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Chloroplast DNA Assorts Randomly in Intraspecific Somatic Hybrids of *Nicotiana debneyi*

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Summary. Plants were regenerated following intraspecific fusion of leaf protoplasts from two naturally occurring genotypes of *Nicotiana debneyi*. The two genotypes differed in the EcoRI fragmentation pattern of chloroplast DNA and in the nuclear-coded phosphoglucomutase (Pgm) isozymes. There was no conscious selection for hybrid genotypes during protoplast culture or plant regeneration. Among 225 plants screened for Pgm, six were identified as nuclear hybrids. Restriction endonuclease and filter hybridisation analysis revealed that the cytoplasms of the hybrids contained one or other but never both parental chloroplast DNAs. The sorting out of chloroplasts was random and complete; the limit of detecting a rare chloroplast-DNA type in a mixture was 0.1%.

Key words: *Nicotiana debneyi* – Somatic hybrids – Chloroplast DNA – Restriction endonucleases – Nucleocytoplasmic incompatibility

Introduction

The analysis of chloroplast organelles in somatic hybrids suggests that in most cases there is rapid, apparently random sorting out to homogeneity for one or other parental chloroplast (Chen et al. 1977; Belliard et al. 1978; Gleba 1978; Aviv et al. 1980; Melchers et al. 1978; Melchers 1980). In other cases there is unidirectional chloroplast segregation (Evans et al. 1980; Maliga et al. 1980).

The interpretation of these results is confounded because of one or more constraints. All involved hybridisation between different species (Chen et al. 1977; Aviv et al. 1980; Evans et al. 1980; Maliga et al. 1980; Melchers et al. 1978) or between parents, one of which had a history of an alloplastic relationship between nucleus and cytoplasm (Belliard et al. 1978; Gleba 1978). The cytoplasmic consequences in these hybrids could be a result of genomic-cytoplasmic interaction. In some cases the plants analysed were all (Belliard et al. 1978) or largely (Aviv et al. 1980) presumptive cytoplasmic (but not nuclear) hybrids. It is difficult to account for the origin of such hybrids let alone interpret the behaviour of the chloroplasts. Deliberate selection to enrich for somatic hybrids (Chen et al. 1977; Melchers et al. 1978; Maliga et al. 1978; Gleba 1978) could also bias the behaviour of chloroplasts. This is particularly pertinent because in all but one case (Chen et al. 1977) at least one of the parents carried a mutation (cytoplasmic – or nuclear-coded) which affected chloroplast function. Additional constraints might also arise from the physiologically different state of the parental protoplasts used. In several cases (Melchers et al. 1978; Maliga et al. 1978; Evans et al. 1980) protoplasts of one parent were derived from suspension culture cells which were achloroplastic, those of the other parent were leaf protoplasts. Aviv et al. (1980) used leaf protoplasts of one parent in which cell division had been inhibited by exposure to X-irradiation (5kR) (Zelcer et al. 1978).

We report on the fate of chloroplast DNA(cp-DNA) in somatic hybrids where the constraints of potential nucleocytoplasmic incompatibility, deliberate selection and physiological dissimilarity of the protoplasts are nonexistent or largely eliminated. The parents used were naturally occurring variants of *N. debneyi* which are polymorphic both for an EcoRI restriction endonuclease site in the cp-DNA and a nuclear-coded isozyme difference (Scowcroft 1979).

Materials and Methods

N. debneyi Genotypes

Details of the distinguishing features of the two *N. debneyi* genotypes employed, TS233 and TS287, are given by Scowcroft (1979). The cp-DNA of TS233 lacked one EcoRI restriction endonuclease site resulting in a 4.95 Mdalton fragment which was absent in TS287 (Fig. 4, tracks a and b). TS287 possessed two additional fragments, 2.60 and 2.38 Mdaltons. The nucleotide sequence homology of the TS233 4.95 fragment with both the 2.60 and 2.38 Mdalton fragments of TS287 was confirmed by filter hybridization with a ^{32}P labelled probe. The nuclear-coded isoenzyme, phosphoglucomutase (Pgm) (E.C.2.7.5.1), had different migration rates in the two genotypes; TS287 and TS233 were homozygous for a fast and slow allele respectively.

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Protoplast Isolation, Fusion, Culture and Plant Regeneration

Protoplasts were readily isolated, by enzymatic digestion of young (3-5 wks) leaves with 2% Cellulysin, 0.5% Macerase (Calbiochem-Behring, Aust.) and 0.5% Driselase (Kyowa Hakko Koygo, Japan) (Scowcroft and Larkin 1980). Protoplast fusion was accomplished by a combination and modification of the polyethylene glycol (PEG) procedure (Kao and Michayluk 1974) and the high Ca^{2+}/high pH procedure (Keller and Melchers 1973). The less dense fractions of leaf protoplasts were used from an isoosmotic Percoll gradient (Scowcroft and Larkin 1980). Protoplasts in the 1.048, 1.039 and 1.029 gm/cm³ fractions were collected, combined and washed with 0.12 M $CaCl_2.2H_20$, 0.3 M mannitol. Drops of protoplast suspension (1×10^5 ml^{-1}) were placed in Falcon petri dishes and the protoplasts allowed to settle for 10 min. An equivalent volume of either 40 or 50% (w/v) PEG1540 (Fluka) in 20 mM $CaCl_2.2H_20$ (unadjusted pH) was added followed after 6 min by 10 volumes of 0.1 M $NaNO_3$, 0.1 M $Ca(NO_3)_2$, 0.02 M glycine, pH 10. After 30 min the protoplasts were washed gently (10 times) with 0.24 M $CaCl_2.2H_20$ and NTK culture medium (Scowcroft and Larkin 1980). The protoplasts which remained had adhered to the dish and were cultured in situ as drops in the dark at 25°C. The culture of the fusion products and plant regeneration followed Scowcroft and Larkin (1980) where protoplasts were cultured in NTK medium which contained Nagata and Takebe (1971) mineral salts, Schenk and Hildebrandt (1972) iron chelate, the sugars, organic acids, vitamins, vitamin-free casamino acids and coconut water of Kao (1977), 1% each of glucose and sucrose, 2 mg/l each of p-chlorophenoxyacetic acid and 6-benzyl amino purine (BAP) and 0.43 M Mannitol.

Shoots and then roots were induced by culturing colonies in the light on Murashige and Skoog (1962) medium containing 1 mg/l BAP and 0.5 mg/l indole acetic acid, or devoid of growth regulators respectively. Shoot cultures derived from stem explants were similarly established.

Cytology and Stomatal Guard Cell Chloroplast Counts

Chromosome numbers of either root tips or pollen mother cells were determined by standard procedures (Collins 1979). Chloroplast number of stomatal guard cells (Butterfass 1960; Chaudhari and Barrow 1976) was determined from leaf epidermal strips to confirm ploidy levels.

Phosphoglucomutase (Pgm) Isozyme Analysis

The isozyme technique used was modified (Brown et al. 1978) from Brewer and Sing (1970).

Cp-DNA Isolation

For the parental genotypes, TS233 and TS287, cp-DNA was isolated from leaves (75 g) of 5-6 week old plants as described by Frankel et al. (1979).

From individual somatic hybrid plants (8-16 weeks after fusion), initially identified by the Pgm analysis, cp-DNA was isolated from 4-6 g of the youngest leaves following 48 h in the dark at 23°C by a modification of Frankel et al. (1979). Chloroplasts were purified on a sucrose step gradient (30%, 45%, 60% sucrose in Buffer B of Frankel et al. (1979); 20,000 rpm, SW50.1 rotor, for 1 h), washed in Buffer B, the pellet suspended in 0.2 ml of 0.4 M NaCl, 20 mM Tris-HCl, pH 7.8, lysed with 0.1 ml 10% (v/v) Sarkosyl in 50 mM Tris-HCl, 20 mM EDTA, pH 7.5, and incubated 10 min at 37°C with 0.1 mg/ml predigested pancreatic RNA'ase. The cp-DNA was phenol extracted and ethanol (70%) precipitated.

Restriction Endonuclease Analysis, cp-DNA Fragment Isolation, ^{32}P Nick-Translation and Filter Hybridisation

Cp-DNA (2-3 μg) was digested with EcoRI (prepared in the Division of Plant Industry) and subjected to electrophoresis in horizontal agarose slab gels essentially as described by Frankel et al. (1979).

The 4.95 Mdalton fragment of EcoRI digests of TS233 was extracted from low melting point agarose (Sea-Plaque Agarose, Marine Colloids, Maine, USA) using hexadecyl trimethyl ammonium bromide as developed by Langridge et al. (1980).

The recovered 4.95 Mdalton fragment (approx. 1 μg) was nick-translated (Rigby et al. 1977) with 25 μCi deoxyadenosine 5'-[α-^{32}P] triphosphate (The Radiochemical Centre, Amersham, UK). The specific activity of the resultant acid precipitable ^{32}P-labelled DNA probe was 1×10^7 cpm/μg DNA.

EcoRI digests of cp-DNA were fractionated in horizontal agarose gels, denatured (Southern 1975) and transferred to nitrocellulose filters (approx. 15 cm²) by a simplified procedure (Wahl et al. 1979) of Southern (1975). Hybridisation of the ^{32}P labelled probe (10^6 counts/filter) to the filters followed the method of Wahl et al. (1979). Hybridization was detected by autoradiography at −70°C.

Results

Plant Regeneration from Fusion Products

From two separate fusion experiments, eighteen fusion droplets were separately established and the derived cultures were maintained separately throughout. Individual colonies each derived from a single protoplast or fusion product were identified as early as 3 weeks after the initiation of culture and were each separately regenerated to produce only one plant. After one plant from each colony was established in the glasshouse, the callus colony remaining was discarded. The six hybrid plants were derived from three different original culture plates. We are thus certain that three and probably all six derived from independent fusion events.

Pgm Identification of Somatic Hybrids

A total of 225 regenerated plants were screened for the Pgm isoenzyme pattern (Table 1a). An interpretation of a typical zymogram is shown in Fig. 1. Two (bands 3 and 4, Fig. 1) of the five bands could be used to distinguish parental and hybrid plants. The segregation patterns for bands 3 and 4 in sexual crosses conform to a model of a single locus with two alleles (Table 1; Scowcroft 1979).

Table 1. Phosphoglucomutase (Pgm) isoenzyme patterns in plants regenerated from protoplast fusion cultures (a), progeny of selfed somatic hybrids (b) and sexual F_2 progeny (c). F and S refer to the fast and slow alleles of TS287 and TS233 respectively

a) Protoplast fusion regenerates						
						Total
Pgm phenotype[a]	F		F = S		S	
Frequency	209		6		10	225
b) Selfed somatic hybrid progeny						
Genotype	FFFF	FFFS	FFSS	FSSS	SSSS	
Phenotype[a]	F	F > S	F = S	F < S	S	
Expected frequency[b]	1	8	18	8	1	36
Oserved frequency						
– Somatic hybrid no. 72	0	3	12	1	0	16
– Somatic hybrid no. 21	0	3	27	3	1	34
c) Sexual F2 progeny						
Phenotype	F		F = S		S	
Frequency	6		35		18	59

[a] F = S, F > S or F < S signifies relative intensity of F and S bands
[b] Expected phenotypic frequencies based on complete quadrivalent association and centromeric segregation

Fig. 1. Diagram of typical zymogram of phosphoglucomutase isozymes for the parental genotypes, TS287 (*a*) and TS233 (*b*), and for plants regenerated after fusion of TS287 and TS233 protoplasts (*c-t*). Most regenerants were TS287 type (*c, d, f-i, k, l, n-s*), rarely TS233 type (*j*) or hybrid (*m, t*; respectively, somatic hybrids 174 and 181 (Table 2)). Presumptive hybrid plants were retested as were those occasional plants (*e*) which were initially unclassifiable. Only bands 3 and 4 were consistently and sufficiently reliable to enable classification of the regenerated plants. Bands 1, 2 and 5 were frequently and variously absent

The great majority (209) carried the fast allele of TS287 and only 10 that of the TS233 parent. This disparate ratio probably resulted from the relatively faster cell proliferation and regeneration rate of TS287 relative to TS233 (Scowcroft and Larkin 1980). Six plants carried both the fast and slow alleles of Pgm. Each was retested to confirm nuclear hybridity. Shoot cultures were established from confirmed hybrid plants.

Cytology and Stomatal Guard Cell Chloroplast Counts

Cytological determination of the chromosome number from either root tip mitoses and/or pollen mother cell metaphases confirmed that each of the six presumptive hybrids had about double the somatic chromosome number of the parents (Table 2). The large number and relatively small chromosome size in root tip mitoses and the occurrence of multivalents in pollen mother cells precluded exact chromosome number determination. The multivalents in the hybrids confirm the expected autotetraploid pairing behaviour.

Table 2. Somatic chromosome number and number of stomatal guard cell chloroplasts in the parents and the six somatic hybrids

	Somatic chromosome number	Chloroplast number
Parents		
TS233	48	18.1 ± 2.3
TS287	48	18.0 ± 2.7
Somatic hybrids		
21	82-97	34.6 ± 3.5
72	78-100	29.3 ± 1.7
174	86-100	31.0 ± 1.0
181	84-98	31.0 ± 1.8
200	82-92	34.0 ± 2.7
215	86-101	36.1 ± 2.8

The number of chloroplasts in the stomatal guard cells of each of the six somatic hybrids was also approximately double that of the two parents, which themselves were identical (Table 2). Based on Butterfass (1960) and Chaudhari and Barrow (1976) this confirms the polyploid nature of the hybrids. Chloroplast numbers determined on the two parents and somatic hybrids 21 and 72 at different times demonstrated the relative consistency of this metric.

Pgm Inheritance

Pgm is inherited in a Mendelian pattern in *N. debneyi*. F_2 segregants from a sexual cross between TS287 and TS233 indicate diploid inheritance (Table 1c), though a χ^2 analysis based on the segregation of two alleles at a single locus, only gave a significance level of between 2%-5%. All the heterozygous F_2 plants from the sexual cross had fast and slow bands of equal relative intensity, thus confirming diploid inheritance.

Segregation from a hybrid autotetraploid yields simplex (FSSS), duplex (FFSS) and triplex (FFFS) genotypes where the relative intensity of the Pgm bands are F < S, F = S and F > S respectively, as well as the quadriplex (FFFF) and nulliplex (SSSS) types (Table 1b). The expected frequency of these five genotypes will be perturbed depending on the degree of quadrivalent formation and the location of the Pgm locus relative to the centromere, parameters about which we have no information. Such pertubations however will not disturb the ratios suffi-

ciently to increase the rare homozygous classes. Both homozygous classes among the progeny of selfed somatic hybrids, 21 and 72, were rare or absent and conversely the duplex heterozygote was frequent (Table 1b).

Relative intensities of F and S Pgm bands were assessed visually and among segregants of the selfed somatic hybrids, 72 and 21, both simplex and triplex types were found. Because of the subjective visual assessment, the excessively frequent duplex heterozygotes may have included indistinct simplex and triplex types.

Morphological Features of the Somatic Hybrids

The leaf morphology of four of the hybrids, 21, 72, 174 and 181 tended to be intermediate between those of the parents (Fig. 2). The leaves of TS233 are distinctly darker green and more ovate than those of TS287. The leaves of the hybrids 200 and 215 were consistently malformed, more spongy and had disordered leaf venation. This tendency to malformation was stable in subsequent plants derived from shoot cultures of hybrids 200 and 215. A number of non-hybrid plants (Pgm patterns like TS287 or TS233) also showed similar leaf malformations.

Somatic hybrids 21, 72, 174 and 181 were fully fertile and set seed whereas 200 and 215 failed to set seed. Flower morphology in these latter two was normal except that the anthers failed to shed mature pollen.

EcoRl Restriction Endonuclease Analysis

The EcoRl analysis revealed that each of the six somatic hybrids contained only one type of chloroplast DNA (Fig. 3). The distinguishing feature in this analysis was the 4.95 Mdalton fragment in TS233 which was absent from TS287 which itself contained two additional fragments of 2.60 and 2.38 Mdaltons.

The hybrids 21, 72, 174 and 181 had a cp-DNA EcoRl fragmentation pattern identical to that of TS287, while those of the hybrids 200 and 215 were identical to that of TS233. As visualised with ethidium bromide, the TS233 4.95 Mdalton fragment could not be seen in 21, 72, 174 and 181 and neither of the two TS287 exclusive fragments, 2.60 and 2.38 Mdaltons, was visible in the fragmentation patterns of 200 or 215. A time course digestion series for TS287 cp-DNA indicated that EcoRl digestion was complete after 10 min incubation. As the standard incubation time was 1.5 h, artifacts arising from incomplete digestion must have been minimal.

Filter Hybridization with ^{32}P Nick Translated Probe

The signals generated by hybridization of the ^{32}P nick-translated TS233 4.95 Mdalton fragment to fractionated EcoRl digests immobilised on nitrocellulose filters are shown in Fig. 4. This confirms the ethidium bromide visualised patterns that the hybrids 21, 72, 181 and 174 contained no admixture of TS233 cp-DNA and that 200 and 215 contained none of TS287 cp-DNA.

In some batches of TS233 cp-DNA, autoradiographs revealed a signal (Fig. 4, track i) representing a DNA fragment of 3.6 Mdaltons, as determined by a comparison with an EcoRl digest of phage λ (see Frankel et al. 1979). A comparable band was not seen in ethidium bromide

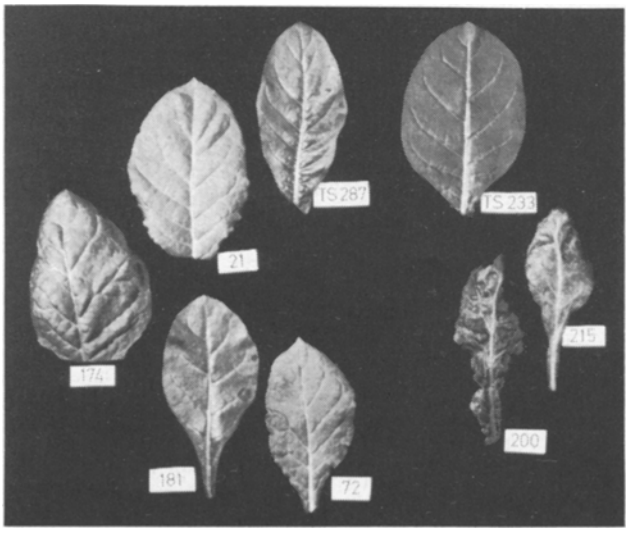

Fig. 2. Fully expanded leaves from young plants of the parents TS287 and TS233 and the six somatic hybrids

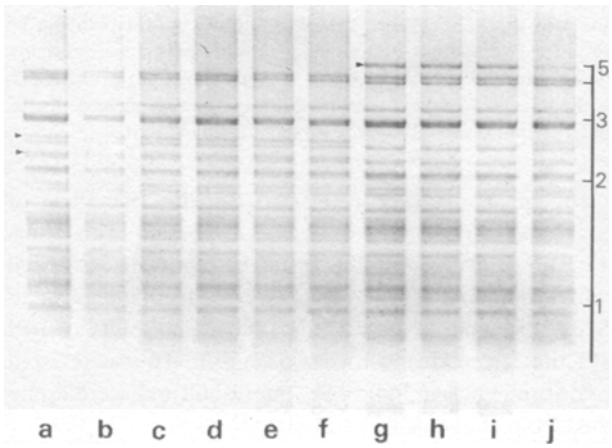

Fig. 3. Fragmentation patterns in agarose gels of EcoRl restriction endonuclease digests of cp-DNA isolated from the parents, TS287 (*a* and *d*) and TS233 (*g*), each of the six somatic hybrids, 72 (*b*), 181 (*c*), 21 (*e*), 174 (*f*), 200 (*h*), 215 (*i*) and an approximately equimolar mixture of TS233 and TS287 cp-DNA (*j*). Arrows indicate the distinguishing 4.95 Mdalton fragment of TS233 and the 2.60 and 2.38 Mdalton fragments of TS287. Molecular weight scale in Mdaltons

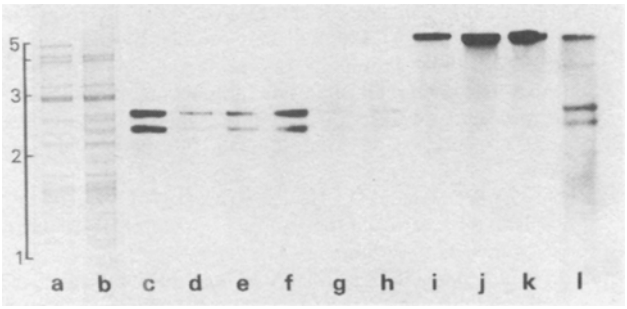

Fig. 4. Autoradiographs (tracks *c* to *l*) following hybridization of ^{32}P nick-translated TS233 4.95 Mdalton EcoRI fragment to nitrocellulose filter transfers of the fractionated EcoRI digests of Fig. 3; TS287 (*c*, *f*), TS233 (*i*); somatic hybrids – 72 (*d*), 181 (*e*), 21 (*g*), 174 (*h*), 200 (*j*), 215 (*k*); equimolar mixture of TS233 and TS287 (*l*). EcoRI fragmentation patterns of TS233 (*a*) and TS287 (*b*) and molecular weight scale in Mdaltons are for reference purposes

stained EcoRI digests (Fig. 4, track a; Fig. 3, track g). This 3.6 Mdalton fragment signal was not generated in autoradiographs of the hybrids 200 and 215. The origin of the additional fragment in the parental TS233 is uncertain. It could represent a partial breakdown product.

Limits of the cp-DNA Analysis

The limits of the EcoRI/filter hybridization assay were determined by a reconstruction experiment in which decreasing amounts of TS287 cp-DNA were included in a standard assay containing 2.5 μg TS233 DNA, and then determining the % (w/w) of TS287 DNA which is the lower limit detectable by the presence of the characteristic 2.6 and 2.38 Mdalton fragments.

No less than 5% TS287 DNA could be detected visually with confidence from ethidium bromide stained gels. As little as 1% TS287 could be detected with confidence from densitometer traces of gel photographic negatives. Prolonged exposure (120 hr) during autoradiography of ^{32}P probed filters was able to detect 0.1% of TS287 cp-DNA. In a standard assay of 2.5 μg cp-DNA this represents 2.5 ηg of contaminating DNA, or for tobacco with a cp-DNA molecular weight of 95 Mdaltons, 1.5×10^7 complete genomes.

Discussion

Among six *N. debneyi* intraspecific somatic hybrid plants, initially identified on the basis of nuclear hybridity, four contained the cp-DNA of one parent and two had that of the other. Such a distribution among six plants statistically conforms to a model of random fixation of one or other cp-DNA type. Filter hybridisation analysis of EcoRI digests indicated that the cp-DNA was at least 99.9% pure with respect to one or other parental type.

An interpretation of random sorting out is considered valid because physiologically similar (same leaf age) protoplasts were used from both parents and the hybrid cell colonies were not deliberately selected. Likewise less inherent bias resulting from nucleo-cytoplasmic interactions would apply to these intraspecific hybrids than for interspecific ones.

We cannot rule out the possibility that hybrid plants containing both *N. debneyi* chloroplasts could be obtained by somatic hybridisation. However, we conclude that such a possibility will be rare. Our result strengthens the claim of the two previous substantive cases (Chen et al. 1977; Melchers, 1980) of random sorting out in genuine nuclear somatic hybrids between different species.

As Chen et al. (1977) pointed out there is a paradox in the random sorting out argument. Using the random genetic drift analogy (Wright 1952), there are too many chloroplasts (200, Possingham 1980) in a mesophyll cell for the chloroplasts to effectively sort out in the 40-50 cell generations which elapsed (in our experiments) from fusion product plating to plant analysis. While observationally there is a rapid reduction in chloroplast number to approximately 20 after three days, this does not take into account an unknown number of proplastids which presumably give rise to chloroplasts when plant differentiation is induced. It is perhaps significant that the number of chloroplasts (10-11) which would permit sorting out in this study is similar to the number (10-20) of proplastids cited by Possingham (1980) as occurring in dividing stem apex cells. This may represent an irreducible minimum for meristematic cells which would also apply to cultured cells and regenerating primordia.

Somatic hybridisation has been advocated as a technique to investigate cytoplasmic genetics and possibly to achieve cp-DNA recombination as apparently occurs for plant mitochondrial DNA (Belliard et al. 1979). The results reported here do not augur well for this. Even in intraspecific somatic hybrids, cytoplasms containing mixed chloroplasts must be judged to be rare (if they occur at all) rather than common. In interspecific hybrids the rarity would be exacerbated. This suggests that complementing chloroplast mutants may be required to enforce chloroplast heterozygosity for a sufficiently long period to permit recombination.

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