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Direct selection for paternal inheritance of chloroplasts in sexual progeny of *Nicotiana*

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Summary. The response of *Nicotiana tabacum* to tentoxin (chlorosis) is inherited with chloroplasts. *N. tabacum* var. *Xanthi*, a tentoxin-resistant line, was used to pollinate tentoxin-sensitive *N. tabacum line 92*, an alloplasmic male-sterile line containing *N. undulata* plastids. The seeds were mutagenized with nitrosomethylurea and germinated in the presence of tentoxin. Two percent of the seedlings had green sectors in their first true leaves. These plants were grown to maturity under non-selective conditions. Homogeneous tentoxin-resistant lines were obtained in the third generation. DNA analysis indicated, however, that selection for paternal plastids, rather than mutagenesis of maternal ones, had occurred in the tentoxin-resistant progeny. Mitochondria, which were not under selection pressure, were inherited maternally as expected. Inheritance of tentoxin-resistant paternal plastids did not require seed mutagenesis. Normally germinated seedlings that were kept under tentoxin selection consistently produced a low level of resistant green sectors in their first true leaves. Thus, normal, low-frequency transmission of paternal plastids in *N. tabacum* can be directly revealed by using tentoxin.

Key words: Tentoxin – Nitrosomethylurea – Maternal inheritance

Introduction

Tentoxin, a cyclic tetrapeptide from the pathogenic fungus *Alternaria alternata (tenuis)*, interacts with the coupling factor of the chloroplast ATPase (Steele et al. 1976) to cause chlorosis in some, but not all, angiosperms. In the genus *Nicotiana*, of 40 species tested, 9 were tentoxin-resistant and 31 tentoxin-sensitive (Durbin and Uchytil 1977a, b). Tentoxin-dependent chlorosis was found to be maternally inherited (Burk and Durbin 1978) and linked to the chloroplast (Aviv et al. 1980; Flick and Evans 1982). It was successfully used as a plastid marker to follow mixed and segregated progeny after interspecific somatic fusions in *Nicotiana* (Fluhr et al. 1984).

A program was initiated in an attempt to define the molecular genetic basis for the tentoxin response. We pollinated a tentoxin-sensitive, alloplasmic male-sterile line of *N. tabacum* with tentoxin-resistant, wild-type *N. tabacum*. The resulting seeds were then mutagenized and tentoxin-resistant progeny sought. Several were obtained; however, upon analysis at the molecular level, it became apparent that resistance was caused by paternal transmission of wild-type chloroplast DNA rather than mutagenesis of the maternal plastid. Rare inheritance of paternal plastids in *Nicotiana* following rescue by tissue culture procedures has previously been demonstrated (Medgyesy et al. 1986). In this report, we show that a normal low level of paternal transmission of plastids in sexual progeny of *N. tabacum* can be directly revealed by selecting for tentoxin resistance.

Materials and methods

Plant material and growth. N. tabacum line 92, an alloplasmic, male-sterile line containing the cytoplasm of a tentoxin-sensitive *N. undulata* (Fluhr et al. 1984), was used as the female parent in all experiments. *Line 92* was routinely hand pollinated by *N. tabacum* var. *Xanthi*, a fertile tentoxin-resistant wild-type species. In some experiments, seeds were mutagenized during imbibition with nitrosomethylurea (Hagemann 1982) as described by Fluhr et al. (1985). In all cases, seeds were germinated on Nitsch (1969) agar supplemented with 10 μg/ml tentoxin (Sigma). After 3 weeks, seedlings with true leaves showing green sectors were transferred to fresh Nitsch agar plates containing 20 μg/ml tentoxin. Seedlings were so maintained for a further 2 months before removal to the greenhouse and growth under non-selective conditions.

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Seedling test for tentoxin sensitivity. Seeds of different *Nicotiana* lines were germinated on Nitsch agar containing 10 to 20 μg/ml tentoxin (Durbin and Uchytil 1977a) as described by Galun (1982). Resistant species germinated as normal green seedlings, while sensitive species germinated as chlorotic (chlorophyll-less) seedlings.

Analysis of chloroplast and mitochondrial DNA. DNA from cells (Mettler 1988), chloroplasts (Fluhr and Edelman 1981), and mitochondria (Aviv et al. 1984) was isolated as described. DNA endonuclease digests were resolved on 0.7% agarose gels and analyzed following ethidium bromide staining or hybridization with chloroplast (*atp*B; Avni 1991) and mitochondrial probes (probe 2; Aviv and Galun 1987) on GeneScreen (NEN) filters (Sambrook et al. 1989).

Results

Selecting for tentoxin resistance following mutagenesis

Pollination of male-sterile *N. tabacum line 92* plants by *N. tabacum* var. *Xanthi* results in tentoxin-sensitive seedlings that turn chlorotic at about 7.5 μg/ml tentoxin. On the other hand, selfed *N. tabacum* var. *Xanthi* seedlings are resistant to tentoxin and remain green at 500 μg/ml tentoxin (Fig. 1). Fluhr (1983) indicated that variegated first true leaves occasionally appeared among chlorotic, mutagenized progeny of *line 92* grown on tentoxin medium, but did not follow the fate of these seedlings. In an attempt to produce a tentoxin-resistant *line 92* plant, we mutagenized 300 seeds with nitrosomethylurea and germinated them in 10 μg/ml tentoxin. One-half of the resulting seedlings had cotyledons with green islands while seven seedlings had first true leaves with green sectors (Table 1A). These seven seedlings were kept in 20 μg/ml tentoxin for another 2 months, then taken off selection and five transferred to the greenhouse. One of the five plants flowered and was pollinated by *N. tabacum* var. *Xanthi.* When the resulting M_1 seeds were germinated on 10 μg/ml tentoxin, most of the seedlings had chlorotic cotyledons. However, one plant had large green sectors in its later leaves and was transferred to the greenhouse. Four hundred M_2 seeds from five different pods of this plant were germinated as before on tentoxin. Most M_2 cotyledons were again chlorotic while a single plant had a completely resistant phenotype in its expanded leaves (Fig. 2). This plant was named 92/*ten*^{R}-1. A second putative mutant, 92/*ten*^{R}-2, was isolated in a separate experiment in the same manner. In this case, 5 out of 300 M_0 seedlings had green sectors in their first true leaves (Table 1A) and a homogeneous, tentoxin-resistant phenotype was obtained in the M_2 generation.

Inheritance of chloroplast and mitochondrial DNA in the tentoxin-resistant progeny

Chloroplast DNA was isolated from the putative mutant lines, 92/*ten*^{R}-1 and 92/*ten*^{R}-2, and compared with that

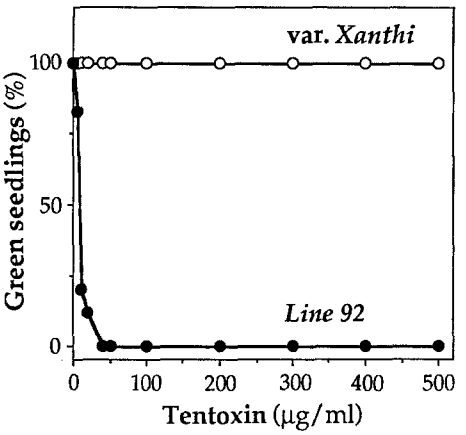

Fig. 1. Tentoxin-dependent chlorosis in *Nicotiana tabacum line 92* versus *N. tabacum* var. *Xanthi.* Seedlings of *N. tabacum line 92* × *N. tabacum* var. *Xanthi* (designated as *Line 92*) and selfed *N. tabacum* var. *Xanthi* (designated as var. *Xanthi*) were germinated in the presence of different concentrations of tentoxin. Forty seeds were used for each point. Cotyledon color (green or chlorotic) was scored after 10 days of growth at 30 μmol/m^2/per second fluorescent light

Table 1. M_0 and F_1 seedlings of *Nicotiana tabacum line 92* × *N. Tabacum* var. *Xanthi* germinated in the presence of 20 μg/ml tentoxin. Seeds were treated as described in Materials and methods

Experiment no.	Seeds	Tentoxin-resistant green spots	
		Cotyledons	1st true leaves
A. Mutagenized seeds			
1	300	150 (50%)	7 (2.5%)
2	300	n.d.	5 (1.5%)
B. Non-mutagenized seeds			
1	2800	n.d.	16 (0.6%)
2	1100	20 (1.6%)	n.d.
3	2160	57 (2.6%)	9 (0.4%)
4	1400	21 (1.5%)	6 (0.4%)

n.d. not determined

from the parents, *line 92* and var. *Xanthi.* The DNAs were digested with *Bgl*I, *Pst*I and *Bam*HI and the restriction fragments were separated on an agarose gel. Unexpectedly, the fragment patterns for chloroplast DNA of the putative mutants 92/*ten*^{R}-1 and 92/*ten*^{R}-2 were in all cases identical to those of var. *Xanthi* the paternal parent, and not *line 92* the maternal parent. This is seen in the ethidium bromide pattern following digestion of chloroplast DNA from 92/*ten*^{R}-1 with *Bgl*I (Fig. 3A). In addition, chloroplast DNA was digested with *Eco*RI and *Cla*I, blotted onto a nylon filter and hybridized with a radioactive probe from the *atp*B gene region (Fig. 3B). Again, the putative mutants (data for 92/*ten*^{R}-1 is shown) had the chloroplast DNA restriction patterns of *N. tabacum* var. *Xanthi.* Thus, lines 92/*ten*^{R}-1 and 92/*ten*^{R}-2 have inherited a paternal, rather than maternal, plastome.

The inheritance of mitochondrial DNA in these same plants was also determined. Total DNA from the puta-

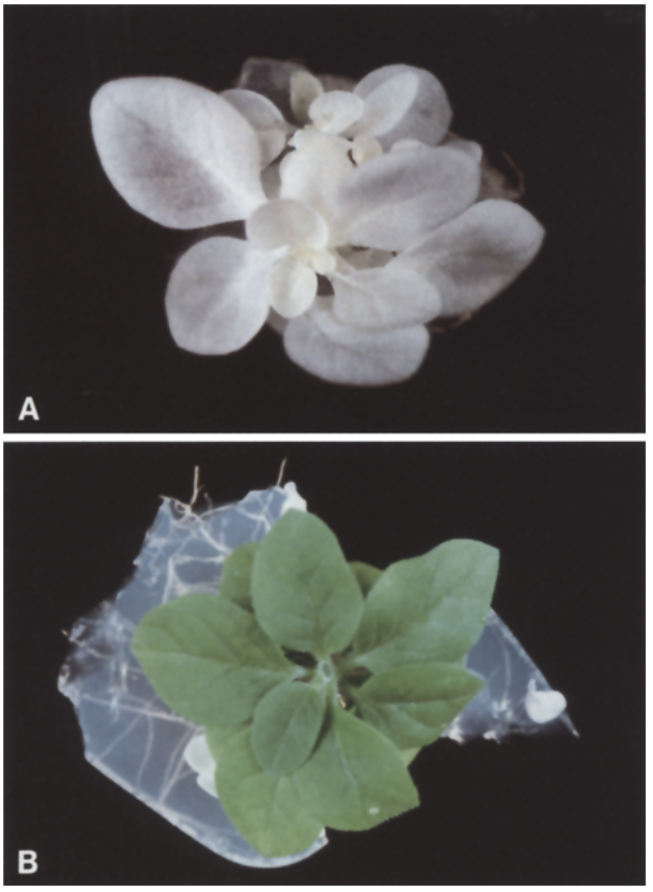

Fig. 2 A, B. Appearance of a tentoxin-resistant *line 92* plant. Surface-sterilized *line 92* seeds were sown and maintained on Nitsch agar containing tentoxin as described in Materials and methods. Photographs were taken after 2 months. **A** *Nicotiana tabacum line 92*, parent plant. **B** *N. tabacum line 92/ten*^{R}-1, M_2 plant showing a completely resistant phenotype in its expanded leaves

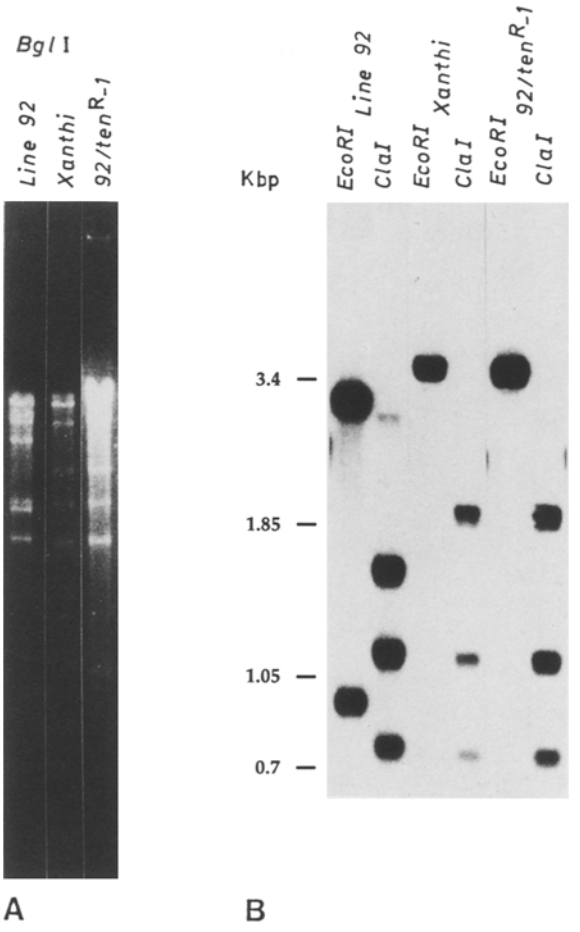

Fig. 3 A, B. Endonuclease restriction patterns of chloroplast DNA. **A** Partially purified chloroplast DNA (5–10 µg) from *Nicotiana tabacum* var. *Xanthi*, *N. tabacum line 92* and 92/*ten*^{R}-1 was digested with *Bgl*I. Fragments were separated on a 0.7% agarose gel, stained with ethidium bromide and photographed. **B** Chloroplast DNA (5 µg) from each of the species in **A** was digested with *Eco*RI or *Cla*I. Fragments were separated on a 0.7% agarose gel, transferred to a GeneScreen (NEN) filter and probed with an *Eco*RI fragment containing the entire *atp*B gene (Avni 1991)

tive mutants and mitochondrial DNA from the parents was isolated. The DNAs were digested with *Pst*I, *Sal*I and *Xho*I, separated on an agarose gel, blotted onto a nylon filter and probed with mitochondrial DNA probe 2 (Aviv and Galun 1987). From the results obtained, it is clear that 92/*ten*^{R}-1 (Fig. 4) and 92/*ten*^{R}-2 (not shown) have inherited the mitochondrial DNA of the maternal parent, *N. tabacum line 92*.

Selecting for tentoxin resistance in non-mutagenized seedlings

The molecular-genetic evidence indicates that in lines 92/*ten*^{R}-1 and 92/*ten*^{R}-2 the chloroplast genome was inherited from the fertile, tentoxin-resistant paternal parent that was routinely used to propagate the infertile, tentoxin-sensitive *line 92* plants. Thus, paternal inheritance, rather than mutagenesis of chloroplast DNA, had apparently caused the plants to become tentoxin-resistant. If this were true, we reasoned that green sectors should be observed under selective conditions without using a mutagen. A large number of seeds (7460), representing several pods from four different batches of var. *Xanthi*-pollinated *line 92* plants, were consequently germinated without mutagenesis on plates containing Nitsch medium and 20 µg/ml *tentoxin*. The results are summarized in Table 1 B. After 3 weeks, an average of 2.1% of the cotyledons indeed were observed to have green islands while 0.5% of the first true leaves had green sectors.

Discussion

In angiosperms, maternal inheritance of plastids is the dominant pattern (Kirk and Tinley-Bassett 1978; Gilham 1978; Sears 1980; Smith 1989). As pointed out by Hagemann (1976), and extensively demonstrated (Corriveau and Coleman 1988), a key factor is the frequent

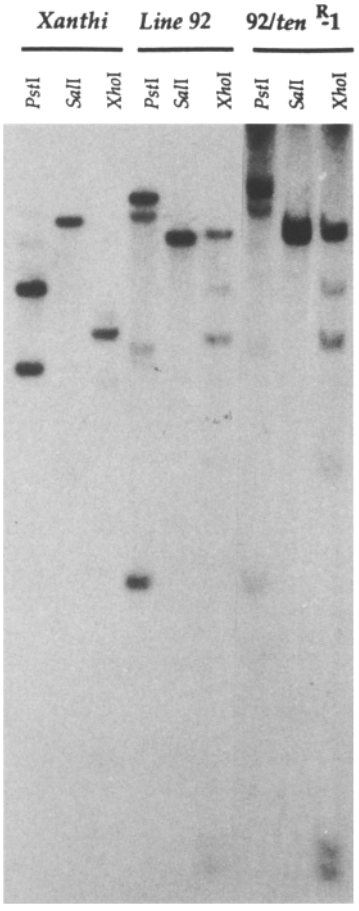

Fig. 4. Endonuclease restriction patterns of mitochondrial DNA. Mitochondrial DNA (5 μg) from *Nicotiana tabacum* var. *Xanthi* and *N. tabacum line 92*, and 25 μg of total DNA from 92/*ten*^{R}-1, was digested with *Pst*I, *Sal*I or *Xho*I. The digested DNAs were electrophoresed on a 0.7% agarose gel, transferred to a GeneScreen filter (NEN) and hybridized with mitochondrial DNA probe 2 (Aviv and Galun 1987)

absence of plastids from the pollen generative cell. Additional factors, such as the relative numbers of maternal and paternal plastids in the zygote, selective elimination of paternally derived zygotic plastids, and differences in rates of replication of plastid types, probably also play a role (Sears 1980; Smith 1989). Frequent biparental (i.e. maternal *or* paternal) inheritance of plastids has been found in a relatively small number of angiosperms (Kirk and Tinley-Bassett 1978). On the other hand, infrequent transmission of male plastids has been noted in about one-third of the angiosperms investigated (Smith 1989). Even so, much of the experimental data is limited by the small population sizes and moderate sensitivities of the marker systems used. Thus, low-frequency paternal transmission of the plastome may be more prevalent than is generally credited.

Several independent studies have led to the conclusion that chloroplasts are inherited in a strictly maternal fashion in *Nicotiana* (reviewed by Kirk and Tinley-Bassett 1978). Nonetheless, Medgyesy et al. (1986) have recently found that paternal plastids can occasionally be inherited in this genus as well. In their study, streptomycin resistance was used as a selective marker to reveal paternal transmission in intraspecific crosses between different lines of *N. plumbaginifolia,* and in interspecific crosses between *N. plumbaginifolia* and *N. tabacum*. Production of callus tissue was an absolute requirement of the system. In the absence of this step, not a single green spot was found on the cotyledons of 6800 seedlings arising from the intraspecific *N. plumbaginifolia* cross germinated in selective conditions (Medgyesy et al. 1986). This is in marked contrast to our results for intraspecific crosses in *N. tabacum*, where 98 tentoxin-resistant green islands were scored on the cotyledons of the 4600 seedlings germinated in selective conditions (Table 1 B). It may be that paternal transmission of plastids is a more frequent phenomenon in *N. tabacum* than in *N. plumbaginifolia*. Alternatively, tentoxin may be a more efficient selective agent than streptomycin for revealing a low density of resistant plastids. At any rate, the use of tissue culture, with its propensity for somaclonal variation, is clearly not a general requirement for obtaining paternal transmission of plastids in *Nicotiana*.

An interesting point arising from our data is the higher percentage of tentoxin-resistant green spots in the cotyledons and true leaves of the mutagenized vs non-mutagenized seedlings (50% vs 2%, respectively, in the cotyledons and 2% vs 0.5% in the true leaves; cf. Table 1). This could have resulted from actual mutagenesis of the *line 92* plastome to tentoxin resistance but loss of the mutation upon removal of the selection pressure (either because the mutant plastid population was low or led to reduced fitness). We have not analyzed this point further.

Mitochondria, ostensibly not under selection pressure in the presence of tentoxin, were maternally inherited in our study as expected. This is in agreement with Medgyesy et al. (1986) and with somatic hybridization experiments that showed that mitochondria and chloroplasts assorted independently during plant growth in *Nicotiana* (Galun et al. 1982; Fluhr et al. 1983).

Nicotiana thus joins the growing number of genera in which low-frequency paternal inheritance of plastids (and/or mitochondria; Neale et al. 1989; Erickson et al. 1989) can be demonstrated upon application of efficient selection pressure following normal sexual crosses. Manipulation of this cryptic potential for variation in the higher plant plastome might help in understanding higher plant plastid recombination, a process that apparently can be activated in *Nicotiana* (Medgyesy et al. 1985; Thanh and Medgyesy 1989).

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References

Aviv D, Galun E (1987) Chondriome analysis in sexual progeny of *Nicotiana* cybrids. Theor Appl Genet 73:821–826

Aviv D, Fluhr R, Edelman M, Galun E (1980) Progeny analysis of the interspecific somatic hybrids: *Nicotiana tabacum*

(CMS) + *Nicotiana sylvestris* with respect to nuclear and chloroplast markers. Theor Appl Genet 56:145–150

Aviv D, Arzee-Gonen P, Bleichman S, Galun E (1984) Novel alloplasmic *Nicotiana* plants by donor recipient protoplast fusion: cybrids having *N. tabacum* or *N. sylvestris* nuclear genome and either or both plastomes and chondriomes from alien species. Mol Gen Genet 196:244–253

Avni A (1991) The molecular genetic basis for tentoxin sensitivity in the higher plant chloroplast. PhD thesis. Weizmann Institute of Science, Rehovot, Israel

Burk LG, Durbin RD (1978) The reaction of *Nicotiana* species to tentoxin. J Hered 69:117–120

Corriveau JL, Colman AW (1988) Rapid screening method to detect potential biparental inheritance of plastid DNA and results for over 200 angiosperm species. Am J Bot 75:1443–1458

Durbin RD, Uchytil TF (1977a) A survey of plant insensitivity to tentoxin. Phytopathology 67:602–603

Durbin RD, Uchytil TF (1977b) Cytoplasmic inheritance of chloroplast coupling factor 1 subunits. Biochem Genet 15:1143–1146

Erickson L, Kemble R, Swanson E (1989) The *Brassica* mitochondrial plasmid can be sexually transmitted. Pollen transfer of a cytoplasmic genetic element. Mol Gen Genet 218:419–422

Flick CE, Evans DA (1982) Evaluation of cytoplasmic segregation in somatic hybrids of *Nicotiana:* tentoxin sensitivity. J Hered 73:264–266

Fluhr R (1983) Molecular genetics of the *Nicotiana tabacum* chloroplast. PhD thesis. Weizmann Institute of Science, Rehovot, Israel

Fluhr R, Edelman M (1981) Physical mapping of *Nicotiana tabacum* chloroplast DNA. Mol Gen Genet 181:484–490

Fluhr R, Aviv D, Edelman M, Galun E (1983) Cybrids containing mixed and sorted-out chloroplasts following interspecific somatic fusion in *Nicotiana*. Theor Appl Genet 65:289–294

Fluhr R, Aviv D, Galun E, Edelman M (1984) Generation of heteroplastidic *Nicotiana* cybrids by protoplast fusion: analysis for plastid recombinant types. Theor Appl Genet 67:491–497

Fluhr R, Aviv D, Galun E, Edelman M (1985) Efficient induction and selection of chloroplast-encoded antibiotic-resistant mutants in *Nicotiana*. Proc Natl Acad Sci USA 82:1485–1489

Galun E (1982) Screening for chloroplast composition in hybrids and cybrids resulting from chloroplast fusion in angiosperms. In: Edelman M, Hallick RB, Chua N-H (eds) Methods in chloroplast molecular biology. Elsevier Biomedical Press, pp 139–148

Galun E, Arzee-Gonen P, Fluhr R, Edelman E, Aviv D (1982) Cytoplasmic hybridization in *Nicotiana:* Mitochondrial DNA analysis in progeny resulting from fusion between protoplasts having different organelle constitutions. Mol Gen Genet 186:50–56

Gilham (1978) Orgenelle heredity. Raven Press, New York, pp 602

Hagemann R (1976) Plastid distribution and plastid competition in higher plants and the induction of plastome mutations by nitroso-urea compounds. In: Bucher TH, Neupert W, Sebald W, Werner S (eds) Genetics and biogenesis of chloroplasts and mitochondria. Elsevier/North-Holland Biomedical Press, Amsterdam, pp 331–339

Hagemann R (1982) Induction of plastome mutations by nitroso-urea compounds. In: Edelman M, Hallick RB, Chua N-H (eds) Methods in chloroplast molecular biology. Elsevier Biomedical Press, pp 119–127

Kirk JTO, Tilney-Bassett RAE (1978) The plastids. Their chemistry, structure, growth and inheritance. Elsevier/North-Holland, Amsterdam, pp 960

Medgyesy P, Fejes E, Maliga P (1985) Interspecific chloroplast recombination in a *Nicotiana* somatic hybrid. Proc Natl Acad Sci USA 82:6960–6964

Medgyesy P, Pay A, Marton L (1986) Transmission of paternal chloroplasts in *Nicotiana*. Mol Gen Genet 204:195–198

Mettler IJ (1988) A simple and rapid method for minipreparation of DNA from tissue culture plant cells. Plant Mol Biol Rep 5:346–349

Neale DB, Marshall KA, Sederoff RR (1989) Chloroplast and mitochondrial DNA are paternally inherited in *Sequoia sempervirens* D. Don Endl. Proc Natl Acad Sci USA 86:9347–9349

Nitsch JP (1969) Experimental androgenesis in *Nicotiana*. Phytomorphology 19:389–404

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning, a laboratory manual. Cold Spring Harbor Laboratory Press, New York, pp 1.101–1.104

Sears BB (1980) Elimination of plastids during spermatogenesis and fertilization in the plant kingdom. Plasmid 4:233–255

Smith SE (1989) Biparental inheritance of organelles and its implications in crop improvement. Plant Breeding Rev 6:361–393

Steele JA, Uchytil TF, Durbin RD, Bhatnager P, Rich DH (1976) Chloroplast coupling factor 1: a species specific receptor for *tentoxin*. Proc Natl Acad Sci USA 73:2245–2248

Thanh ND, Medgyesy P (1989) Limited chloroplast gene transfer via recombination overcomes plastome-genome incompatibility between *Nicotiana tabacum* and *Solanum tuberosum*. Plant Mol Biol 12:87–93

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