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Regeneration of cytoplasmic male sterile protoclones of *Nicotiana sylvestris* with mitochondrial variations

X. Q. Li[1,*], P. Chetrit[1], C. Mathieu[1], F. Vedel[1], R. De Paepe[2], R. Remy[1], and F. Ambard-Bretteville[1]

[1] Génétique Moléculaire des Plantes, UA 115, Université Paris-Sud, F-91405 Orsay, France
[2] Génétique et Physiologie du Développement des Plantes, CNRS, F-91190 Gif/Yvette, France

Summary. One fertile and two male-sterile diploid plants were regenerated from the same callus after two cycles of protoplast culture from fertile *Nicotiana sylvestris*. Genetic studies indicated that the male-sterile trait was under cytoplasmic control. Progenies of each regenerated plant possessed different mitochondrial (mt) DNA restriction patterns. Both cms protoclone types were characterized by specific mtDNA deletions. In addition, a 40 kD mitochondrially encoded polypeptide is lacking in the cms plants.

Key words: Somaclonal variation – Mitochondrial DNA – Protein synthesis

Introduction

Occurrence of genetic variation in plants obtained by "in vitro" culture has been described in most of the species capable of regeneration (Larkin and Scowcroft 1981). These somaclonal changes affect various characters such as morphology, fertility, antibiotic resistance, hormone susceptibility and auxotrophy. Most of the variation is under nuclear genetic control but some cases of maternal inheritance are reported, i.e. antibiotic resistance (Maliga et al. 1975; Cseplo and Maliga 1984), herbicide resistance (Cseplo et al. 1985) and albinism (Day and Ellis 1984), controlled by the plastid genome. In numerous crop species, cytoplasmic male sterility (cms) used in the production of F_1 hybrid seed, results from incompatibility between nuclear and mitochondrial (mt) genomes (Leaver and Gray 1982; Levings 1983; Hanson and Conde 1985; Pring and Lonsdale 1985). Reversion to fertility may result from changes occuring either in the nuclear (restorer genes), or in the mt genome. Cases of reversion from male sterility to fertility after tissue culture have been reported in sugarbeet (Lichter 1978) and maize (Gengenbach et al. 1977; Brettell et al. 1980), but never the contrary. In maize, reversion to fertility was correlated with significant changes in mtDNA (Gengenbach et al. 1981, Kemble et al. 1982). Variations in mtDNA have also been detected among potato somaclones (Kemble and Shepard 1984), in suspension cultures of maize (McNay et al. 1984) and tobacco (Grayburn and Bendich 1987) and in wheat embryogenic callus (Rode et al. 1987).

In *N. sylvestris*, a diploid species which appears to be the female parent of the allotetraploid tobacco, *N. tabacum* (Gray et al. 1974; Bland et al. 1985), protoplast culture induces an unusually high level of variability. About 50% of the diploid plants regenerated from a botanical pure line (T) contain mutations of nuclear recessive genes governing leaf colour and shape, germination and fertility (Prat 1983). The other regenerated plants are apparently unmodified. When a second cycle of protoplast culture was carried out with the progeny of such a normal plant, most of the regenerated plants appeared to contain mutations in either recessive, semi-dominant or cytoplasmic genes (Li 1987). We report here the regeneration of cms plants after two cycles of protoplast culture from a fertile *N. sylvestris* botanical line. These plants are characterized by specific mtDNA and polypeptide variations.

Materials and methods

Plant material. Two cycles of leaf mesophyll protoplast culture were performed as follows (Fig. 1): a first cycle was carried out from young plants of a botanical line of *N. sylvestris*, obtained

Permanent address: Department of Biology, Peking University, Beijing, China

Offprint requests to: F. Vedel

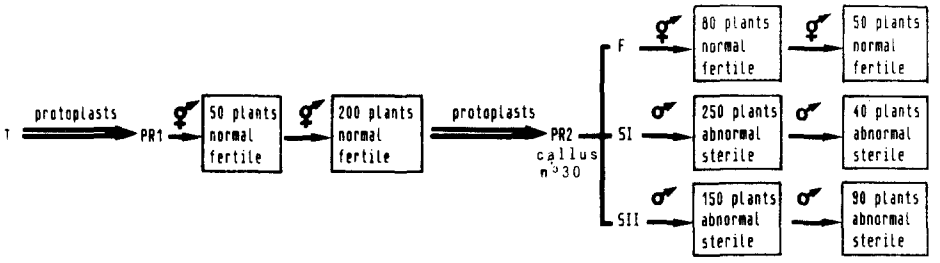

Fig. 1. Obtainment and genetic analysis of plants regenerated through two successive cycles of protoplast culture. Plant regeneration; PR1: first cycle; PR2: second cycle. Sexual progenies; ♂ fertile plants as males; ♀ self-pollination. In the case of PR2-SI, 250 plants coming from 4 first generation crosses and 40 plants coming from 4 second generation crosses were examined. In the case of PR2-SII, 150 plants coming from 2 first generation crosses and 90 plants coming from 1 second generation cross were examined

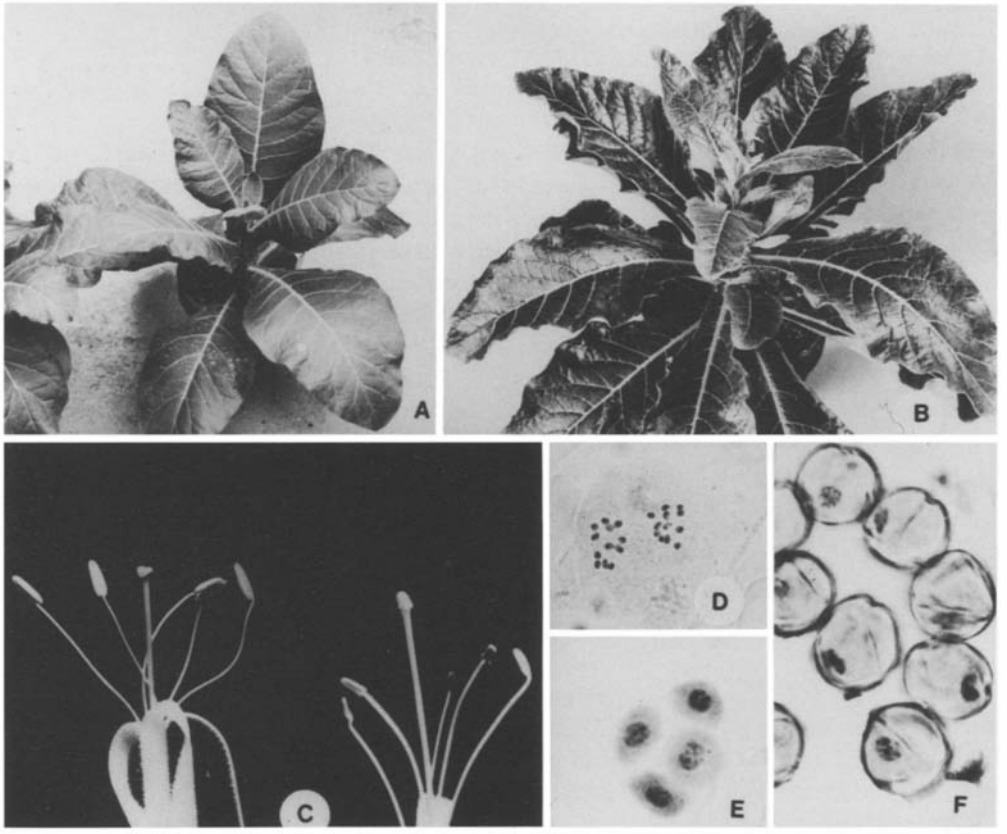

Fig. 2A–F. Morphological and cytological characterization of the PR2 cms plants. **A** and **B** leaf morphology of original line plant and male sterile plant respectively. **C** floral morphology of original line plant (*left*) and male sterile plant (*right*). Cytological observation of male gametogenesis in male sterile indicates that microsporogenesis was normal at least up to microspore formation: **D** metaphase II with 2n = 24 chromosomes; **E** regular tetrad stage; **F** microspores

from the Institut du Tabac, Bergerac, France, and maintained by self-pollination in the Gif Phytotron greenhouses. The plantlets were grown in sterile conditions on agar medium, and the protoplast culture was performed as described by Prat (1983). The regenerated plants were called PR1. The second cycle of culture was carried out with plants belonging to the second generation of selfing of a diploid PR1 plant, morphologically normal and fully fertile. The regenerated plants were called PR2.

Mitochondrial DNA isolation and analysis. MtDNA was purified from parental and regenerated lines as previously published (Kolodner and Tewari 1975). DNA restriction and agarose gel electrophoresis, isolation of DNA fragments, Southern transfer, nick translation and hybridization except for the yeast var1 probe were carried out as previously described (Vedel and Mathieu 1983). In the case of the yeast var1 probe (Hudspeth et al. 1982), hybridization was carried out in 45% formamide at 38 °C. Washes were in 2 x SSC for 30 min at room temperature.

Polypeptide synthesis by isolated mitochondria. Mitochondria were isolated from young leaves of plants at the rosette stage, according to the method of Boutry et al. (1984) and incubated

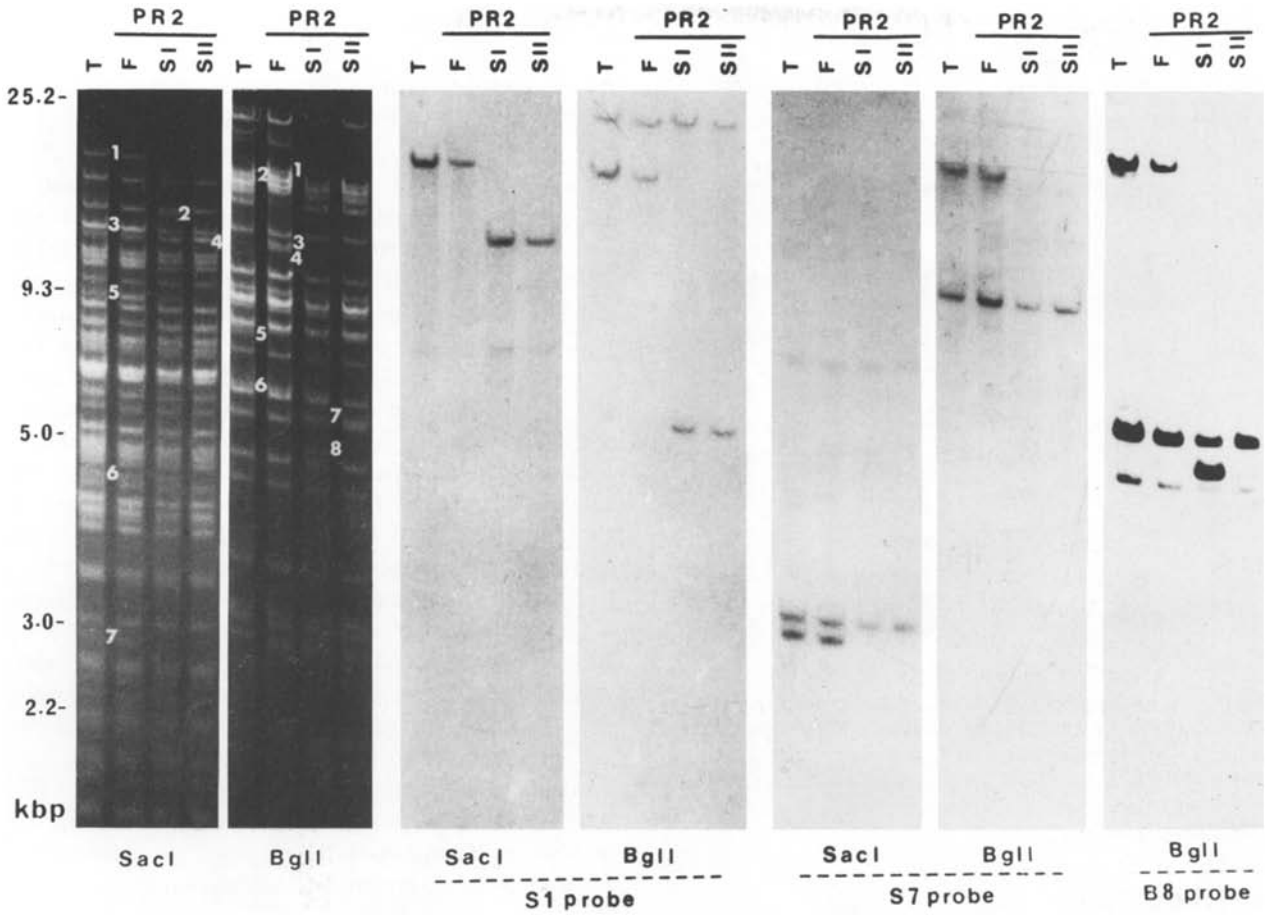

Fig. 3. 0.7% agarose gel electrophoresis of mtDNA SacI and BglI digests from fertile (T, PR2-F) and cms (PR2-SI and SII) lines. Specific fragments are numbered according to their size (molecular weights are given in results). Autoradiographs were obtained after hybridization between SacI and/or BglI mtDNA fragments and three specific fragments used as probes. S1: 16.7 kb SacI fragment; S7: 2.7 kb SacI fragment; B8: 5.05 kb BglI fragment

for 60 min in a translation medium containing ^{35}S methionine (Forde and Leaver 1980) and erythromycin, a potent inhibitor of plastid translation (Newton and Walbot 1985). The synthesized polypeptides were fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, dried and autoradiographed.

Results

Regeneration and characterization of PR2 cms plants

Three diploid plants were regenerated from the n° 30 callus of the second cycle of protoplast culture. The first plant (PR2-F) was morphologically normal and fertile; between the two other plants, closely linked on the callus, one (PR2-SI) was abnormal from the beginning, the second (PR2-SII), was initially normal but became progressively abnormal during development. At flowering time, PR2-SI and SII appeared similar in that they had curvated leaves, small carved flowers, and were male sterile (Fig. 2). Two generations of selfing of the fertile PR2-F produced only normal, fertile plants, while crosses of PR2-SI and SII as females with the original line (T) or fertile PR1 and PR2 plants as males gave only plants with the same leaf and flower alterations. Cytological observation of male gametogenesis in the sterile plants showed that pollen degenerated before anthesis. Crosses between PR2-SI and SII sterile plants as females with fertile plants as males showed that the male-sterile trait was maternally inherited. Thus PR2-SI and SII were cms plants with specific morphological abnormalities. Association of cms with alterations of leaves and flowers is common in *Nicotiana* (Hanson and Conde 1985).

mtDNA changes in the PR2 plants

There is evidence that cms is mitochondrially encoded in *Nicotiana* species (Belliard et al. 1979). The comparison between mtDNAs of the original T line and first generation progenies of PR2 plants was therefore undertaken. Native mtDNAs of T and PR2 lines did not

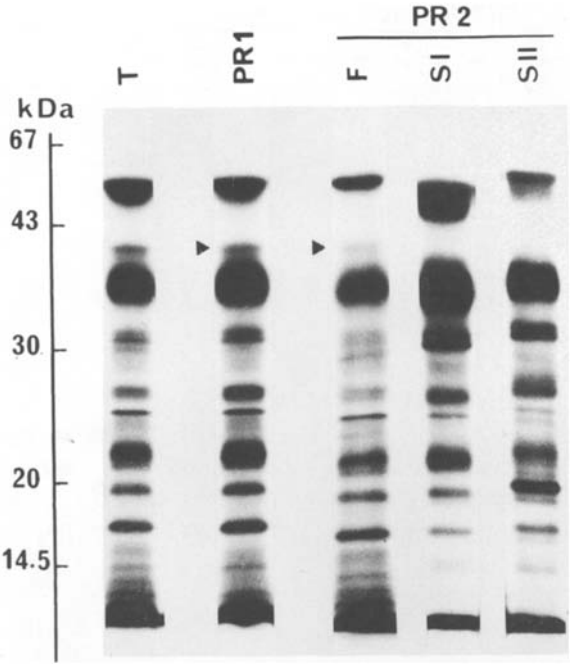

Fig. 4. Autoradiographs obtained after polyacrylamide gel electrophoresis of polypeptides synthesized by mitochondria from *N. sylvestris* original line T and regenerated lines PR1, PR2-F, PR2-SI and PR2-SII. Mt polypeptides were labelled with ^{35}S methionine. A major difference is the lack of a 40 kD polypeptide (▸) in the cms profiles

contain visible plasmids but were different from one another when digested by various restriction enzymes. MtDNA variations are clearly shown by SacI and BgII digests (Fig. 3). Three types of changes are observed: loss of parental bands, occurrence of new bands and stoichiometric variations. MtDNAs of PR2 second generation progenies were identical to those of first generation. No changes were detected in the PR1 mtDNA (not shown).

SacI enzyme gave identical mtDNA restriction patterns among fertile lines T, PR1 and PR2-F. Both cms lines PR2-SI and SII have lost parental fragments S1 (16.7 kb) and S7 (2.7 kb) and show similar stoichiometric decrease of fragment S3 (11.4 kb) and an increase of fragment S4 (11 kb). PR2-SII mtDNA can be distinguished from PR2-SI by the absence of fragments S5 (8.4 kb) and S6 (4.6 kb). PR2-SI possesses fragment S2 (12.6 kb) that is absent in other lines. Concerning BgII patterns, fragment B2 (16.4 kb) is lost in both cms lines but not in fertile PR2-F; fragment B5 (7.5 kb) is absent in the three PR2 lines and fragment B6 (6.3 kb) is lost in PR2-SII only. New BgII fragments are seen in PR2 lines: B1 (17.1 kb), B3 (11.3 kb) and B4 (11.1 kb) in PR2-F, B7 (5.8 kb) in both cms lines and B8 (5.05 kb) in PR2-SI only.

No changes were detected in PR1 and PR2 ct DNAs using the restriction enzymes SalI, HpaII and EcoRI.

Evidence for mtDNA deletions in the cms plants

To characterize further the mtDNAs variations in the PR2 plants and their possible relation to male sterility, fragments S1 and S7, specific to fertile lines T and PR2-F, and fragment B8, specific to PR2-SII, were used as probes in hybridization experiments (Fig. 3). S1 hybridizes with itself in fertile patterns and with S4 (11 kb) in cms patterns. In BgII patterns, S1 shows homology with the largest BgII fragment (25 kb) common to all lines, and with either B2 (16.4 kb) in fertile or B7 (5.8 kb) in cms lines. S7 (2.7 kb) is deleted in both cms lines; this is confirmed in BgII digests, where S7 hybridizes with B2 (16.4 kb) in fertile lines. S7 also hybridizes with a 3.2 kb SacI fragment and a 9.3 kb BgII fragment in all four lines. B8 (5.05 kb) hybridizes with itself in PR2-SI, with B2 (16.4 kb) in both fertile lines and with two fragments (5.7 kb and 4.8 kb) common to all lines.

S1, S7 and B8 are homologous to the B2 fragment suggesting that this fragment may be the target sequence for mt rearrangements related to cms in PR2 plants. Among these events we detected at least two deletions of 5.7 kb (i.e. the size difference between S1 and S4) and 2.7 kb (S7). So far, we have no evidence for deletions in mtDNA of fertile regenerated plant, PR2-F. Additional experiments are needed to locate some of the mtDNA variations on a B2 map and to determine if repeated elements are involved in these variations.

mt polypeptide variations among fertile and cms lines

To determine if mtDNA changes in *N. sylvestris cms* lead to alterations in gene expression, "in organello" protein syntheses were performed. Several variations occurred in the cms lines. A 40 kD polypeptide present in fertile T, PR1 and PR2-F was absent in both cms PR2-SI and SII profiles in all leaf and plant developmental stages (Fig. 4). This polypeptide has the same size as the yeast mitochondrially encoded var1 protein, which is part of the mt small ribosomal subunit (Terpstra et al. 1979). In plants, a protein of similar size, called R1 was previously reported (Leaver et al. 1985) but was not associated with cms.

The stoichiometry of a polypeptide of approximately 28 kD in PR2-SI is different from fertile lines. Another variation concerns a polypeptide of about 19 kD in size specific to both cms. A polypeptide of similar size was previously found in cms *N. debneyi* (Boutry et al. 1984). Under similar conditions, namely pyruvate and malate substrate, presence of erythromycin and darkness, isolated chloroplasts did not synthesize polypeptides. This shows that chloroplast contamination was not responsible for the variations among mt polypeptide patterns.

Gene mapping experiments

Variations in hybridization patterns were not found with the maize coxII and cob genes (gift from Pr. C. J. Leaver, Edinburgh, Scotland) or with wheat 26S rDNA gene (gift from Dr. Falconet, Orsay, France) (data not shown). To see if mtDNA reorganisations in the var1 region could be correlated with the absence of the 40 kD polypeptide in the cms mt profiles, we hybridized *N. sylvestris* mtDNA blots with a yeast var1 probe (gift from Pr. R. A. Butow, Dallas, TX, USA).

Preliminary results indicated that hybridization patterns of all PR2 lines were different from the original T line. Labelled fragments distinguishing fertile from sterile lines were S1 (16.7 kb) and B2 (16.4 kb) in fertile and S4 (11 kb) and B7 (5.8 kb) in sterile patterns. However, we cannot be sure that we have detected a var1-like sequence in the *N. sylvestris* mt genome. Hybridization under low stringency conditions between the yeast var1 probe, about 90% A–T, and *N. sylvestris* mtDNA may be due to non specific hybridization to A–T rich regions rather than gene homology.

Discussion

Our results reveal several new findings:

a. Regeneration of diploid cms plants by "in vitro" culture has not been reported. Cseplo and Maliga (1984) described regeneration of male-sterile *N. sylvestris,* but all plants were aneuploid.

b. Somaclonal mt variation was previously obtained in potato, wheat and maize (see introduction). In Nicotiana species, cytoplasmic variation induced by anther culture (Matzinger and Burk 1984) and mtDNA variation in cybrids (Belliard et al. 1979) and in cell suspension (Grayburn and Bendich 1987) have been reported. However, mtDNA variation was not described in plants regenerated from Nicotiana protoplast culture in homoplasmic conditions.

c. In different species, fertile lines were previously distinguished from cms lines by differential polypeptide syntheses in isolated mitochondria (Forde and Leaver 1980; Boutry et al. 1984). In this study we have observed mt polypeptide variation among *N. sylvestris* protoclones, namely specific loss in cms plants of a polypeptide, the size of which is similar to yeast var1 gene product.

A correlation between cms and molecular data remains to be ascertained. Further analyses are needed to demonstrate that: first, mtDNA deletions and rearrangements are responsible for cms; second, that regenerated plants are completely homoplasmic. It is not clear how homoplasmy was achieved during the regeneration of these three plants. PR2-SII was probably heteroplasmic at its regeneration, and became homoplasmic during development.

Whatever the case, if the only genetic changes are in the mt genome, it is clear that the isolation of three homoplasmic, or even nearly homoplasmic, PR2 plants different from each other and from the PR1 plant, is very unlikely, and would require a strong selection against either normal mtDNA molecules or cells containing the normal mitochondria. This would be surprising considering the reduced growth rate of cms plants. Another hypothesis is that mtDNA reorganisations are controlled by a nuclear gene and that changes have accumulated during "in vitro culture" and development, leading to complete substitution of normal mtDNA molecules by abnormal ones. In yeast, nuclear genes are known to control mtDNA petite mutations (Ephrussi and Hottinger 1951) and splicing of mRNA (Faye and Simon 1984), and, in *Podospora,* nuclear genes probably act on the mtDNA rearrangements accompanying senescence (Vierny et al. 1982). The possibility of nuclear genes controlling mtDNA rearrangements during protoplast culture is under investigation.

On the other hand, the region of the maize mt genome encoding α-subunit of the ATPase gene appears highly variable among the fertile and cms lines (Small et al. 1987). These authors suggested that substoichiometric fragments carrying this gene are part of mtDNA molecules occurring in low copy number relative to the rest of the mt genome, because of a lower replication rate. According to this hypothesis, in vitro culture may be able to induce different replication rates among mtDNA subgenomic molecules because of selection pressure. Some populations of molecules would not be affected whereas others would suffer a decrease in their replication rate until completely eliminated. Such mechanisms may explain the regeneration of mtDNA variants after protoplast culture, whereas no apparent changes occurred between *N. sylvestris* and *N. tabacum* mtDNAs during evolution (Bland et al. 1985).

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