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Nicotiana tabacum mutants with chloroplast encoded streptomycin resistance and pigment deficiency

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Summary. Callus of *Nicotiana tabacum* SR1, a mutant with maternally inherited streptomycin resistance, was induced from leaf sections. Callus pieces were mutagenised with N-ethyl-N-nitrosourea and inoculated onto a shoot-induction medium on which calli are normally green. White callus sectors were observed in the mutagenised cultures, and white and variegated shoots were regenerated from these sectored calli. The SR1-A10 line regenerated a chimeric shoot with white leaf margins. The chimeric shoot was grafted onto a normal green rootstock, grown into a flowering plant in the greenhouse, and crosses were made. The SR1-A15 line was crossed using flowers formed on albino plants grown in sterile culture. Pigment deficiency was maternally inherited in both lines. Physical mapping of the chloroplast genome of the SR1-A15 mutant by SalI, PstI and BamHI restriction endonucleases did not reveal any difference between the SR1-A15 and the parental SR1 chloroplast genomes.

Key words: *Nicotiana tabacum* – Plastome mutants – Cell culture – Pigment deficiency – Physical mapping

Introduction

Flowering plants, such as *Nicotiana tabacum,* contain several chloroplasts and each chloroplast contains several identical chloroplast genomes (Bedbrook and Kolodner 1979; Herrmann and Possingham 1980). In the absence of an appropriate selection scheme, attempts to recover chloroplast recombinants in flowering plants have failed (Kutzelnigg and Stubbe 1974; Fluhr et al. 1984; Chiu and Sears 1985; for a more detailed discussion see Maliga and Fejes 1985).

Available chloroplast markers in flowering plants include resistance to the antibiotics streptomycin and lincomycin (Maliga 1984b), to triazine herbicides (Arntzen and Duesing 1983; Cseplo et al. 1985), tentoxin (Durbin and Uchytil 1977), and maternally inherited pigment deficiency (Kirk and Tilney-Basset 1978). Of these markers, antibiotic-resistance mutations have been shown to be selectable in cell culture (Maliga 1984b) and therefore suitable for the recovery of chloroplast recombinants. Experience with *Chlamydomonas,* a unicellular alga, suggests that recovery of chloroplast recombinants by selection for two antibiotic resistance markers may be difficult since interactions among altered ribosome components in the antibiotic resistance mutants could affect the viability of specific marker combinations (discussed in Metz and Geist 1983). Selection for chloroplast recombinants seemed feasible using a single antibiotic resistance marker, however, if expression of the resistance could be prevented by a second independent chloroplast mutation. Recovery of recombinant chloroplast genomes was anticipated by screening for the expression of the antibiotic resistance marker from the double mutant as the result of chloroplast recombination. Such mutants exist in *Chlamydomonas* (Sager 1977). This paper describes the isolation of double chloroplast mutants in the flowering plant *N. tabacum.*

Streptomycin resistance in *Nicotiana* cell culture is defined as the ability to form green callus on a selective medium (Maliga 1984b). Derivatives of the maternal streptomycin-resistant mutant, *Nicotiana tabacum* SR1 (Maliga et al. 1973, 1975), have been isolated in which greening is prevented by a second, maternally inherited

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mutation conferring pigment deficiency. One of the lines described in this paper, SR1-A15, has been used to recover a chloroplast recombinant (Medgyesy et al. 1985).

Materials and methods

Callus culture and plant regeneration

Calli were induced from leaves of diploid (2n = 4X = 48) plants of *N. tabacum* SR1 on RMO medium (Maliga et al. 1973, 1975). On this medium calli are normally green and form shoots. Plants were obtained by rooting the shoots on RM medium containing MS salts (Murashige and Skoog 1962), 3% sucrose and 0.6% agar. The cultures were incubated at 28 °C. Leaf callus was induced in dark. Mutagenised calli, which were screened for white sectors, were illuminated for 16 h at 1,000 lx, and albino calli and plants were illuminated for 16 h at 100 lx. Cell culture procedures have been summarized in a technical paper (Maliga 1984a).

Mutagenic treatment of calli

Dark-grown leaf calli (about 20 mg each) were transferred to liquid RMO medium containing 0.1 or 1.0 mM N-ethyl-N-nitrosourea (NEU, filter sterilized) and incubated in the dark for 24 h. After treatment the calli were rinsed in RMO medium.

Testing of seedlings for pigment deficiency

The seeds were surface sterilized and germinated on RM medium (Maliga 1984a). Seedlings were incubated at 28 °C and illuminated at 100 lx for 16 h.

Mapping of chloroplast DNA

N. tabacum SR1 chloroplast DNA (cpDNA) was prepared by the method of Kolodner and Tewari (1975). Leaves were collected from plants grown in sterile culture. The DNAse treatment of the chloroplast preparation was omitted. DNA from SR1-A15 callus was isolated as described by Chilton et al. (1982). DNA was digested by restriction endonucleases as recommended by the supplier, electrophoresed in horizontal agarose gels (0.7% agarose) in Tris-acetate buffer. DNA was transferred from agarose to nitrocellulose according to Maniatis et al. (1982). For probing SalI fragments of SR1 cpDNA, cloned in the plasmid pBR322 were used (Table 3). SalI clones were kindly provided by Erzsebet Fejes (Medgyesy et al. 1985). Plasmid DNA was CsCl purified, labeled with ^{32}P-dCTP using a BRL nick translation kit, and ethanol precipitated. Hybridization was carried out as described by Thomasow et al. (1980). Blots were exposed to Kodak X-Omat film for 2 h to 2 days with two intensifying screens at −70 °C.

Results

Isolation of albino cell lines

Calli grown from leaf sections in the dark were white. They turned green after transfer to light. In cultures of 900 mutagenised calli, six albino or pale green clones were identified after treatment with 0.1 mM NEU, and fifteen after treatment with 1.0 mM NEU. The same number (900) of controll calli yielded no pigment-deficient lines. The two lines in which seed transmission of pigment deficiency was studied, SR1-A10 and SR1-A15, derive from the sample mutagenized with 1.0 mM NEU.

Five calli from which albino shoots have regenerated were repeatedly subcultured and inspected for chimeric shoots. Chimeric shoots with green and white sectors were obtained in three clones. One of them, SR1-A15, was a periclinal chimera with white leaf margins (Fig. 1A).

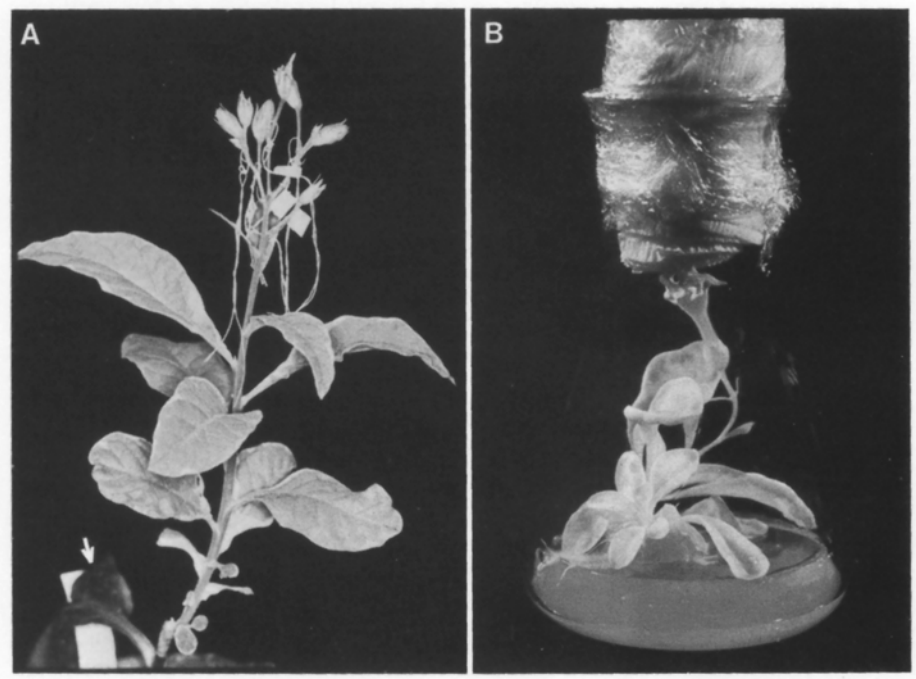

Fig. 1. Grafted SR1-A10 shoot (**A**) and an SR1-A15 plant in sterile culture (**B**). Note differences in leaf colour of chimera and rootstock. *Arrow*, Fig. 1A, points to a leaf on the rootstock

Table 1. Inheritance of pigment deficiency in the SR1-A10 line

Cross	Capsule no.	No. of seedlings	Color of seedlings		
			White	Green	Variegated
SR1-A10 (selfed)	1	210	210	0	0
	2	152	151	1	0
	3	272	215	57	0
	4	64	63	1	0
	5	215	215	0	0
	6	166	166	0	0
Subtotal:		1,079	1,020	59	0
SR1 (selfed)	seeds pooled	1,027	0	1,027	0
SR1-A10 ♀ × SR1 ♂	1	141	141	0	0
	2	97	94	1	2
	3	223	233	0	0
	4	196	196	0	0
	5	284	241	43	0
	6	65	64	1	0
	7	17	17	0	0
Subtotal:		1,033	986	45	2
SR1 ♀ × SR1-A10 ♂	1–15, pooled	2,458	0	2,458	0

Table 2. Inheritance of pigment deficiency in the SR1-A15 line

Cross[a]	Capsule no.	No. of seedlings	Color of seedlings		
			White	Green	Variegated
SR1-A15 (selfed)	1	9	9	0	0
SR1-A15 ♀ × SR1 ♂	1	12	12	0	0
	2	7	7	0	0
Subtotal:		19	19	0	0
SR1 ♀ × SR1-A15 ♂	1	13	0	13	0

[a] Data on selfed SR1 seedlings are included in Table 1

Inheritance of pigment deficiency in the SR1-A10 line

Chimeric SR1-A10 shoots were grafted onto a normal green rootstock in the greenhouse and were grown to flowering (Fig. 1 A). Under greenhouse conditions there was a tendency for the loss of the mutant cells in the chimeric shoots. Seeds were collected after selfing, and reciprocal crosses with green *N. tabacum* SR1 plants. The seeds were surface sterilized, germinated in sterile culture, and scored for pigmentation (Fig. 2) as bulk samples or as a group from the same seed capsule (Table 1).

Inheritance of pigment deficiency in the SR1-A15 line

Flowers formed on albino SR1-A15 plants in sterile culture after subculturing the shoot tips a few times (Fig. 1 B). These flowers were fertile and were suitable for crossing with greenhouse plants. The number of viable seeds formed in a capsule was low (Table 2). Seeds derived from selfing and crosses were germinated, and the seedlings were scored for pigmentation. Data indicate maternal inheritance of pigment deficiency in this line (Table 2).

Mapping of the SR1-A15 chloroplast genome

The *N. tabacum* chloroplast genome has been mapped with several restriction endonucleases, including SalI, PstI and BamHI (Seyer et al. 1981; Fluhr et al. 1983; Tassopulu and Kung 1984; Hildebrand et al. 1985). The SR1-A15 chloroplast genome has been mapped for these sites by probing blots of appropriate total cellular DNA digests with *N. tabacum* SalI cpDNA fragments (Fig. 3). The number and size of hybridizing SalI/PstI, and of BamHI fragments are listed in Table 3. As a

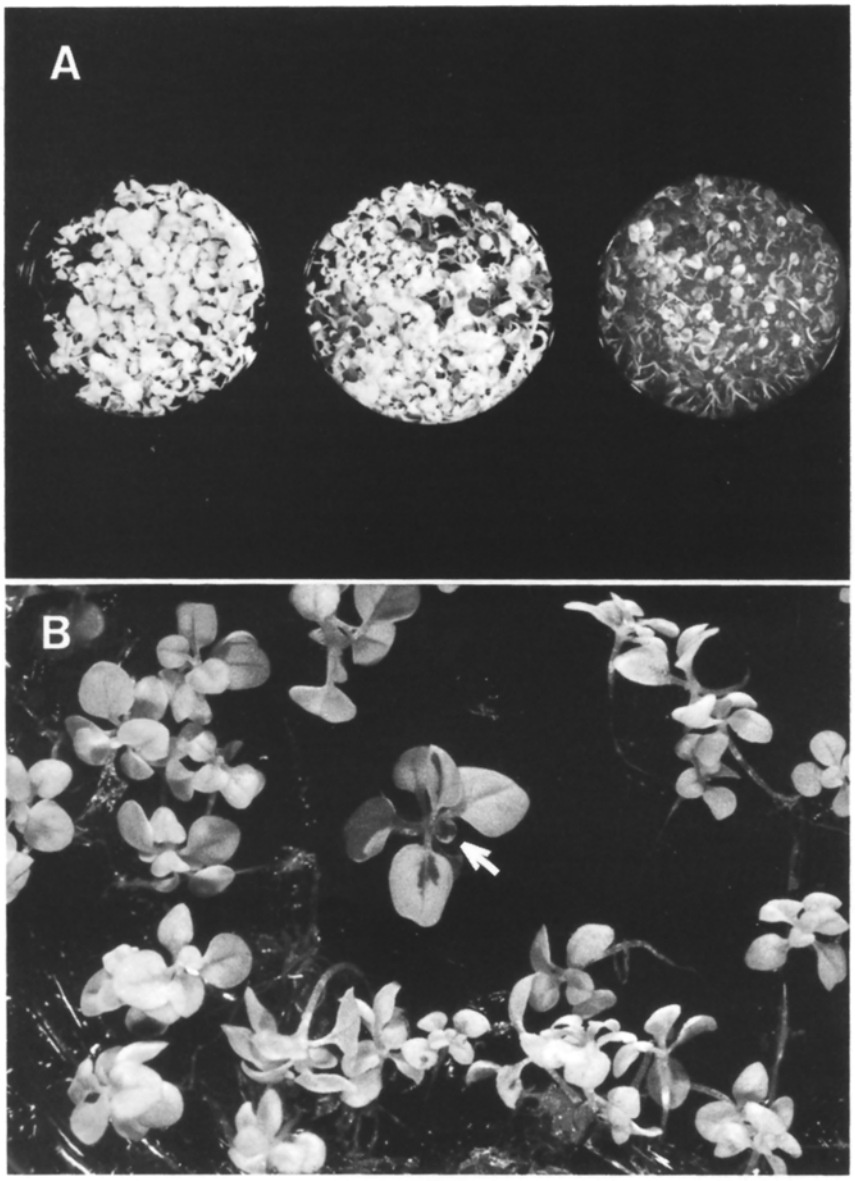

Fig. 2 A, B. Inheritance of pigment deficiency in the SR1-A10 line. The chimera was used as maternal parent in crosses with the SR1 mutant (**A,** *left* and *center* Petri plates and **B**) or as pollinator (**A,** *right* Petri plate). *Arrow* points to a variegated seedling (**B**)

reference, digests of SR1 cpDNA were run alongside the SR1-A15 total cellular DNA digests, as shown for probing with SalI fragments 2 and 5 on Fig. 3. The chloroplast genomes of SR1-A15 and SR1 mutants could not be distinguished by this approach. The map derived from data shown in Table 3 and from published information is shown in Fig. 4.

Discussion

A simple system, involving mutagenic treatment of callus and shoot regeneration has been exploited to recover maternally inherited, pigment-deficient mutants. The mutagene, NEU, was choosen because it was shown to be effective in inducing maternal pigment mutations (Hagemann 1982).

Albino shoots of the SR1-A10 and the SR1-A15 mutants are light sensitive and would not survive after grafting in the greenhouse. Regeneration of a periclinal chimera in line SR1-A10 made crosses very convenient since the chimeric shoots developed well after grafting in the greenhouse. Germ cells in *Nicotiana* derive from the second histogenic layer of the shoot apex, the same layer from which the cells giving the coloration of the leaf margin derive. Perfect periclinal chimeras behaved in crosses as if they consisted only of mutant cells (Burk et al. 1964). Indeed, some of the seed capsules gave exclusively white seedlings when selfed, or when used as the maternal parent in crosses with green SR1 plants

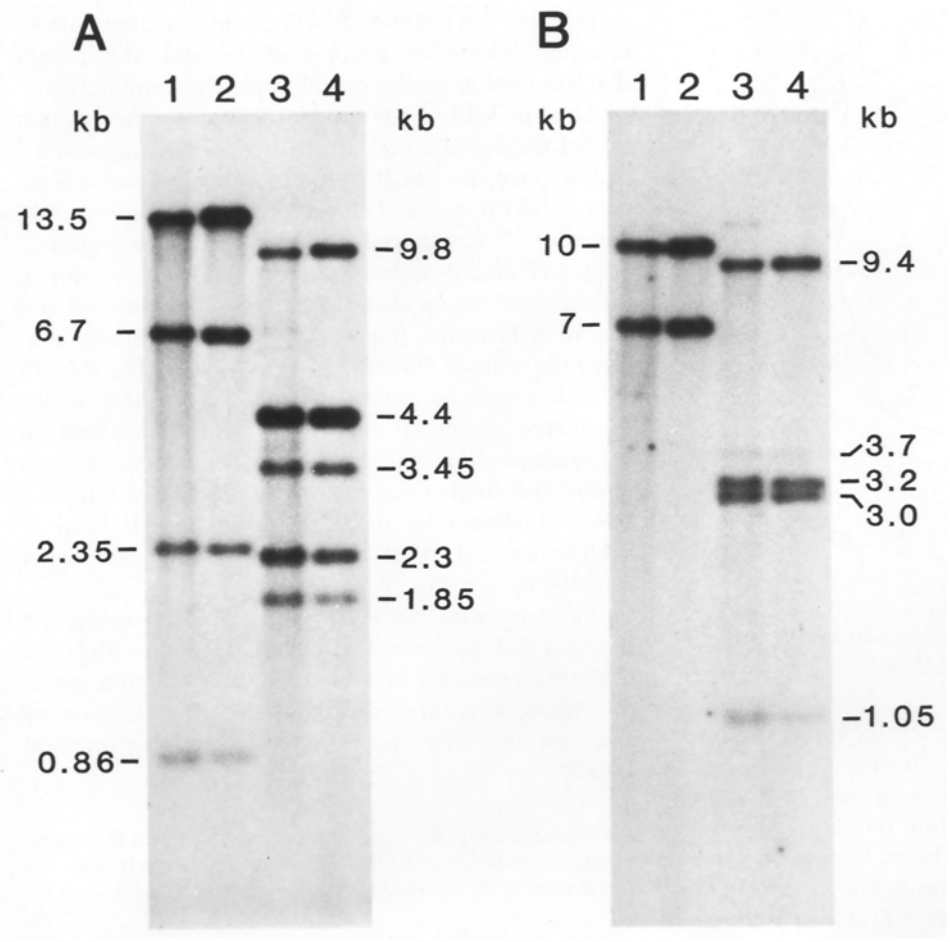

Fig. 3A, B. Identification of cpDNA fragments in the SR1-A15 line. Total cellular DNA from SR1-A15 callus (*lanes 1, 3*), and SR1 cpDNA (*lanes 2, 4*) was digested with SalI and PstI (*lanes 1, 2*), and with BamHI (*lanes 3, 4*), and probed with SR1 cpDNA SalI fragment 2 (**A**) and 5 (**B**)

Table 3. Chloroplast DNA fragments identified in the SR1-A15 line by hybridizing radiolabeled SalI fragments cloned from the SR1 chloroplast genome

Probe	Size (kbp) of fragments[a] hybridizing in digests of	
	SalI/PstI	BamHI
SalI 1	25 (Pl), 19 (P3B)*, 1.26 (P3B), 1.88 (P7)	19 (B2), 7 (B5), 5.3 (B6), 3.3 (B12), 1.2 (B24), 0.46 (B28)
SalI 2	13.5 (P2A), 6.5 (P6), 2.4 (P4), 0.84 (P12)	9.8 (B3), 4.4 (B9, B10), 3.45 (B11A) 2.3 (B18), 1.85 (B21)
SalI 3	9 (P3A), 8.2 (P5), 2.8 (P7), 2.4 (P10), 1.45 (P11)*	9.2 (B4), 5.7 (B6), 5.4 (B8), 3.7 (B11B), 3 (B16), 2.95 (B17), 1.1 (B25), 1.04 (B26)
SalI 4	25 (P1)*, 19 (P3B), 1.7 (P7)	19 (B2), 7.3 (B5), 5.5 (B6), 3.2 (B12), 1.07 (B24), 0.46 (B28)
SalI 5	10 (P3A), 7 (P4)	9.4 (B4), 3.7 (B11A), 3.2 (B14), 3 (B15), 1.05 (B27)
SalI 6	8.8 (P2A), 7.3 (P2B)	20 (B1), 10.5 (B3), 3.3 (B13), 2.3 (B19), 2.05 (B20), 1.3 (B22), 1.25 (B23)
SalI 7	8 (P5), 2.9 (P7), 2.5 (P10)*, 1.4 (P11), 0.5 (P9)	5.3 (B6), 5 (B7), 2.8 (B16), 1 (B25), 0.93 (B26)
SalI 8	8 (P2B), 2.6 (P8)	21 (B1)
SalI 10	2.3 (P9), 0.58 (P8)	20 (B1), 5.15 (B7)

[a] Map position of fragment on Fig. 3 is given in prenthesis. Border fragments hybridizing from opposite unique region – repeat junction are marked by asterisks

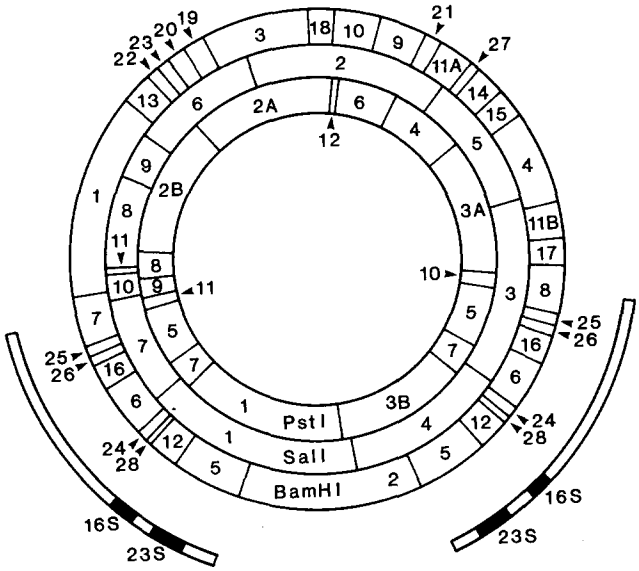

Fig. 4. Restriction map of the *N. tabacum* cpDNA for the enzymes PstI, SalI and BamHI. Note that BamHI sites generating smaller fragments than 500 bp are not shown

(Table 1). Other capsules gave green seedlings in non-Mendelian ratios indicating that the second histogenic layer was a mosaic of cells with wild-type or mutant plastids. Variegated seedlings in the progeny indicated heteroplastidic cells in the germ layer. Green seedlings were obtained exclusively, however, when the SR1-A10 line was the pollinator. These data (Table 1) indicate maternal inheritance of pigment deficiency in the SR1-A10 line (for discussion of criteria see Hagemann 1982).

Flowers on the SR1-A15 plants in culture were fertile and seed could be obtained from reciprocal crosses with greenhouse plants. Data indicate that pigment deficiency in that line is controlled by a maternally inherited mutation (Table 2).

The conclusion that pigment deficiency in lines SR1-A15 and SR1-A10 is a chloroplast trait is based on the demonstration of maternal inheritance, an accepted proof for the location of maternally inherited pigment deficiency (Kirk and Tilney-Basset 1978; Metz and Geist 1983). Recovery of a chloroplast recombinant using line SR1-A15 (Medgyesy et al. 1985) supports the localization of pigment deficiency in the chloroplasts. It suggests, furthermore, that the mutation leading to pigment deficiency did not affect the streptomycin resistance locus.

Nonphotosynthetic mutants of *Chlamydomonas reinhardtii* (Myers et al. 1982), *Triticum aestivum* (Day and Ellis 1984), and *Hordeum vulgare* (Day and Ellis 1985) were found to carry a vastly altered chloroplast genome with deletions, isertions and rearrangements. In other nonphotosynthetic mutants no (Day and Ellis 1984) or only minor (Gordon et al. 1980) changes were detected in the cpDNA. It was of interest therefore to map the chloroplast genome of the SR1-A15 mutant that was used in studies on chloroplast recombination.

Data in Table 3 allowed clarification of the position of PstI fragments 1 and 3B relative to SalI fragments 1 and 4 since the small SalI/PstI fragment hybridized only to SalI fragment 1 (Table 3; Salts et al. 1984). PstI fragment 12 was placed in the large unique region as originally described by Fluhr et al. (1983) and not in the repeated region (Salts et al. 1984). The number and size of hybridizing fragments in BamHI digests confirms the map of Hildebrand et al. (1985). The data in Table 3 could be interpreted, however, only if we considered recent information (Sugita et al. 1984) on the exact position of junctions between a large inverted repeat and single-copy regions in tobacco chloroplast DNA. It should be noted that the BamHI map of Hildebrand et al. (1985) contains only fragments bigger than 500 bp.

Mapping with the PstI, SalI or BamHI restriction enzymes did not reveal any changes in the SR1-A15 chloroplast genome. Pigment deficiency therefore could be due to a point mutation or a small deletion or inversion that remained undetected by the method used.

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