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Metabolic complementation for a single gene function associated with partial and total loss of donor DNA in interspecific somatic hybrids

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Summary. We report here on the obtainment of interspecific somatic, asymmetric, and highly asymmetric nuclear hybrids via protoplast fusion. Asymmetric nuclear hybrids were obtained after fusion of mesophyll protoplasts from a nitrate reductase-deficient cofactor mutant of *N. plumbaginifolia* with irradiated (100 krad) kanamycin-resistant leaf protoplasts of a haploid *N. tabacum*. Selection for nitrate reductase (NR) and/or kanamycin (Km) resistance resulted in the production of three groups of plants (NR^+, NR^+Km^R, and NR^-Km^R). Cytological analysis of some hybrid regenerants showed the presence of numerous tobacco chromosomes and chromosome fragments, besides a polyploid *N. plumbaginifolia* genome (tetra- or hexaploid). All the regenerants tested were male sterile but some of them could be backcrossed to the recipient partner. In a second experiment, somatic and highly asymmetric nuclear hybrids were obtained after fusion of mesophyll protoplasts from the universal hybridizer of *N. plumbaginifolia* with suspension protoplasts of a tumor line of *N. tabacum*. Selection resulted in two types of colonies: nonregenerating hybrid calli turned out to be true somatic hybrids, while cytological analysis of regenerants obtained on morphogenic calli did not show any presence of donor-specific chromosomes. Forty percent of the hybrid regenerants were completely fertile, while the others could only be backcrossed to the recipient *N. plumbaginifolia*. Since the gene we selected for is not yet cloned, we were not able to demonstrate the transfer of genetic material at the molecular level. However, since no reversion frequency for the nitrate reductase mutant is known, and due to a detailed cytological knowledge of both fusion partners, we feel confident in speculating that intergenomic recombination between *N. plumbaginifolia* and *N. tabacum* has occurred.

Key words: Protoplast fusion – Highly asymmetric nuclear hybrids – Intergenomic recombination

Introduction

In recent years several methods for somatic gene transfer in plants have been developed. If the gene of interest has been cloned, advanced transformation systems, based on bacterial or viral vectors (e.g., *Agrobacterium tumefaciens, A. rhizogenes,* direct gene transfer into protoplasts, microinjection, etc.), may provide an efficient way of introducing it into a desired recipient genome. However, if previously isolated genes are lacking or if complex and uncharacterized multigenic traits have to be transferred, somatic cell fusion via protoplast fusion may represent a good alternative method. Protoplast fusion also enables the production of novel genotypes between sexually incompatible plant species. Fusion products between phylogenetically remote species generally eliminate chromosomes of one of the parental genomes, thereby creating asymmetric hybrids that contain, in addition to a complete recipient genome, a few chromosomes derived from the donor.

In order to increase the number of highly asymmetric hybrids and to direct the process of chromosome elimination, several authors used irradiated donor protoplasts. The irradiation inactivates the cell, thereby inducing chromosomal breaks and leading to chromosome fragments that can also be transferred into the recipient genome. Restoration of chlorophyll synthesis in carrot and *Datura* was described by Dudits et al. (1980) and by Gupta et al. (1984), respectively. Gupta et al. (1982) and Somers et al. (1986) reported evidence on the restoration of nitrate reductase deficiencies in *Nicotiana*. The number of genes transferred by the protoplast fusion method is difficult to estimate since, in most cases, numerous donor chromosomes are transferred to the recipient genome. Highly asymmetric hybrids containing only one or a few donor chromosomes were only rarely described (Dudits et al. 1980; Bates et al. 1987).

The aim of our work was the production of asymmetric hybrids characterized by a partial or total elimination of donor chromosomes. Therefore, two types of fusions, using irradiated and nonirradiated donor protoplasts, were carried out between *Nicotiana* species. The selection of hybrids was facilitated by the presence of two selectable markers, i.e., resistance to kanamycin and metabolic complementation of nitrate reductase deficiency of the recipient.

Material and methods

Plant material

Nitrate reductase-deficient mutant plants (NR^-) from *Nicotiana plumbaginifolia* (cnx_{20} a molybdenum restorable cofactor mutant $2n=2x=20$) and its derivative, the universal hybridizer T_{116}/A_1 [NR defective (NR^-) and kanamycin resistant (Km^R)], were aseptically grown on Murashige and Skoog medium (1962) supplemented with 10 m*M* ammonium succinate and 0.22 m*M* Na_2MoO_4 (Negrutiu et al. 1983).

Haploid ($2n=2x=24$) kanamycin-resistant (Km^R) *Nicotiana tabacum* cv petit havana SR_1 was obtained after anther culture of transformed *N. tabacum* protoplasts (Paszkowski et al. 1984). These plants were cultured on Murashige and Skoog medium (1962) without hormones.

Clone A_{14} of *Nicotiana tabacum* was obtained from a tumoral strain isolated by Morel (1948) and found to be a crown-gall tobacco line (Mouras et al. 1987), since it contained specific *A. tumefaciens* T-DNA regions of the T_i-plasmid. This clone was maintained on hormone-free Murashige and Skoog medium (1962).

Isolation, fusion, and culture of protoplasts

Mesophyll protoplasts were isolated from 4- to 6-week-old plants of T_{116}/A_1, cnx_{20} and SR_1 as described by Negrutiu et al. (1986). Protoplasts of the tumor line suspension A_{14} were isolated as described by Gleba et al. (1978). The suspension was grown in Murashige and Skoog medium (1962) without hormones and was subcultured every 3 days.

Two independent fusion experiments were carried out: (1) In experiment A, nitrate reductase-deficient cnx_{20} protoplasts (recipient) were fused with irradiated (100 krad $Cobalt^{60}$ source) protoplasts of a haploid kanamycin-resistant tobacco plant (SR_1, donor). (2) In experiment B, nitrate reductase-deficient kanamycin-resistant protoplasts of T_{116}/A_1 (recipient) were fused with nonirradiated suspension protoplasts of line A_{14} (donor).

Fusions were carried out as described by Menczel et al. (1982). Electrofusion experiments were carried out as described by Tempelaar and Jones (1985). Protoplasts were further cultured in K_3 medium and subsequently diluted in selection medium MD_N or MD_S with or without 25 mg/l kanamycin sulfate (Negrutiu et al. 1983, 1986), according to the experiment. After 1 month, visible calli were transferred to solid selection medium and regenerated as described elsewhere (Installé et al. 1985). Control experiments were cultured under the same conditions.

Cytological analysis

Chromosome spreads were obtained from callus material and from root tips of regenerated plants as described by Pynacker and Ferwerda (1984). Alternatively, metaphase plates were prepared according to the protoplast method as described by Mouras et al. (1978).

Genetic analysis

Hybrid regenerants with a well-developed rooting system were immediately transferred into the greenhouse, while the rest of the regenerants were grafted onto wild-type *N. tabacum* plants in order to improve their development. Self-pollinations and backcrosses to the wild-type recipient partner were made. Seeds were collected and sown on two selective media containing macroelements of Murashige and Skoog (1962) (without nitrate), vitamins, and 1% sucrose. Selection medium 1 was supplemented with 10 m*M* KNO_3 and selection medium 2 with 10 m*M* of $KClO_3$. Medium 1 allows the selection of plantlets capable of metabolizing nitrate as a sole source of nitrogen, and must therefore be considered as having an active nitrate reductase enzyme. Medium 2, supplemented with $KClO_3$, allows the selection of nitrate reductase-deficient plantlets.

Tumor test

The oncogenic character of line A_{14} is expressed as the possibility of forming tumors whenever grafted onto wild-type plants. Therefore, pieces of callus and pieces of tissues obtained from regenerated somatic hybrids were grafted onto wild-type *N. tabacum* plants. Grafts were made as described by Limasset and Gautheret (1950).

Enzymatic assay of neomycin phosphotransferase activity (NPTII)

The NPTII activity in fusion products of experiment A was detected using the in situ gel assay developed by Reiss et al. (1984), and adapted for plant tissue as described by Paszkowski et al. (1984).

Results

Selection of hybrids

Numeric data for fusion experiment A [nitrate reductase-deficient *N. plumbaginifolia* (cnx_{20}) fused with irradiated kanamycin-resistant haploid tobacco] are given in Table 1.

The presence of two selectable markers, i.e., nitrate reductase and kanamycin resistance, allowed us to use different selection strategies: selections were performed either for metabolic complementation of the nitrate reductase gene alone, of the kanamycin gene alone, or of both markers at once. This selection strategy allowed us to obtain three groups of regenerated plants (Table 1):

(1) nitrate reductase-resorted (NR^+) and kanamycin-sensitive (Km^S); (2) nitrate reductase-deficient (NR^-) and kanamycin-resistant (Km^R); (3) nitrate reductase-restored (NR^+) and kanamycin-resistant (Km^R).

Numeric data for fusion experiment B (universal hybridizer of *N. plumbaginifolia* fused with tumor line A_{14} of tobacco) are also given in Table 1. After 2 months on selection medium, two types of hybrid colonies were observed: white to green friable calli (40% of the colonies) which did not exhibit organogenesis at all and resembled callus of the parental tumor line A_{14}; the other 60% of the colonies were fully green and frequently (70%–80%) were able to regenerate into plants. From the population

Table 1. Data on hybridization and regeneration response between *Nicotiana plumbaginifolia* and irradiated and nonirradiated *N. tabacum* somatic cells

Fusion type	No. of parental cells $\times 10^5$	Selection medium	No. of colonies on selective medium	Positive regeneration response	Plant phenotype + characteristics						
					recipient type			aberrant type			
					NR^+Km^R	NR^+	NR^-Km^R	NR^+Km^R	NR^+	NR^-Km^R
Exp. A											
$cnx_{20} + SR_1$ irradiated	N_P	N_T									
(A) PEG	1.95	3.25	MD_N	178	93	1	6	–	21	65	–
	1.95	3.25	MD_S Km25	51	13	1	–	–	11	–	1
	2.67	1.33	MD_N Km25	57	14	4 (5)	–	–	10 (9)	–	–
(B) EF	9.75	9.75	MD_N	199	88	6	5	–	16	61	–
	9,75	9.75	MD_S Km25	133	57	–	–	13	12	–	32
	6.4	2.2	MD_N Km25	37	16	not determined					
Exp. B											
$T_{116}/A_1 + A_{14}$	20	15	MD_S Km25	760	560	not detemined					

N_P: *Nicotiana plumbaginifolia*
N_T: *Nicotiana tabacum*
MD_N: selection medium with NO_3^- as a sole nitrogen source
MD_S: selection medium with 10 m*M* NH_4 succinate
Km_{25}: 25 mg/l kanamycin sulfate
PEG: polyethylene glycol-induced fusion
EF: electrofusion AC 1MHz–8V (80 V/cm), DC 1,500 V/cm, 3 × 20 µs
NR^-: nitrate reductase-deficient
NR^+: nitrate reductase-restored
Km^R: kanamycin resistant

Fig. 1. Typical metaphase plate of *N. plumbaginifolia* (cnx_{20}) + *N. tabacum* (SR_1) hybrids (exp. A). *N. tabacum* chromosomes are indicated by *arrows*

of regenerants, only those with a phenotype similar to the recipient (T_{116}/A_1) were further propagated. Regenerants with an aberrant phenotype were discarded because they grew very slowly. Control experiments did not result at all in the production of resistant colonies on selection medium.

Cytological analysis

Analysis of chromosome sets was greatly facilitated because metaphase chromosomes of each parent are clearly distinguishable (Mouras et al. 1986). *Nicotiana plumbaginifolia* chromosomes are all telocentric, except one pair which is subtelocentric, while *N. tabacum* chromosomes are meta-, submeta-, and telocentric.

A typical metaphase plate from a regenerant obtained in fusion experiment A is shown in Fig. 1. All hybrids studied possessed polyploïd (tetra- to hexaploïd level) chromosome sets of *Nicotiana plumbaginifolia,* i.e., the recipient partner. Hybrid metaphase plates also contained a relatively large number of tobacco chromosomes or chromosome fragments. Their number ranged between 8 and 16 (i.e., 33%–66%) of the haploid tobacco genome.

Metaphase plates of nonorganogenic calli obtained in experiment B contained between 80 and 135 chromosomes per plate (Fig. 2A). These chromosome numbers can be interpreted as being a sum of the two parental genomes (*Nicotiana tabacum* + *N. plumbaginifolia*). Twenty-five regenerated hybrid plants were analyzed cytologically. About 15 plates per individual were counted. All plants analyzed had a constant chromosome number of 40 (Fig. 2B). All chromosomes were of the recipient partner *Nicotiana plumbaginifolia;* no typical tobacco chromosomes could be identified in any plate.

Genetic analysis

All the regenerants from experiment A that had been transferred into the greenhouse had a hybrid phenotype

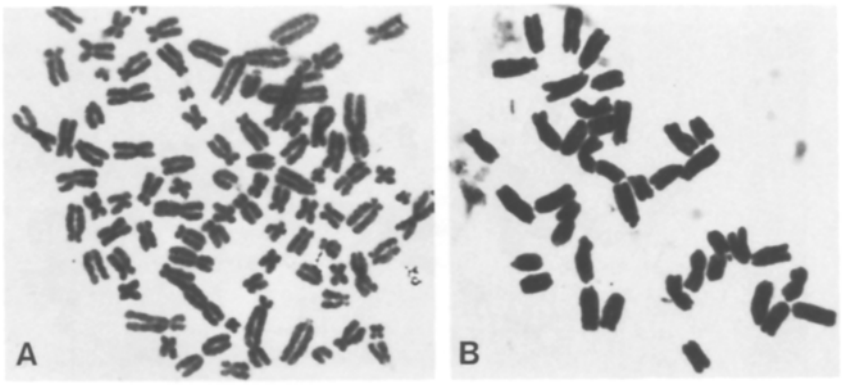

Fig. 2 A and B. Typical metaphase plates of hybrid calli or regenerants obtained after fusion of mesophyll cells of *N. plumbaginifolia* with suspension cells of a tobacco tumor line (A_{14}). **A** Metaphase plate of nonorganogenic calli with 2n = 87 chromosomes. **B** Metaphase plate of hybrid regenerants with 2n = 40 chromosomes of the *Nicotiana plumbaginifolia* type

Table 2. Germination response and segregation for the NR-marker from seeds obtained after selfing of different hybrid regenerants of exp. B

Media No. hybrids	Germination medium + $KClO_3$ 10 m*M*			Germination medium + KNO_3 10 m*M*		
	No. of NR^+	No. of NR^-	% NR^- plants	No. of NR^+	No. of NR^-	% NR^- plants
1B	118	4	3.2%	–	–	–
18	606	17	2.7%	421	10	2.3%
20	235	4	1.6%	390	4	1.0%
21	303	2	0.6%	481	2	0.4%
7	–	–	–	412	6	1.4%
13	–	–	–	159	3	1.8%
1B × 21	–	–	–	74	3	3.8%
1B × 13	–	–	–	106	5	4.5%

Index:
(–) test not realized because of insufficient number of seeds

with abnormalities, especially in flower structure. All of them were male sterile but some could be backcrossed to diploïd wild-type *Nicotiana plumbaginifolia*. In all cases, seed setting and seed germination were very poor. Segregation ratios (NR^+ and kanamycin resistance) were slightly aberrant from typical Mendelian segregations (data not shown). These F_1 plants are currently being investigated.

All 20 regenerated plants from experiment B that were grafted reached maturity and produced numerous flowers. Only 8 of them were completely fertile; the others could easily be backcrossed to the wild type. The collected seeds germinated in a range of 90%. Results of the germination tests on selective media are given in Table 2. The segregation ratios (NR^+/NR^-) do not correspond with a typical monogenic or digenic Mendelian segregation. The number of NR-deficient plants is very low and varies between 0.4% and 3% in different hybrids. The two selection media gave similar results, as expected.

Tumor test

After about 4 months responses of the grafting tests were evaluated. Grafts of calli resulted in the development of the callus as tumors (crown galls) of variable sizes for different lines (Fig. 3). This capability of forming tumors indicates that T-DNA is present in these lines. Southern hybridization showed the existence of T-DNA in different copy numbers in independent clones. In contrast, grafts of pieces of tissue taken from regenerated plants never led to the formation of tumors when grafted onto wild-type *N. tabacum* plants.

NPTII-activity

Plants regenerated from experiment A under kanamycin selection pressure and subsequently grown without selection were tested for neomycin phosphotransferase II activity. The autoradiogram is shown in Fig. 4. Some regenerants still contain NPTII activity while others do not. The enzyme in the hybrids migrates slower than the control protein because the donor partner *N. tabacum* contains a fusion protein that is 23 amino acid residues longer (Paszkowski et al. 1984). The results from the NPTII assay show that after regeneration a further stabilization of the plants occurs, i.e., further chromosome elimination may occur at random, without selection pressure.

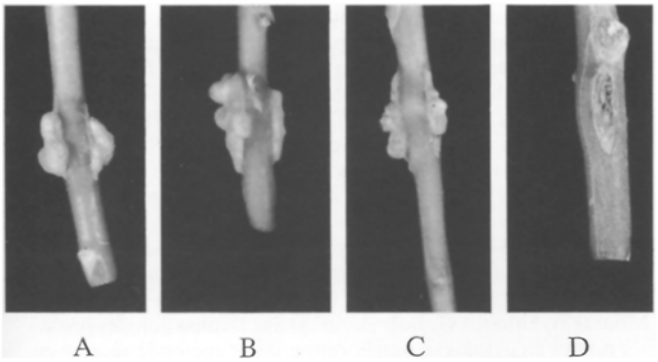

Fig. 3A–D. Graft responses of unorganized hybrid calli and tissue pieces of hybrid regenerants. **ABC** Tumor formation from unorganized calli. **D** Regenerated hybrid – no sign of tumor formation was observed 4 months after the graft test

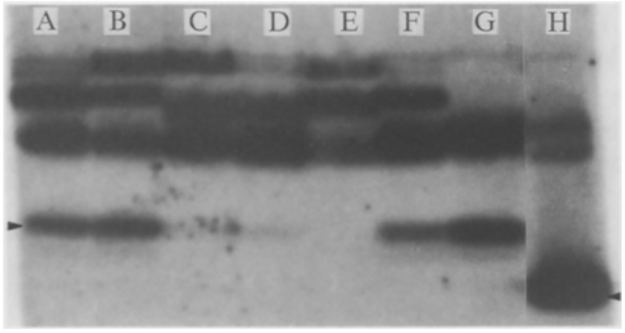

Fig. 4. Autoradiogram of neomycin phosphotransferase II (*arrow*) activity in *Nicotiana plumbaginifolia* + *N. tabacum* hybrids. *Lanes A–G*: different *Nicotiana plumbaginifolia* + *N. tabacum* hybrids. All hybrids tested were regenerated on kanamycin medium and subsequently cultured without kanamycin. Four out of seven lines still show NPTII activity. *Lane H*: positive control. *N. tabacum* transformant

Discussion

Treatment of plant cells with lethal doses of irradiation and subsequent use in fusion experiments resulted in the production of asymmetric hybrids. The irradiation seems to direct the process of chromosome elimination but can not be used to control the extent of eliminated donor material (Gleba et al. 1988). So far there is no direct evidence of gene introgression in plants from protoplast fusion experiments. The fusion experiments described in this work resulted in somatic hybrids that can be classified into three groups:

(1) true somatic hybrids that contain the two parental genomes (exp. B); (2) partial somatic hybrids in which numerous donor chromosomes are present besides a polyploïd receptor genome (exp. A); (3) highly asymmetric hybrids in which an infinite portion of the donor genome is present besides the recipient genome at the tetraploid level (exp. B).

The use of irradiation for directing the process of chromosome elimination has been described by several authors. Dudits et al. (1980) regenerated carrot plants with one extra parsley chromosome, after fusion of untreated *Daucus carota* protoplasts with irradiated parsley protoplasts. Gupta et al. (1984), in fusion experiments between nonirradiated protoplasts of an albino mutant of *Datura* with irradiated *Physalis minima* protoplasts, transferred a few (1–3) *Physalis* chromosomes into recipient cells. Bates et al. (1987) created highly asymmetric fertile hybrids between *N. tabacum* and *N. plumbaginifolia*, and recently Gleba et al. (1988) showed the intergeneric transfer of a few chromosomes from *Atropa* to *Nicotiana*. Nevertheless, highly asymmetric hybrids were rarely described, and the chromosome composition of the regenerants obtained in exp. A, e.g., clearly shows that irradiation can increase but not control the process of chromosome elimination. In addition, in the above mentioned experiments no clear evidence of integration of specific donor genes into the host genome is given, since donor chromosomes were always present in variable numbers.

Therefore, the regenerants of exp. B are very interesting since their chromosome composition is identical to the recipient *N. plumbaginifolia*. All the regenerated hybrids are tetraploids without any trace of specific donor chromosomes. These regenerants seem to have eliminated all donor chromosomes but are nevertheless corrected for the nitrate reductase deficiency of the recipient partner. Since no reversion frequency is known for the NR-deficient mutant used in this experiment and since the correction frequency for the nitrate reductase function is much higher than the theoretical reversion frequency of the cnx-marker (Dirks et al. 1986), the correction of the auxotrophic mutant T_{116}/A_1 for the nitrate reductase function can only be due to the transfer of genetic material from the donor line A_{14} to the recipient.

Since the *cnxA* gene has not yet been cloned, it is impossible to show molecular evidence for the transfer of genetic material. Nevertheless, our results allow us to speculate that intergenomic recombination has occurred between *N. plumbaginifolia* and *N. tabacum*. We were able to demonstrate this phenomenon due to a detailed cytological knowledge of both fusion partners. Indeed, as shown by Mouras (1982) and Mouras et al. (1986) the karyotypes of both partners are known and showed very dissimilar chromosome morphology. This knowledge allowed us to clearly analyze the hybrid regenerants at the cytological level.

In the case of fusions between partners with very similar or indistinguishable chromosomes, it is very difficult to identify the chromosomes of each partner in a given hybrid. In such cases only chromosome counts can be made, but this gives no information about the occurrence of recombinogenic events between recipient and

donor genome. To prove the existence of recombinogenic events in somatic hybrids obtained via protoplast fusion and to confirm the previously mentioned results, we are currently analyzing fusion hybrids that have a genetic marker that can be traced at the molecular level. Protoplast fusion holds promise for transferring small amounts of genetic material between different plant species.

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