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A light sensitive recipient for the effective transfer of chloroplast and mitochondrial traits by protoplast fusion in *Nicotiana*

P. Medgyesy, R. Golling* and F. Nagy

Institute of Plant Physiology, Biological Research Center, Hungarian Academy of Sciences, P.O. Box 521, H-6701 Szeged, Hungary

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Summary. A light sensitive mutant was used as a recipient in the transfer of chloroplasts from a wild-type donor. Gamma irradiated (lethal dose) mesophyll protoplasts of *Nicotiana gossei* were fused with mesophyll protoplasts of a *N. plumbaginifolia* line carrying light sensitive plastids from a *N. tabacum* mutant. After fusion, colonies containing wild-type plastids from the cytoplasm donor were selected by their green colour. Most of the regenerated plants had *N. plumbaginifolia* morphology, but were a normal green in colour. The presence of donor-type plastids was confirmed by the restriction pattern of chloroplast DNA in each plant analysed. These cybrids were fully male sterile with an altered flower morphology typical of certain types of alloplasmic male sterility in *Nicotiana*. The use of the cytoplasmic light sensitive recipient proved to be suitable for effective interspecific transfer of wild-type chloroplasts. The recombinant-type mitochondrial DNA restriction patterns and the male sterility of the cybrids indicated the co-transfer of chloroplast and mitochondrial traits.

Key words: Cytoplasm transfer – Light sensitive plastids – Male sterility – *Nicotiana* – Protoplast fusion

Introduction

Protoplast fusion provides a new approach in transferring cytoplasmic organelles between plant species as compared to the conventional breeding methods (Galun and Aviv 1983).

An increase in the ratio of cybrids (new nucleus-cytoplasm combinations) in the fusion derived clones was achieved by irradiating the cytoplasm donor protoplasts to inactivate their nuclei (Zelcer et al. 1978). On the other hand, selection for the cytoplasm to be transferred, using a selectable cytoplasmic mutation, made it easy to isolate fusion-derived cell lines with the desired cytoplasm (Medgyesy et al. 1980). Using a combination of these methods, cytoplasms of *N. tabacum*, *N. megalosyphon* and *N. sylvestris* have effectively been transferred into *N. plumbaginifolia*, used as a model species in our laboratory (Menczel et al. 1982, 1983; Cséplo et al. 1984). These transfer experiments were based on selection for streptomycin or lincomycin resistant plastids derived from the lethally irradiated donor protoplasts. The small number of mutants of such types (Maliga 1984), however, limits the range of available cytoplasm donors.

In this paper we report on the use of a cytoplasmic light sensitive *N. plumbaginifolia* line as a recipient, which made possible the efficient transfer of chloroplasts from a wild-type donor. The selection was based on the greening ability of wild-type plastids from the irradiated donor protoplasts in the heterokaryon derived clones.

In the experiment of Menczel et al. (1983) the selection for streptomycin resistance (a chloroplast trait) resulted in the co-transfer of male sterility (a mitochondrial trait) of the cytoplasm donor. In our experiment the co-transfer of chloroplasts and a mitochondrial trait (alloplasmic male sterility) has also been observed. The role of the various selection systems in this phenomenon is discussed.

Materials and methods

Plant material

Nicotiana gossei (2n = 36) plants were maintained on RM salts (Murashige and Skoog 1962) with 2% sucrose, solidified with 0.6% Bacto-Agar (RM medium), under culture room condi-

* On leave from: Department of Genetics, Section of Biosciences, Martin Luther University, Domplatz 1, DDR-4020 Halle/S., German Democratic Republic

tions (1,000 lx/16 h; 70% rel. hum.; 28 °C). The light sensitive *N. plumbaginifolia* Np(LS1)41 (2n=20) line, containing plastids of a cytoplasmic light sensitive *N. tabacum* mutant (LS1), was produced by protoplast fusion (Sidorov et al. 1981). These plants were maintained on RM medium supplemented with thiamine, 1 mg/l and 1-naphthaleneacetic acid, 0.1 mg/l, in dim light (100 lx) at 25 °C. For protoplast isolation young, light green leaves were used.

Protoplast isolation

Protoplasts were isolated from leaves as previously described (Menczel et al. 1981). Protoplasts of *N. gossei* were irradiated with a dose of 200 Jkg^{-1} of ^{60}Co gamma rays (0.066 Jkg^{-1} s^{-1} dose rate) according to the procedure of Menczel et al. (1982).

Protoplast fusion

Two drops of the protoplast mixture (1:1; $1-2\times10^6$ pp/ml) were placed into a plastic dish (3.5 cm in diameter) and the protoplasts were allowed to settle (15 min). One drop of PEG 40 solution (40% polyethylene glycol, MW=6,000; 50 mM $CaCl_2$; 0.3 M glucose) was then gently added. Three to five min later a drop of filter sterilized W10 solution (66 mM $CaCl_2$; 10% dimethyl sulfoxide; 0.4 M glucose; 30 mM NaOH-glycine buffer; pH=10.5; freshly prepared) was added. Twenty minutes later two drops of W10 solution, and after an additional 20 min, four drops of K_3 medium (Nagy and Maliga 1976) with 0.4 M glucose, were added. After 20 min the liquid over the protoplasts was diluted with 1 ml K_3 medium. Thirty minutes later it was replaced by fresh K_3 medium without mixing of the protoplasts.

Culture conditions

Cultures were kept in dim light (100 lx) at 25 °C and were diluted every 7th–10th day with K_3 medium containing gradually decreasing concentrations (0.4, 0.3 and 0.2 M) of glucose (30–40× final dilution). Six weeks after fusion the colonies were transferred into RMOP medium (RM salts; thiamine, 1 mg/l; myo-inositol, 100 mg/l; 6-benzyladenine, 1 mg/l; 1-naphthaleneacetic acid, 0.1 mg/l) with 0.2 M glucose (Menczel et al. 1982), solidified with 0.6% Bacto-Agar, at a final density of about 1,000 colonies per plate (10 cm in diameter). The plates were kept in light (1,000 lx/16 h) at 28 °C. Within two months green colonies appeared against the background of white ones. The former were isolated and grown for a month on the same medium but with 2% sucrose. Calli with shoot primordia were subcultured onto RM medium. Shoots were rooted and maintained on P medium (RM salts with 2% sucrose, but one-fifth of the original concentration of KNO_3, NH_4NO_3 and $MgSO_4$).

Chromosome counting

Root tips of regenerated plants were treated with colchicine (0.05%; 3 h), fixed in ethanol-acetic acid solution (3:1; 24 h; 4 °C) and stained with the standard acetocarmine method (for a protocol see Collins 1979).

Restriction analysis of chloroplast and mitochondrial DNA

Chloroplasts and mitochondria were isolated from leaves of aseptically cultured plants and were purified on sucrose step-gradients. Chloroplasts were treated by EcoRI restriction endonuclease and the DNA fragments were separated by agarose gel electrophoresis as in previous experiments (Menczel et al. 1981). Mitochondrial DNA was extracted, purified by CsCl-ethidium bromide gradient centrifugation, digested by SalI restriction endonuclease and the fragments were separated by agarose gel electrophoresis as previously described (Nagy et al. 1983).

Codes for the parental and isolated lines

The codes of the lines indicate the nuclear genetic constitution (Np=*N. plumbaginifolia*), the origin of the cytoplasm (in brackets, e.g. gos=*N. gossei*), and the isolation number of the cell line.

Results and discussion

Production and characterization of cybrids

After fusion of gamma irradiated (lethal dose) mesophyll protoplasts of *N. gossei* with mesophyll protoplasts of the cytoplasmic light sensitive *N. plumbaginifolia* Np(LS1)41 line, 200 green colonies were isolated from 1.0×10^4 colonies (2%). Most of the plants regenerated from the green clones could be identified by their morphology both in sterile culture and in the greenhouse as *N. plumbaginifolia* (Fig. 1). The regenerated plants, with a few exceptions, contained pure populations of donor-type chloroplasts, representing cases where organelle segregation had been completed before plant regeneration. White and variegated shoots appeared only in a very few clones, in contrast to other experiments (Fluhr et al. 1984; Gleba et al. 1985) where 60% of the fusion derived clones produced white and variegated shoots. In the present experiment the

Fig. 1. Morphology of the parental and cybrid plants. From *left to right: N. plumbaginifolia;* Np(gos)29; *N. gossei*

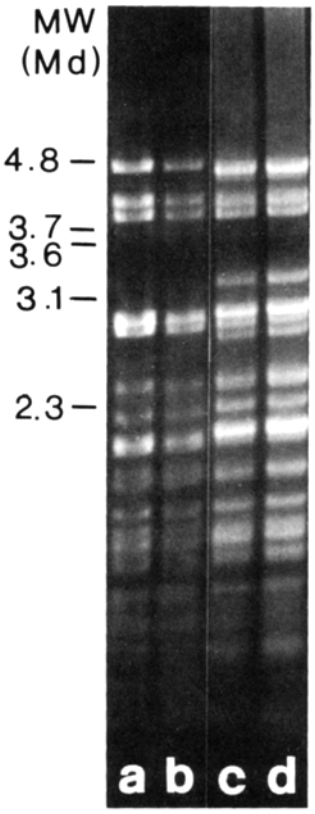

Fig. 2. EcoRI restriction patterns of chloroplast DNA of the parental and cybrid plants. *a N. tabacum; b* Np(LS1)41; *c* Np(gos)29; *d N. gossei.* Molecular weights were estimated using EcoRI fragments of λ phage DNA

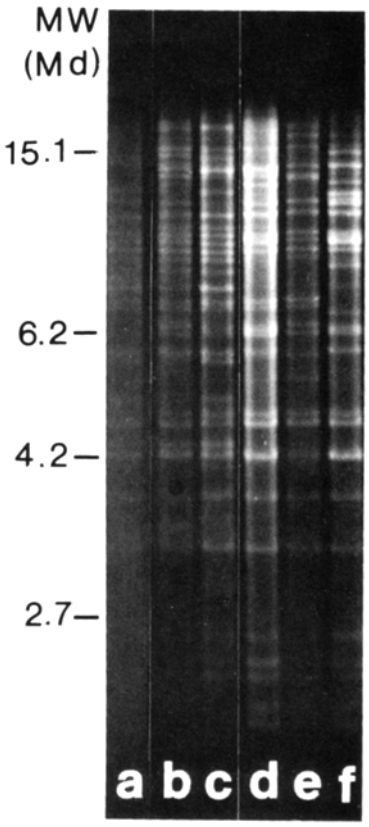

Fig. 3. SalI restriction patterns of mitochondrial DNA of the parental and cybrid plants. *a N. gossei; b* Np(gos)6; *c* Np(gos)5; *d* Np(LS1)41; *e N. plumbaginifolia; f N. tabacum.* Molecular weights were estimated using HindIII fragments of λ phage DNA

prolonged cell culture period under greening conditions might provide a selection advantage for the wild-type chloroplasts. The chromosome numbers of the green *N. plumbaginifolia* plants were diploid (in 12 clones) or tetraploid (in 5 clones). Tetraploidy is a common phenomenon in tissue culture (Bayliss 1980). The combination of irradiation and a chloroplast marker resulted in a high ratio of cybrids as found in similar fusion experiments (Menczel et al. 1982, 1983; Cséplő et al. 1984; Aviv et al. 1984a). Using the same selection system and the same light sensitive line as recipient, chloroplasts of *N. knightiana* and *N. undulata* have also been effectively transferred into *N. plumbaginifolia* (paper in preparation).

In previous fusion experiments selection for streptomycin or lincomycin resistant plastids, derived from lethally irradiated donor protoplasts, was used to achieve effective chloroplast transfer (Menczel et al. 1982, 1983; Cséplő et al. 1984). In the present fusion experiment the recipient carried a mutation (light sensitivity of the plastids), allowing selection of cell lines with wild-type plastids of the irradiated donor protoplasts. In this selection scheme any species having chloroplasts compatible with the nucleus of the recipient species may be used as a chloroplast donor. An additional advantage is that the presence of small amounts of recipient-type plastids may be revealed in the regenerated plants as variegation. It is notable, that cytoplasmic chlorophyll deficient mutants can be produced at a high frequency without any tissue culture process (Hagemann 1982; Hosticka and Hanson 1984).

Co-transfer of chloroplast and mitochondrial traits

EcoRI restriction analysis of chloroplast DNA of five randomly chosen diploid clones confirmed the presence of unchanged donor-type plastids (Fig. 2). Mitochondrial DNA of each of these clones showed unique SalI restriction pattern (Fig. 3) different from each other and from those of both parents, as it was found in other experiments, where heteroplasmic fusion combinations were used (Belliard et al. 1979; Nagy et al. 1981; Galun et al. 1982; Nagy et al. 1983). These cybrids showed various alterations in flower morphology (Fig. 4A–C) and were fully male sterile. The stamens in the individual flowers had stigmoid or petaloid morphology, or were antherless, or were completely missing. Plants from the individual clones had either a certain combination, or one of these flower types. In some clones all or a part of the flowers had split corolla. All the flower parts were shortened. Pollination of the plants

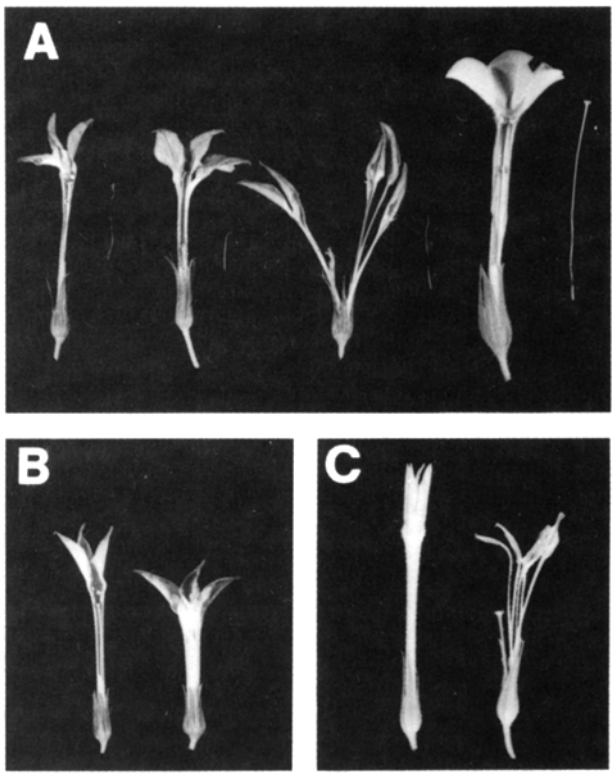

Fig. 4 A–C. Flower morphology of the parental and cybrid plants. The flowers are cut open. From *left to right:* **A** *N. plumbaginifolia;* Np(gos)6: antherless stamens and slightly or deeply split corolla; *N. gossei.* **B** *N. plumbaginifolia;* Np(gos)29: petaloid stamens. **C** *N. plumbaginifolia;* Np(gos)10: no stamen and deeply split corolla

by wild-type *N. plumbaginifolia* gave a normal seed set. Male sterility was in the same way expressed in the F_1 progeny (10 plants checked from each cross). The observed alterations in flower morphology of the Np(gos) cybrids closely resembled certain alloplasmic male sterile types in *Nicotiana* (Gerstel 1980). Alloplasmic male sterility in *Nicotiana* seems to be the consequence of incompatibility between the nucleus and the alien mitochondria (Belliard et al. 1979; Galun et al. 1982). Similarly, recombinations in mitochondrial DNA of the Np(gos) cybrids, which is characteristic of heteroplasmic fusions (Nagy et al. 1983), may be responsible for the male sterility with various flower alterations.

In the present experiment the co-transfer of chloroplast and mitochondrial traits is probably the consequence of the selection procedure used, since in the absence of any selection for cytoplasmic traits, chloroplasts and alloplasmic male sterility segregated independently in fusion derived clones (Belliard et al. 1978; Aviv and Galun 1980; Glimelius et al. 1981; Bonnett and Glimelius 1983; Aviv et al. 1984a, b). It has been shown (Menczel et al. 1983), that selection for streptomycin resistance (a chloroplast trait) of the donor resulted in 100% co-transfer of male sterility. One of the possible explanations was that the streptomycin resistance mutation, used as a selectable chloroplast marker (greening ability on streptomycin containing medium), was located on mitochondrial DNA as well. In the experiment reported here, which used selection for greening ability of wild-type donor chloroplasts (against the background of light sensitive plastids), however, co-transfer of the mitochondrial trait (alloplasmic male sterility) was also observed. Previously irradiated plastids were suggested to have preferential fusion transmission (Sidorov et al. 1981), but irradiation had no effect on the transfer of male sterility (Aviv and Galun 1980; Aviv et al. 1984a). It seems that in both cases the selection for one of the parental plastid populations resulted in co-transfer of (at least a part of) the corresponding mitochondrial genome. Supposing a co-operation between chloroplasts and mitochondria existed, which would facilitate their mutual reproduction, the long stringent selection for a pure plastid population at the cellular level (prior to plant regeneration) might explain the 100% co-transfer phenomenon. Using short selection periods (e.g. during shoot regeneration from the fusion derived calli) for one of the parental plastid populations, an independent segregation or only a tendency for co-transfer of the male sterility was detected (Fluhr et al. 1983, 1984, 1985; Aviv et al. 1984a).

The co-transfer of chloroplast and mitochondrial traits, which has also been verified with streptomycin and light sensitive *N. tabacum* recipients (paper in preparation), focuses attention on the genetic and metabolic co-operation between cytoplasmic organelles.

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