (five clones tested). The demonstration of chloroplast transfer and the presence of N. tabacum plastids in the N. plumbaginifolia plants was confirmed by chloroplast DNA fragmentation patterns after EcoRI digestion.

PROTOPLAST fusion offers the possibility of one-step transfer of organelles between plant species, replacing the tedious procedure involving repeated back-crosses. Organelle transfer by protoplast fusion is based on the segregation of nuclei in the primary fusion products, and subsequent segregation of organelles resulting in new combinations of nuclei and organelles. Transfer of chloroplasts has been achieved using this method (Belliard et al. 1978; Aviv and Galun 1980; Medgyesy, Menczel and Maliga 1980; Sidorov et al. 1981) as has male sterility (Zelcer, Aviv and Galun 1978) or fertility (Aviv and Galun 1980).

Reported values of nuclear hybrid formation in interspecific fusion products are close to 100% (Kao 1977; Gleba and Hoffmann 1978; Medgyesy, Menczel and Maliga 1980). Efficient chloroplast transfer, therefore, may be
carried out only if nuclear fusion is prevented and the nucleus of the organelle donor is eliminated. Irradiation has been used in mammalian cells for this purpose (Harris 1972), and the feasibility of this approach has also been demonstrated in higher plants (Aviv and Galun 1980; Sidorov et al. 1981). In studies on plants, however, only one dose was applied.

In this paper we report experiments using different doses to establish the optimal conditions for chloroplast transfer. We also investigated whether the irradiated nucleus is eliminated as a whole or whether a few chromosomes are retained from the irradiated cells. The possibility of chromosome transfer from irradiated protoplasts has been demonstrated (Dudits et al. 1980).

Chloroplasts were to be transferred from N. tabacum into N. plumbaginifolia nuclear background. N. plumbaginifolia is used in our laboratory as a model species in cellular genetic studies (Maliga et al. in press). As donor, an N. tabacum mutant, SR1, was used because it contains chloroplasts coding for a maternally-inherited streptomycin-resistant mutation (Maliga, Breznovits and Marton 1975; Menczel et al. 1981).

Protoplasts of the donor were irradiated before fusion with 60Co γ-rays using doses higher than LD100. Such high doses prevented formation of colonies from the resistant N. tabacum cells, so heterokaryon-derived clones containing SR1 plastids could be selected by their streptomycin resistance. Recovery of plastids from inactivated protoplasts in the primary fusion products has been demonstrated in other systems (Aviv and Galun 1980; Medgyesy, Menczel and Maliga 1980). The resistant clones were characterized with respect to plastids and nuclear genetic constitution at the regenerated plant level, to determine which treatment gave the highest frequency of N. plumbaginifolia plants with SR1 chloroplasts.

MATERIALS AND METHODS

Protoplast isolation and fusion: Mesophyll protoplasts of N. tabacum SR1 and N. plumbaginifolia were isolated from plants grown in sterile culture and fused by polyethylene glycol treatment as described (Menczel et al. 1981).

Inactivation of protoplasts: Protoplasts were suspended in W5 solution (125 mM CaCl2; 155 mM NaCl; 5 mM KCl; 5 mM glucose; pH = 5.6) at a density of about 10^5 per ml in polycarbonate centrifuge tubes (Nalgene Labware) and irradiated using a 60Co source. The doses used were 50 and 120 J kg^-1 (dose rate 0.042 J kg^-1 sec^-1) and 210 and 300 J kg^-1 (0.07 and 0.11 J kg^-1 sec^-1, respectively). The LD50 of 60Co irradiation for N. tabacum protoplasts is about 20 J kg^-1 at a dose rate of 0.042 J kg^-1 sec^-1 (V. A. Sidorov, personal communication). Inactivation of protoplasts by iodoacetate (10 mM) was carried out as described (Medgyesy, Menczel and Maliga 1980).

Culture media and conditions: Protoplasts were cultured in dim light (100 lx) in K3 medium (Nagy and Maliga 1976) containing 0.4 M glucose. Selection of streptomycin-resistant colonies and tests for the resistance of regenerated plants were carried out at a higher light intensity (3000 lx) using the Linsmaier and Skoog’s RM medium (Linsmaier and Skoog 1965) with 1.0 mg benzyladenine plus 0.1 mg naphthaleneacetic acid per liter (RMOP medium) solidified with 0.8% agar (Difco). The concentration of streptomycin sulphate was 1 g per liter in both cases. Plants were regenerated on RMOP or RMB (RM plus 1.0 mg 1^-1 benzyladenine) medium without streptomycin. The shoots were rooted and maintained on P
medium (RM salts but with one fifth of the original concentration of KNO₃, NH₄NO₃ and MgSO₄; 3% sucrose). Cultures were incubated at 28° and illuminated 16 hr per day.

Analysis of esterase isoenzymes: Crude extracts were prepared from leaves of sterile plants kept in the dark 16 hr before use. Leaf tissue (0.2 g) was homogenized in 1 ml ice-cold extraction buffer (0.1 M Tris; 15 mM 2-mercaptoethanol; 2 mM cysteine; 2 mM MgCl₂; 1 mM EDTA; 20% glycerol, pH = 8.0). The homogenate was centrifuged at 15,000 × g for 15 minutes. Supernatant (200 µl) was layered on polyacrylamide gels and separated according to a modified version of Maurer's disc electrophoretic method (MAURER 1971). The upper gel (4%) contained 0.01 M Tris-H₃PO₄ buffer (pH = 5.5), the lower gel (6%; 7 cm long) was buffered with 0.05 M Tris-HCl (pH = 7.5). In both gels cross linking was 2%. The reservoir buffer contained 1.0 g Tris and 5.5 g 5,5'-diethylbarbituric acid per liter (pH = 7). Electrophoresis was carried out for 1.5 hr at 4° with a constant current of 4 mA per tube. The gels were stained at 37° in 0.1 M phosphate buffer (pH = 6) containing 0.4 mg Fast Blue RR salt and 0.2 mg α-naphtylacetate per ml (BREWBAKER et al. 1968).

Cytology: Chromosomes in colchicine-treated (0.5%, 3 hr) root tips were stained by the standard acetocarmine technique.

Analysis of chloroplast DNA: Chloroplast DNA (cpDNA) was fragmented by the EcoRI restriction endonuclease and the fragments analyzed by agarose gel electrophoresis as described by MENCZEL et al. (1981).

RESULTS

Establishment of the resistant clones: N. tabacum SR1 protoplasts were irradiated with four different doses (MATERIALS and METHODS). Irradiation with these doses prevented colony formation although protoplasts regenerated cell walls and some of them underwent the first cell division.

The irradiated protoplasts were fused with N. plumbaginifolia protoplasts by polyethylene glycol treatment. Aliquots of the same protoplast mixture (1:1), not subjected to fusion treatment, were also cultured and served as controls for the inactivation by irradiation. To get comparable data in the absence of irradiation, iodoacetate treated SR1 protoplasts were fused using the same protocol. This treatment also prevents cell division (WRIGHT 1978; MEDGYESY, MENCZEL and MALIGA 1980).

Small colonies obtained after two weeks in culture in K3 medium were plated into RMOP medium containing 0.4 M glucose and 1 g l⁻¹ streptomycin. Three weeks later they were replated onto the same selective medium but with

TABLE 1

<table>
<thead>
<tr>
<th>Dose (J kg⁻¹)</th>
<th>Frequency of resistant colonies (percent)*</th>
<th>Mixed control</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEG treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>18.3 (12,300)</td>
<td>0 (68,400)</td>
</tr>
<tr>
<td>50</td>
<td>22.0 (14,290)</td>
<td>0 (36,500)</td>
</tr>
<tr>
<td>120</td>
<td>6.9 ( 7,140)</td>
<td>0 ( 5,280)</td>
</tr>
<tr>
<td>210</td>
<td>4.0 ( 7,050)</td>
<td>0 ( 9,280)</td>
</tr>
<tr>
<td>300</td>
<td>5.7 ( 1,850)</td>
<td>0 ( 1,400)</td>
</tr>
</tbody>
</table>

* The total number of colonies are in parentheses.
† SR1 protoplasts were inactivated by iodoacetate.
TABLE 2

Distribution of plant types in the clones

<table>
<thead>
<tr>
<th>Dose (J kg⁻¹)</th>
<th>Number of clones studied</th>
<th>No. clones regenerating indicated plant types</th>
<th>Frequency of plastid transfer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H</td>
<td>H and Nt</td>
</tr>
<tr>
<td>0‡</td>
<td>100</td>
<td>69 (5)</td>
<td>3 (0)</td>
</tr>
<tr>
<td>50</td>
<td>38</td>
<td>10 (0)</td>
<td>0</td>
</tr>
<tr>
<td>120</td>
<td>41</td>
<td>15 (0)</td>
<td>0</td>
</tr>
<tr>
<td>210</td>
<td>33</td>
<td>5 (0)</td>
<td>0</td>
</tr>
<tr>
<td>300</td>
<td>35</td>
<td>9 (0)</td>
<td>0</td>
</tr>
</tbody>
</table>

* H, hybrid; Nt, N. tabacum; Np, N. plumbaginifolia. Number of clones in which sensitive, or both sensitive and resistant plants were obtained is in parentheses.
† Frequency of regenerating clones in which N. plumbaginifolia plants with N. tabacum chloroplasts were obtained.
‡ SR1 protoplasts were inactivated by iodoacetate.

judgement of the resistance phenotype was made after retesting the clones on 0.2 M glucose. Resistant colonies containing SR1 chloroplasts turned green, while the sensitive ones remained white and showed retarded growth. Final the same selective medium. The frequency of resistant colonies is given in Table 1.

*Regeneration and classification of plants:* Plants could be regenerated in 70–90% of the resistant clones (Table 2) by shoot induction on RMOP or RMB media in the absence of streptomycin and subsequent rooting on P medium. Three to four plants were regenerated from each clone. The plants were classified as parental types or somatic hybrids by their characteristic leaf esterase isoenzyme patterns (Figure 1). Identification of N. plumbaginifolia + N. ta-

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**Figure 1.**—Leaf esterase isoenzymes of the parents and regenerated plants. A) N. tabacum; B) somatic hybrid; C) resistant N. plumbaginifolia segregant; D) N. plumbaginifolia.
Plastid transfer using protoplasts

*Arabidopsis* somatic hybrids and their properties will be described in more detail in a separate paper. Data concerning the various clones are summarized in Table 2. Esterases of every plant derived from a particular clone were studied only if differences in morphology suggested the presence of more than one genotype. Streptomycin resistance of the regenerates was tested by culturing leaf pieces on a selective medium, where resistant sections form green callus while those containing sensitive chloroplasts are white (Figure 2).

Plants from 57 clones were grown to maturity in the greenhouse. Diploid regenerates were identical with the parents, except for two that had altered petal morphology. Tetraploids were very similar to diploids but more robust.

Chromosome numbers in the *N. plumbaginifolia* regenerates: The analysis of esterases did not exclude the possibility that the *N. plumbaginifolia* regenerates contained a few chromosomes from the irradiated *N. tabacum* donor. For this reason, chromosome numbers were counted in several *N. plumbaginifolia* regenerates. In the plants, the original diploid (*2n = 2X = 20; Figure 3*), or tetraploid chromosome numbers were found (Table 3).

Inheritance of streptomycin resistance: After transfer, maternal inheritance of the SR1 plastids was expected in the resistant *N. plumbaginifolia* plants. These plants were fertile and gave many seeds both in crosses and after selfing. Maternal inheritance of streptomycin resistance was found in each of the five clones in which selfs (500 seedlings) and F₁ and reciprocal F₁ seedlings (500 each) were tested (Figure 4).

In these five clones, and in plants representing seven additional clones, the

![Figure 2](image-url)

FIGURE 2.—Streptomycin resistance test with leaf sections of the regenerated plants. A) sensitive *N. plumbaginifolia* parent; B, C and D) resistant *N. plumbaginifolia* regenerates.
Figure 3.—Morphology (A) and a metaphase plate (B) of a resistant diploid (2n = 20) N. plumbaginifolia plant.

The parental nuclei fused to yield hybrids in the majority of fusion products after iodoacetate treatment of donor protoplasts. The proportion of N. plumbaginifolia nuclear segregants was consequently low (one of 74; Table 2). Irradiation significantly increased the frequency of this type of nuclear segregant. This effect was dependent on the radiation dose, the maximum value being 84% at 210 J kg⁻¹ (Table 2). A high frequency (13 of 15) of nuclear segregants was also reported by AVIV and GALUN (1980) who used 42 J kg⁻¹ (5 kR) X-ray irradiation in their experiment. The frequency (44%) obtained at the lowest dose (50 J kg⁻¹) agrees well with data published from this laboratory (SIDOROV et al. 1981).

The N. plumbaginifolia regenerates had the original diploid, or tetraploid chromosome numbers. Appearance of tetraploid plants may be the result of

<table>
<thead>
<tr>
<th>Dose (J kg⁻¹)</th>
<th>Diploid</th>
<th>Number of clones Te.raplo.d</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>5</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>210</td>
<td>13</td>
<td>10</td>
<td>23</td>
</tr>
<tr>
<td>300</td>
<td>7</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>(Total)</td>
<td>25</td>
<td>18</td>
<td>43</td>
</tr>
</tbody>
</table>
spontaneous polyplody known to occur frequently in cultured plant cells (Bayliss 1980), or of fusion of more than two protoplasts (e.g., Smith, Kao and Combatti 1976; Melchers and Sacrístán 1977). No aneuploids were found among the plants studied cytologically (43 clones); therefore the elimination of irradiated donor nuclei was complete in these cases.

The formation of \textit{N. plumbaginifolia} nuclear segregants did not necessarily result in chloroplast transfer in each case. In some clones, sensitive plants were obtained from resistant calli. This could be due to the maintenance of sensitive plastids in phenotypically resistant cells. The proportion of clones giving sensitive plants decreased with increasing doses of radiation and was practically zero at higher doses. Improved competitive ability of irradiated plastids may be the explanation for this previously observed phenomenon (Sidorov et al. 1981).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Seedling test for streptomycin resistance. Seeds were germinated in distilled water containing 1 g l$^{-1}$ streptomycin sulphate. A) resistant \textit{N. plumbaginifolia} seedlings with SR1 plastids; B) sensitive \textit{N. plumbaginifolia} seedlings. Note differences in pigmentation.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Identification of \textit{N. tabacum} and \textit{N. plumbaginifolia} plastids by the EcoRI restriction pattern of cpDNAs. A) \textit{N. plumbaginifolia}; B) \textit{N. tabacum}. Note differences in the regions indicated.}
\end{figure}
Mixed plastid populations could not be detected in the sexual progeny of the five plants studied. Seedlings obtained from selfing and from crosses using the resistant plants as female (500 plants tested) were all resistant to streptomycin. Lack of plastid heterogeneity was also found in a previous transfer experiment (Medgyesy, Menczel and Maliga 1980).

The high doses of radiation must have induced several mutations in the irradiated cells. Nevertheless, among 57 clones, there were only two in which deviations from normal morphology could be detected in regenerated plants. Since many clones with the same nucleus–organelle combination can be produced in a single experiment, the relatively infrequent genetic alterations do not limit the applicability of this transfer technique.

Fusion of enucleated protoplasts (cytoplasts) with protoplasts has also been suggested for efficient organelle transfer (Lörz and Potrykus 1980). The system described in this paper appears to be superior, since only 10% chloroplast transfer was obtained by fusing SR1 cytoplasts to N. plumbaginifolia protoplasts (Maliga et al., in press). Irradiation combined with iodoacetate treatment was also shown to be suitable for the transfer of chloroplasts (Sidorov et al. 1981), and possibly of other cytoplasmic organelles, without the use of selectable genetic markers. Data in this paper should be helpful in choosing the optimal dose in such transfer experiments.

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LITERATURE CITED


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