

## Organization and Expression of the Mitochondrial Genome in the *Nicotiana sylvestris* CMSII Mutant

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Manuscript received February 1, 1998

Accepted for publication June 16, 1998

### ABSTRACT

Previous analyses suggested that the *Nicotiana sylvestris* CMSII mutant carried a large deletion in its mitochondrial genome. Here, we show by cosmid mapping that the deletion is 60 kb in length and contains several mitochondrial genes or ORFs, including the complex I *nad7* gene. However, due to the presence of large duplications in the progenitor mitochondrial genome, the only unique gene that appears to be deleted is *nad7*. RNA gel blot data confirm the absence of *nad7* expression, strongly suggesting that the molecular basis for the CMSII abnormal phenotype, poor growth and male sterility, is the altered complex I structure. The CMSII mitochondrial genome appears to consist essentially of one of two subgenomes resulting from recombination between direct short repeats. In the progenitor mitochondrial genome both recombination products are detected by PCR and, reciprocally, the parental fragments are detected at the substoichiometric level in the mutant. The CMSII mtDNA organization has been maintained through six sexual generations.

THE mitochondrial (mt) genome in higher plants is larger and more complex than in other eukaryotes (Schuster and Brennicke 1994). According to cosmid mapping, it may be represented as a single circular map, the so-called master molecule (Palmer and Shields 1984). This molecule often contains repeated sequences, some of which are able to recombine, generating subgenomic molecules in various amounts, depending on the balance between their recombination and replication rates (Lonsdale *et al.* 1988). However, neither the master genome nor the predicted subgenomic molecules have been physically identified in any species, and images from electron microscopy and pulsed-field electrophoresis suggest that most of the mt genome may consist of linear molecules (Backert *et al.* 1995; Oldenburg and Bendich 1996). A particular mt genome is thus characterized by both the nature and the relative stoichiometry of its molecules. From the molecular analysis of different maize cytotypes, Small *et al.* (1987) proposed that mtDNA evolution could result from changes in the proportions of substoichiometric molecules. However, experimental evidence for such an evolutionary mechanism is scarce, as mt genomes are in general stably inherited in a maternal fashion. Some cases of spontaneous changes have been described, for example, those associated with the reversion to fertility of cytoplasmic male sterile (CMS) plants in CMS-S maize (Schardl *et al.* 1985; Small *et al.* 1988) and CMS bean

(Mackenzie *et al.* 1988; Mackenzie and Chase 1990). Mitochondrial reorganizations have often been observed in *in vitro* cultured cells (Ozias-Akins *et al.* 1988; Shirzadegan *et al.* 1991; Kanazawa *et al.* 1994) and, more rarely, in the regenerated plants themselves (Vitart *et al.* 1992; Hartmann *et al.* 1994). A well-characterized system is the *in vitro*-induced reversion of the maize Texas CMS (Rottmann *et al.* 1987). A few examples of nuclear-directed changes of mt organization have been described, for example, in maize (Newton *et al.* 1990), bean (Mackenzie *et al.* 1988; He *et al.* 1995), and Arabidopsis (Martinez-Zapater *et al.* 1992).

In most variant systems analyzed so far, recombination events have been involved in the observed mtDNA structural changes (Belliard *et al.* 1979; Young and Hanson 1987; Fauron *et al.* 1990), suggesting recombination and differential replication of subgenomic molecules are indeed implicated in the evolution of mt genomes. Concerning the reversion of the CMS-T cytoplasm in maize, Fauron *et al.* (1990) proposed a model implicating two recombination events, one occurring frequently *in planta*, and a second rare event that occurred during the *in vitro* culture. We have previously described several mtDNA reorganizations in *Nicotiana sylvestris* mutants regenerated by protoplast culture from a fertile T line (Li *et al.* 1988; Vitart *et al.* 1992). Two of these mt mutants, named CMSI and CMSII, regenerated from the same protoplast-derived callus, presented similar morphological abnormalities and male sterility. Restriction patterns and Southern hybridizations suggested the presence of deletions in the mtDNA of both mutants, more than 50 kb in CMSII and ~15 kb in CMSI (Chétrit *et al.* 1992). The deletions were associated with two

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recombination events, *rec1* found in both CMSI and CMSII, and *rec2* specific to CMSI. For each recombination event, only one of the recombinant fragments could be detected by Southern hybridization, while the other recombinant fragment and parental fragments were undetectable. Target repeats of both recombinations have been characterized. Repeats of 102 bp (EMBL number X96741) were found at the recombining sites of *rec1* (Gutierrez *et al.* 1997a; P. Chétrit and S. Gutierrez, unpublished results), while *rec2* occurred between two short repeats of 65 bp (P1a *et al.* 1995). At last, several expression changes common to both CMSI and CMSII have been found. First, the complex expression pattern obtained using a probe overlapping one of the *rec1* sites was reduced to a single signal in the mutants (Chétrit *et al.* 1992). Second, the NAD7 polypeptide, a subunit of the first complex of the respiratory chain (complex I), could not be detected in either mutant (P1a *et al.* 1995; Gutierrez *et al.* 1997b). In addition, NAD9 and the nuclear-encoded 38-kD complex I subunit could not be detected, suggesting some misassembly of the complex. This complex I defect results in a dramatic decrease in its activity, and this has been postulated to account for reduced growth and sterility.

The aim of this article is to address the following points: (1) What is the extent and gene content of the mtDNA deletion in CMSII, and are the changes in the expression of *nad7* solely responsible for the abnormal phenotype? (2) Is the *rec1* recombination only involved in appearance of the deletion, did it specifically occur during the *in vitro* culture or did it preexist in the progenitor line, and what is the stability of the CMS II mtDNA organization through sexual generations?

## MATERIALS AND METHODS

**Plant material:** The fertile *N. sylvestris* T line is a botanical line provided by the Institut des Tabacs (SEITA, Bergerac, France) and maintained by self-pollination. The cytoplasmic male sterile (CMSII) line was regenerated after leaf mesophyll protoplast culture from the T line (Li *et al.* 1988) and maintained by backcrossing with T plants as male. Plants were grown in a greenhouse under a 16-hr photoperiod at 24° (day) and 17° (night).

**Mitochondrial DNA isolation:** mtDNA was isolated from young leaves as described in Chétrit *et al.* (1992).

**Mitochondrial RNA isolation and analysis:** Leaf mRNAs were prepared from sucrose gradient-purified mitochondria (Stern and Newton 1985). RNA electrophoresis, Northern transfer, and hybridization were performed according to Sambrook *et al.* (1989).

**Mitochondrial gene probes:** The heterologous probes used were *atpA* (Oenothera, Schuster *et al.* 1986), *atp6* (maize, Dewey *et al.* 1985), *atp9* (*N. sylvestris*, P1a *et al.* 1991), *coxI* and *coxIII* (Oenothera, Hiesel *et al.* 1987), *coxII* (maize, Fox and Leaver 1981), *cob* (maize, Dawson *et al.* 1984), exons B, C, and E of *nad1* (wheat, Chapelaine and Bonen 1991), exon D of *nad1* (wheat, Haouazine *et al.* 1993), exon 3 of *nad4* (wheat, Lamattina and Grienenberger 1991), *nad4L* (*Arabidopsis thaliana*, Brandt *et al.* 1992), all exons of *nad5* (wheat, Pereira de Souza *et al.* 1991), *nad6* (wheat, Haouazine *et al.*

1993), *nad9* (wheat, Lamattina *et al.* 1993), *rps12* (wheat, Gualberto *et al.* 1988), *orf25* (maize, Dewey *et al.* 1986), *rnm26* (wheat, Falconet *et al.* 1985), and *rnm18* (wheat, Falconet *et al.* 1984). *Nicotiana sylvestris nad1/A*, *nad3*, and *nad7* coding sequences were cloned in the laboratory.

**Cosmid libraries:** Cosmid libraries of T mtDNA were prepared using the *SacI* site of a modified pHC79 cosmid vector as previously described (Chétrit *et al.* 1992). Two T cosmid libraries were used (clones named 1–400 and T1–T350). Cosmid DNAs were prepared according to Birnboim and Doly (1979).

**Colony hybridization and cosmid mapping:** Screening of cosmid libraries was performed according to Grunstein and Hogness (1975), and clones were mapped using the *SacI* restriction enzyme (Boehringer Mannheim, Indianapolis).

**DNA analysis:** DNA restriction, agarose gel electrophoresis, Southern transfer on Hybond N+ (Amersham, Arlington Heights, IL) membranes, and hybridization using radiolabeled probes were performed as described in Sambrook *et al.* (1989). mtDNA restriction fragments used as probes for screening of cosmid libraries were purified from preparative agarose gels according to Zhu *et al.* (1985) and labeled using random priming kits (Boehringer).

**cDNA probe:** Ten micrograms of T mtRNA was incubated at 37° for 30 min in 40 µl of a mixture containing 200 units of reverse transcriptase (Stratascript) with the appropriate 10× buffer, 4 mm pd(N)6 (random hexanucleotide primers provided by Pharmacia, Piscataway, NJ), 0.5 mm dATP, dGTP, and dTTP, 12 units of RNase Inhibitor (Promega, Madison, WI), and 5 µl of [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci/mmol, Amersham). After ethanol precipitation, the reaction product was used as a probe on Southern filters carrying *SacI*-restricted cosmid DNA.

**Cloning and sequence analysis:** DNA fragments were subcloned into pBluescript KS+ vector (Stratagene, La Jolla, CA) as described in Sambrook *et al.* (1989) and sequenced using the T7 sequencing kit (Pharmacia).

**PCR amplification:** PCR experiments were performed according to the following procedure: 20–50 ng of DNA template was mixed with 50 pmol of each primer, 0.2 mm each dNTP, one unit of *Taq* DNA polymerase (Appligene), and the *Taq* polymerase buffer in a total volume of 25 µl. Forty amplification cycles were performed, each cycle consisting of 1 min at 92°, 3 min at the primer annealing temperature, and 1 min at 72°. PCR products were electrophoresed in a 1% agarose gel, and Southern transfer and hybridization were performed as in Sambrook *et al.* (1989). mtDNAs of plants corresponding to six sexual generations of the T and CMSII lines (1989–1995) were used as templates in the PCR experiments. Sequence of the oligonucleotides: O1-5' ACTAGTGAGAGGGCAAAAAT T3'; O2-5' AACAGCTATGTACATTTTTTTTTT3'; O3-5' ACC GCAGATGGTAGTCTGG3'; O4-5' CGTTAGCGGACCGACT TGAC3'; O5-5' ATTGAAAACGTCGAAGTAAG3'; O6-5' TGA GCAACTCAAGCG3'; O7-5' TATATAGGCTCGCTC3'.

## RESULTS

**Characterization of the CMSII mt deletion:** Previous results suggested that the reorganization of the CMSII mt genome was associated with a homologous recombination event named *rec1*, involving two *SacI* restriction fragments of 19 and 5.4 kb (Chétrit *et al.* 1992). The 19- and 5.4-kb *SacI* fragments were undetectable by Southern hybridization in the CMSII mtDNA, whereas a recombinant 11.8-kb *SacI* fragment appeared. The reciprocal recombinant fragment, 12.6 kb in size, was

detectable in neither the fertile nor the mutant line. In addition, six other *SacI* fragments were undetectable in CMSII. From these results, it was proposed that CMSII mtDNA carried a large deletion of about 50 kb, located between the two target sites of *rec1* (Chétrit *et al.* 1992). To confirm this model, we realized a partial physical map of the T mt genome by the overlapping cosmid method, using as initial probes the 5.4-kb *SacI* fragment and a 9-kb *SacI* fragment located near the 19-kb *SacI* fragment. The results of the walking experiments are presented in Figure 1A. Parental fragments of *rec1* were found to be located 60 kb apart, delimiting a region named  $\delta$  containing 15 *SacI* fragments. Several coding sequences are located between the *rec1* target sites. First, the  $\delta$  region contains the 12.3- and 2.9-kb *SacI* fragments previously found to carry the four exons of the *cis*-spliced *nad7* gene coding for the NAD7 subunit of complex I (Pla *et al.* 1995). Second, an *orf87-nad3-nad1/A* cluster is located in the 5.4-kb *SacI* fragment (Gutierrez *et al.* 1997a), the *rec1* target site being located within the *nad3-nad1/A* intergenic region (Figure 1A). Third, the 4.3- and 1.6-kb fragments showed homology with *rrn26* and with exon B of *nad1*.

To determine if the  $\delta$  region was deleted in CMSII, cosmids containing all fragments spanning this region were used as probes on T and mutant Southern blots (Figure 1B). The six fragments located between the 19-kb *SacI* fragment and the 5.1-kb *SacI* fragments in the T75 cosmid (0.6, 2.9, 12.3, 9, 6.8, and 4.8 kb; 36 kb total) were undetectable in CMSII. In contrast, the nine adjacent fragments located between the 5.1-kb and the 5.4-kb fragments (5.1, 1.7, 1.5, 2, 2.8, 4.3, 1.6, 1.4, and 3.5; 24 kb total) were visible in CMSII using T75, T162, and T158 cosmids as probes. Screening T libraries with the 1.7-kb (from T75) and the 1.6-kb (from T158) fragments as probes showed that these fragments are in fact present in the T genome (Figure 2) in several cosmid organizations: (1) the 1.6-kb probe detected two additional groups of overlapping cosmids also containing the 2- to 3.5-kb fragments (Figure 2A). A first group carried *nad3* closely associated to *rps12* as already described in Gutierrez *et al.* (1997a), while fragments of the other group showed homology with *coxII* and *atp6* (Figure 2B); (2) using the 1.7-kb probe, we found a single additional series of cosmids, containing a region homologous to *cob* (Figure 2C).

All three alignments were found in CMSII cosmid libraries (data not shown), which could explain presence of the corresponding fragments in CMSII mtDNA, even if the whole  $\delta$  region is deleted. Such a deletion can be explained by considering the fact that, in the progenitor mtDNA, the 102-bp *rec1* repeats (arrows in Figure 1A) are in direct orientation on a molecule called P. Recombination between these repeats would give rise to two recombinant subgenomic molecules, the C molecule carrying the 11.8-kb *SacI* recombinant fragment and the  $\Delta$  molecule carrying the 12.6-kb recombi-

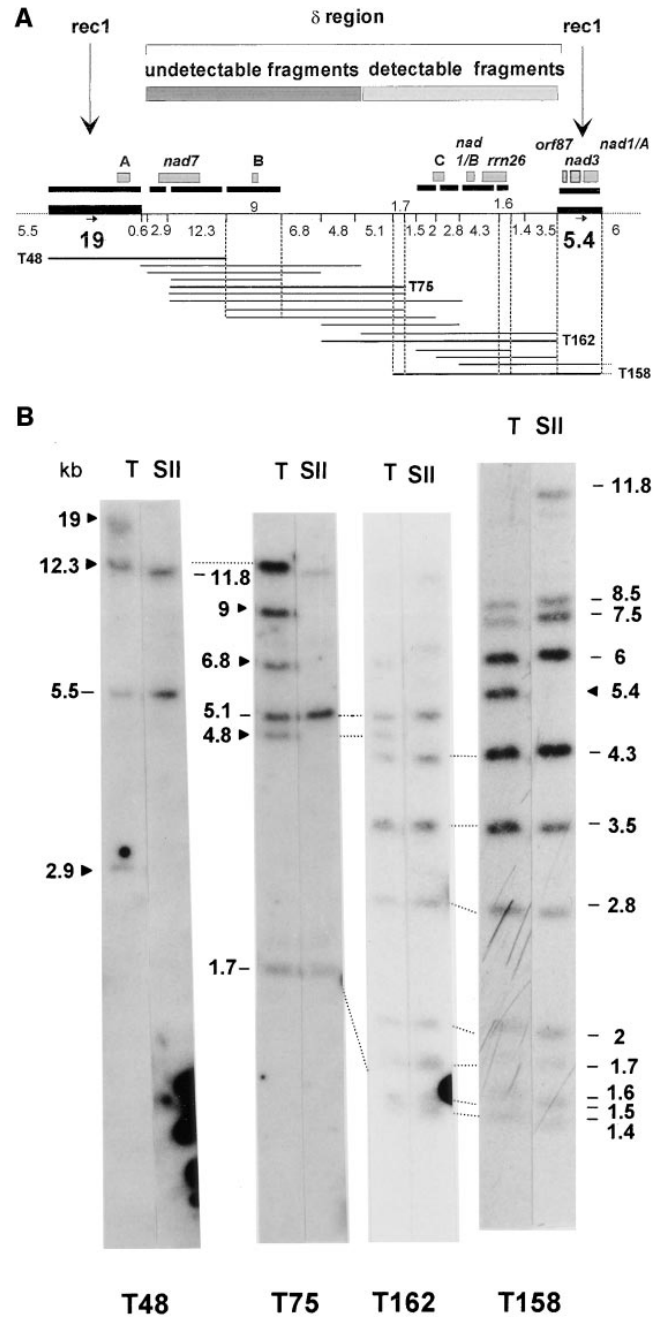


Figure 1.—Cosmid mapping of the  $\delta$  region located between the two parental fragments of *rec1*. (A) Partial map obtained after screening the fertile mtDNA cosmid libraries with the 5.4-, 9-, 1.7-, and 1.6-kb *SacI* fragments as probes. Horizontal arrows, 102-bp direct repeats involved in *rec1*; black boxes, potentially transcribed regions determined using a T cDNA probe; gray boxes, regions homologous to known mt genes; A, B, and C, *SacI* subfragments homologous to expressed regions of unknown function. (B) Hybridization of T48, T75, T162, and T158 cosmid DNA on fertile (T) and CMSII (SII) mtDNA *SacI* restriction patterns.

nant fragment (Figure 3). The progenitor T line would contain only the P molecule, while the CMSII would only contain the C molecule. The  $\Delta$  molecule, 72.6 kb in size and carrying the  $\delta$  region, would be absent in

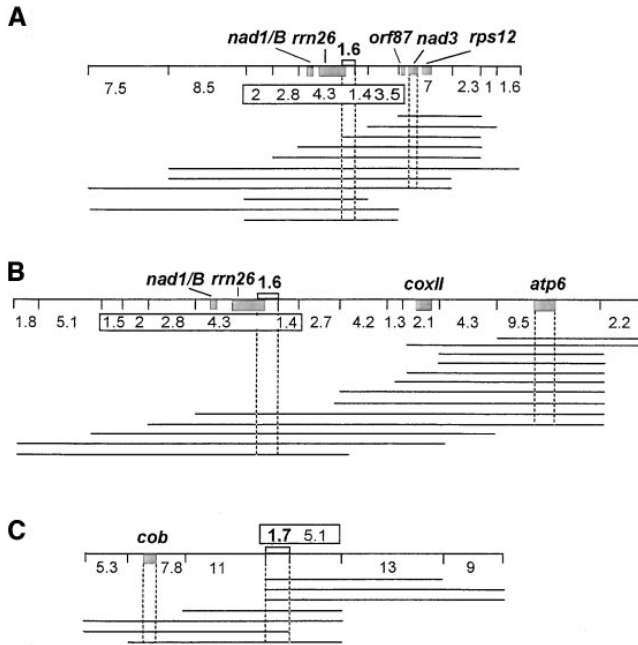


Figure 2.—Additional groups of overlapping T cosmids containing the 1.6- (A and B) and 1.7-kb (C) fragments (white boxes). Fragments of the  $\delta$  region detectable in the CMSII mtDNA (24 kb total) are boxed. Gray boxes represent known mt genes or exons located within these cosmid groups.

both lines. According to this model, in CMSII, the progenitor P genome has been replaced by the C subgenome lacking the  $\delta$  region. However, due to the presence of several repeated regions in the T mtDNA, the *nad7* gene only appears to be lost in CMSII.

#### Transcriptional analysis of the CMSII mt genome:

Results of Northern experiments are shown in Figure 4. As previously found in CMSI (P1a *et al.* 1995), *nad7* transcripts are undetectable even after overexposure in CMSII. In contrast, although the *orf87-nad3-nad1/A* cotranscripts present in the T line (Gutierrez *et al.* 1997a) are lost following deletion of the *orf87-nad3* region, these last sequences are still transcribed from the *orf87-nad3-rps12* cluster, and transcripts corresponding to *nad1/A*, which is not part of the deletion, remain present (data not shown). Similarly, as expected from their repetition in T mtDNA regions maintained in CMSII, *rrn26* and exon B of *nad1* gave the same patterns in both T and CMSII. We checked for other expressed sequences in the region deleted in CMSII. Southern hybridizations using a cDNA probe corresponding to total T mtRNAs revealed that in addition to the above fragments, the 2.8-, 2-, 9-, and 19-kb *Sad* fragments contained transcribed regions (data not shown). The adjacent fragments of 2 and 2.8 kb gave the same major signals when used as probes on T transcripts, suggesting that both fragments are overlapped by a single unidentified ORF called C (Figure 1A), the sequence of which was not determined. As expected from the presence of these two fragments in CMSII (Figure 2), no transcription changes as compared to T could be detected using

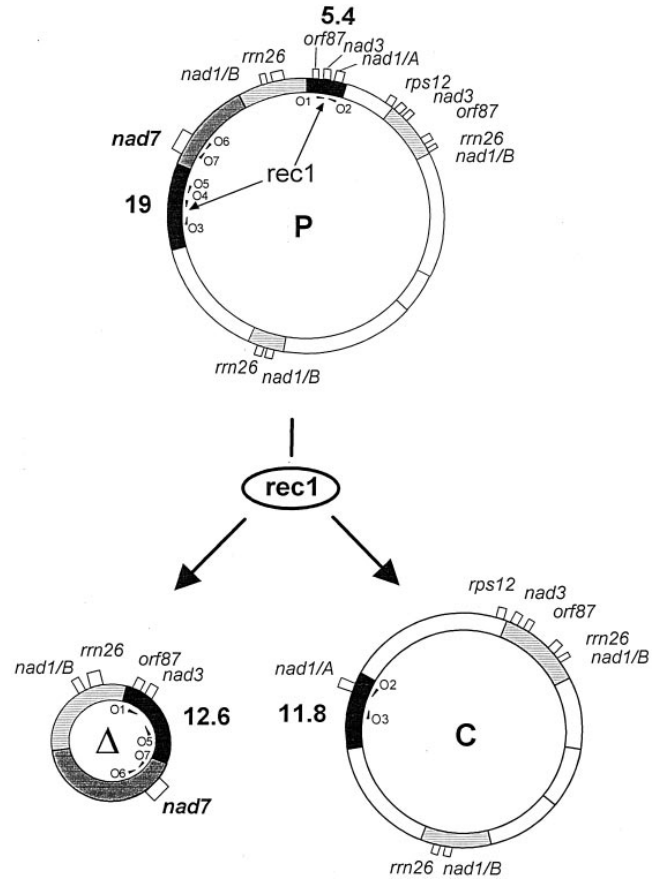


Figure 3.—Model of the *rec1* recombination. Two subgenomic molecules, C and D, are generated through the *rec1* recombination between repeats in direct orientation (horizontal arrows in Figure 1A) in the P molecule. The black regions are the *Sad* fragments involved in *rec1*: the parental 19- and 5.4-kb fragments in P and the recombinant 11.8- and 12.6-kb *Sad* fragments in C and D, respectively. The T mt genome contains only the P molecule, while the CMSII mt genome contains only the C molecule, in which the  $\delta$  region located between the 19- and 5.4-kb *Sad* parental fragments is lacking. Gray areas, *Sad* fragments of the  $\delta$  region present in unique copy in the P molecule. Striped areas, repeated fragments. White boxes, known genes carried by these fragments. Arrowheads, location of primers designed for amplification of *rec1* parental and recombinant fragments in PCR experiments (see Figure 6).

them as probes (Figure 4). The transcribed regions carried by the 19- and 9-kb fragments were located in two subfragments called A (2-kb *ApaI/Clal* subfragment) and B (1.8-kb *BamHI/HindIII* subfragment), respectively. The genomic sequences (GenBank accession numbers 72588 and 72609) are interrupted by several stop codons and show no significant similarity with any known mt genes or ORFs. Although the 19- and 9-kb *Sad* fragments are undetectable in the mutant mtDNA, the complex transcription patterns obtained using subfragments A and B as probes did not significantly differ between T and CMSII (Figure 4), suggesting total or partial duplications of the A and B regions in T and their presence in CMSII. This hypothesis was further

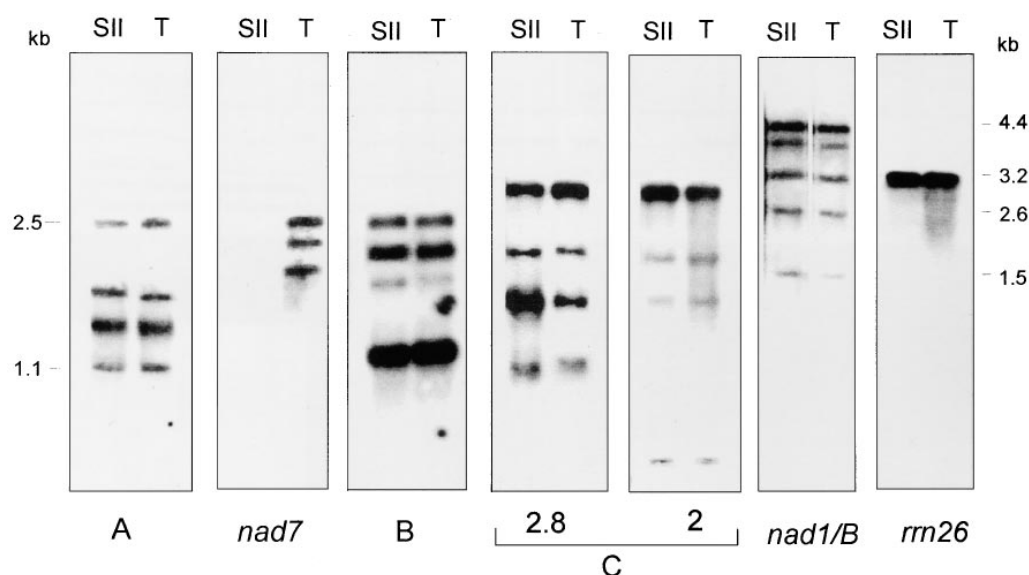


Figure 4.—Transcription analysis of the  $\delta$  region: Comparison between T and CMSII Northern profiles using mt genes and ORFs found in the  $\delta$  region as probes: *nad7*, *rm26*, *nad1/B*, and A, B, and C subregions.

supported by the use of A and B as probes on T Southern blots. Two or three signals were revealed with all three restriction enzymes used, the stronger signals corresponding to the 19- and 9-kb *SacI* fragments (Figure 5). In conclusion, transcriptional changes corresponding only to *nad* sequences could be shown in CMSII.

**PCR analysis of parental and recombinant fragments of rec1:** The origin of the mt genome reorganization in CMSII was investigated. Two main possibilities were considered: either the rec1 event was induced by the *in vitro* culture or it preexisted in the parent line, but at a very low level as the recombinant fragments were not detectable by Southern hybridization. Reciprocally, we wondered whether the parental fragments of rec1 could

be present at a substoichiometric level in CMSII. In order to answer these questions, PCR experiments were performed with the two lines using primers specific to parental and recombinant fragments (see materials and methods; Figures 3 and 6). The stability of both T and CMSII mtDNA organizations was tested over six sexual generations, with several plants per generation. Results from three of the generations tested are presented in Figure 6.

In all T mtDNAs analyzed, regions specific to 11.8- and 12.6-kb recombinant fragments were amplified by PCR (Figure 6B). Reciprocally, regions specific to both parental fragments and the 12.6-kb recombinant fragment were found in CMSII whatever the generation tested (Figure 6, A and B). No amplification products were obtained in any control experiments. As PCR reactions are not quantitative in our conditions, the variation observed in the different amplifications was not representative of differences between samples, and no changes through sexual generations could be demonstrated in either cytotype. To check for the presence of the *nad7* gene at a substoichiometric level in CMSII, additional PCR experiments were carried out using primers designed for the 12.3-kb *SacI* fragment (Figure 6C). A PCR product of the expected size was obtained, strongly suggesting that the *nad7* gene is maintained at a low level in CMSII, as was previously found for CMSI (P1a *et al.* 1995). This was confirmed by RT-PCR experiments, revealing the presence of edited *nad7* mature transcripts in either CMS (P1a *et al.* 1995; P. Chétrit and S. Gutierrez, unpublished results).

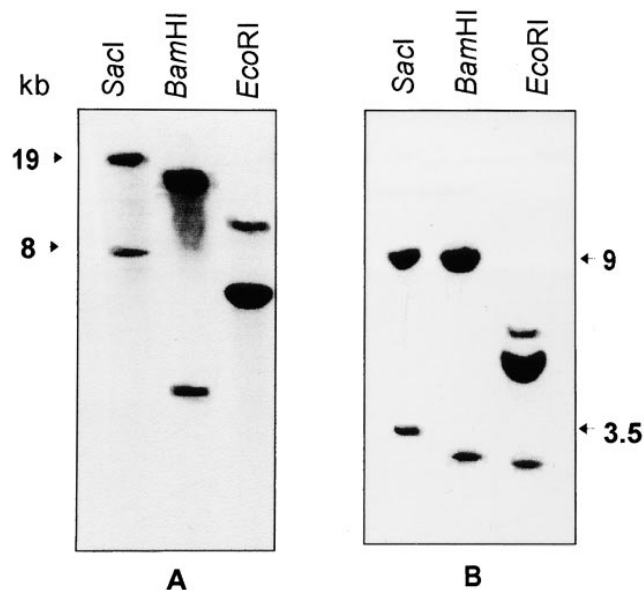


Figure 5.—Southern hybridizations of A (2-kb *ApaI/ClaI* subfragment) and B (1.8-kb *BamHI/HindIII* subfragment) on T mtDNA digested by *SacI*, *BamHI*, and *EcoRI* restriction enzymes.

## DISCUSSION

The aim of this work was to characterize the alterations in structure and expression of the CMSII mt genome. The results presented here allow us to propose a model for the transition from T to CMSII mtDNA

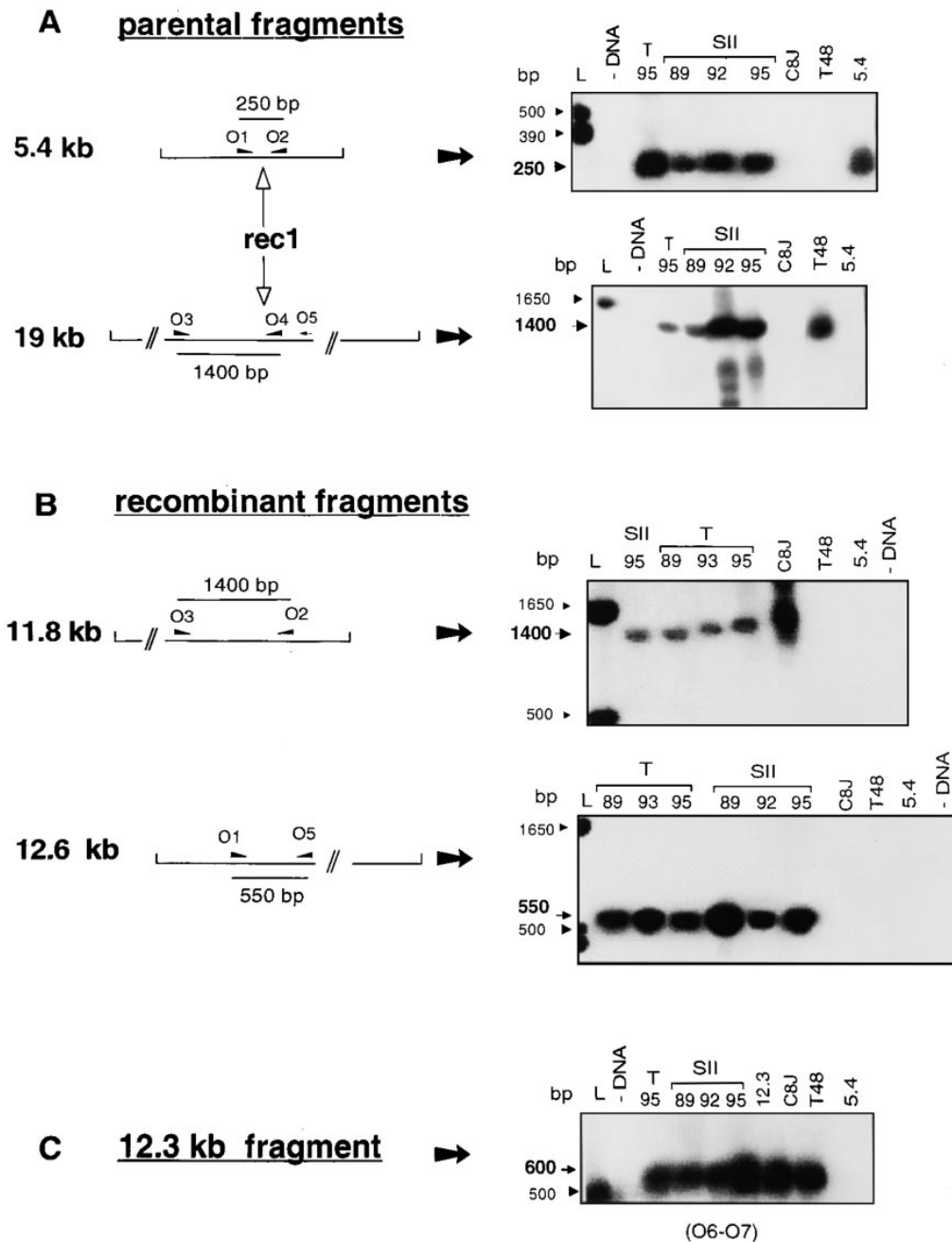


Figure 6.—PCR amplification of parental and recombinant fragments in T and CMSII mtDNAs. PCR experiments were performed on different templates using primers specific to (A) the 5.4- and 19-kb *rec1* parental fragments (primers O1–O2 and primers O3–O4, respectively), to (B) the 11.8- and 12.6-kb recombinant fragments (primers O2–O3 and primers O1–O5, respectively), and to (C) the 12.3-kb fragment undetectable by hybridization in the CMSII line (primers O6–O7). Primers are also located on the map in Figure 3. The blotted amplification products were hybridized with the corresponding restriction fragment as a probe, except for the 12.6-kb recombinant fragment for which the 5.4-kb parental fragment was used as probe. Schematic position of the primers in the restriction fragments and expected sizes of PCR products are indicated. PCR experiments were carried out using mtDNAs of several generations of the T (years 1989, 1993, and 1995) and CMSII lines (years 1989, 1992, and 1995) as templates. –DNA, negative control performed without DNA template in the reaction. Negative and positive controls were also carried out using the parental and/or recombinant fragments as templates: the 5.4-kb parental fragment (5.4), the T48 cosmid containing the 19-kb fragment (T48), the CMSII C8J cosmid carrying the 11.8-kb fragment (C8J, Chétrit *et al.* 1992), and the 12.3-kb fragment (12.3). L, 1-kb Ladder (GIBCO BRL, Gaithersburg, MD).

organization, involving *rec1* as a unique recombination event and resulting in expression changes of only *nad* genes.

**Structural organization of the CMSII mt genome: a subgenomic molecule has emerged as the new “master” molecule in the mutant:** Cosmid mapping showed that the two parental *Sad* fragments involved in *rec1*, 19 and

5.4 kb in size, respectively, are physically linked and separated by a 60-kb region called  $\delta$  (Figure 1). The region  $\delta$  contains two types of *Sad* fragments: some are detectable in CMSII (24 kb total) and others are not (36 kb total). We showed that the fragments still found in CMSII are in fact present in at least two copies in T mtDNA. These results strongly suggest that the whole

$\delta$  region is deleted in the mutant mtDNA. This deletion can be explained by considering the fact that in the progenitor mtDNA, the *rec1* repeats are in direct orientation in a molecule called P (Figures 1 and 3). Recombination between these repeats would give rise to two recombinant subgenomic molecules called C and  $\Delta$ . According to this model, in CMSII, the progenitor P genome would be replaced by the C subgenome in which the  $\delta$  region is lacking.

Whether the whole mt genetic information of *N. sylvestris* is carried by the P molecule or by a putative multipartite structure generated by recombining repeats is not known. Such uncertainty is typical for all plant mt genomes, even when they have been fully mapped. For instance, Yamato *et al.* (1992) suggested that the rice mt genome may be represented by two circular molecules instead of a master circle carrying a large duplication, and in *Petunia*, it was proposed that *atp9* and *pcf*, the chimeric gene associated with sterility, could be carried by two different molecules (Folkerts and Hanson 1991). In *N. sylvestris*, three long repeats, sharing 12 kb in common and containing sequence homology to *rrn26* and *nad1/B*, are present, but no recombinant borders were found. Short repeats involved in the emergence of new mtDNA organizations after protoplast culture were found to be located close to the *rec1* target sites: (1) a 404-bp repeat involved in the *rec3* recombination was found in the so-called U organization (Vitart *et al.* 1992) at  $\sim$ 1 kb from *rec1* in the 19-kb *Sad* fragment; (2) a 65-bp repeat involved in the *rec2* event specific for the CMSI organization (Pl a *et al.* 1995) is carried by the 12.3-kb *Sad* fragment. A short motif of 6 bp (TGCAAG) is shared by the three repeats, but whether this sequence could be the target site for recombination is not known. A 6-bp motif has also been suggested to be involved in homologous recombination in maize (Newton *et al.* 1990). Whatever the case, it appears that in *N. sylvestris* several sets of short repeats exist that are potentially able to produce new mtDNA organizations after *in vitro* culture.

**Origin and stability of the CMSII mt genome:** Southern and cosmid analyses suggested that the  $\delta$  region is completely absent in CMSII mtDNA. However, PCR experiments revealed the presence of *rec1* parental fragments in the mutant and both recombinant fragments in the T line. Although PCR amplifications are sometimes artefactual as recombinant fragments may be produced *in vitro* ("jumping PCR"), the fact that *rec1* parental and recombinant fragments were systematically found in different experiments strongly favors the hypothesis that they are also present *in vivo*. Presence of the  $\delta$  region at the substoichiometric level in CMSII could also be deduced from two other experiments: (1) a PCR product of the expected size was obtained using primers designed for the 12.3-kb *Sad* fragment carrying the *nad7* gene, and (2) RT-PCR experiments revealed the presence of edited mature *nad7* transcripts in either

CMS mutant. However, such transcripts are at levels too low to produce detectable amounts of the NAD7 polypeptide (Pl a *et al.* 1995; P. Chétrit and S. Gutierrez, unpublished results).

Taken together, these results strongly suggest that all parental and recombinant molecules involved in *rec1* are present in T and CMSII, the two genomes differing only in the proportions of these molecules. Small *et al.* (1987) originally proposed that stoichiometric variations could be a mechanism for plant mt genome evolution. This has since been confirmed by experimental situations generally also associated with *in vitro* culture (Shirzadegan *et al.* 1991; Hanson and Folkerts 1992; Hartmann *et al.* 1994; Kanazawa *et al.* 1994). However, to our knowledge, a symmetrical situation as described here, where both parental and recombinant fragments are present in wild-type and mutant plants, has not been reported yet, except in the *N. sylvestris*U mt organization generated by *rec3*, where substoichiometric fragments are detectable by Southern hybridization (Vitart *et al.* 1992). In the other cases described to date, only one of the recombinant fragments could be found, even when PCR experiments were performed (Hartmann *et al.* 1994; Kanazawa *et al.* 1994).

The question remains as to how such sudden changes in molecule stoichiometry may occur during culture and be maintained in the regenerated plants. This may result from either higher recombination rates or preferential replication of certain subgenomic molecules, or both mechanisms. It is likely that the P molecules are not yet the major replicating form in CMSII, as they are present in less than 1% of the normal stoichiometry, as judged by reconstruction experiments (data not shown). Differences in rates of replication of mt molecules have been suggested to account for the reversion to fertility in CMS bean (Janska and Mackenzie 1993). In this species, loss or maintenance of the *pvs* sequence associated with CMS is under the control of the *Rf* gene (He *et al.* 1995), and in *A. thaliana*, a mutation at the *CHM* locus promotes mtDNA reorganizations (Martinez-Zapater *et al.* 1992). A still unidentified nuclear mutation could account for mt changes in the CMSII protoplast-derived plant (a particularly high nuclear mutation rate was observed in protoplast-derived plants. Prat 1983; De Paepe *et al.* 1990), but not for the maintenance of the mutant organization during sexual reproduction. Indeed, CMS plants were backcrossed with the T line as male, and after six generations they should possess a near isogenic wild-type nuclear genome. A possible alternative would be the presence of an unidentified mtDNA mutation, either preventing efficient replication of the P molecule or favoring that of the C subgenome. Whatever the case, the new equilibrium has been maintained through sexual reproduction. No phenotypic reversion was observed among the hundreds of CMSII plants examined, none of them presented heterogeneous leaf sectors or produced fertile flowers.

Several dozen of these plants (on average a dozen per generation) were chosen at random for Southern analysis: the parental fragments were never detected in any experiment, whatever the tissue analyzed (leaf, bud, or anthers) or the stage of plant development, from seedlings to flowering plants. In addition, several plants from each generation were analyzed by PCR; regions specific to parental fragments were amplified in all samples analyzed. Thus, as substoichiometric fragments never disappeared (PCR experiments) nor became detectable by Southern hybridization, they must be maintained in the same average range, less than 1% of the normal stoichiometry as judged by reconstruction experiments (data not shown). Given that some plant cells contain only a few hundred mt genomes (Lamppa and Bendich 1984), one would expect substoichiometric molecules to be rapidly lost from some cells after a few divisions, assuming random segregation of mtDNA between daughter cells. Female gametes derived from these fully homoplasmic cells would give rise to offspring free of the substoichiometric molecules, which was never observed, in contrast with the segregation observed in maize NCS plants (Newton *et al.* 1989). These observations strongly suggest that substoichiometric molecules are continuously produced by recombination (and then the recombinants greatly outnumber parentals in mutant CMSII).

**The only unique gene that is deleted in CMSII mtDNA is *nad7*:** Results presented here show that the  $\delta$  region deleted in CMSII contains the whole *cis*-spliced *nad7* gene (P1a *et al.* 1995) and the *orf87-nad3* part of an *orf87-nad3-nad1/A* cotranscription unit carried by the 5.4-kb *Sad* fragment (Gutierrez *et al.* 1997a). As expected, *nad7* transcripts and the *orf87-nad3-nad1/A* cotranscripts are lacking, while a single signal likely to correspond to *nad1/A* alone remains present (Chétrit *et al.* 1992; P. Chétrit and S. Gutierrez, unpublished results). All the other genes and putative ORFs, namely *orf87-nad3*, *rnn26*, and *nad1/B* exon present in the  $\delta$  region are duplicated in T mtDNA regions maintained in CMSII, from which they are still expressed. Repetition of *rnn26* is typical in higher plant mtDNA (Falconet *et al.* 1985), while other duplications, such as that of *nad3* and *nad1/B*, seem to be specific to *Nicotiana* (Gutierrez *et al.* 1997a). No other mt transcriptional changes have been found in CMSII using the mt probes listed in materials and methods (results not shown). It is also unlikely that the deletion contains unique tRNAs, because CMS and T *in organello* mt synthesis patterns only present a limited number of differences, while absence of any tRNA would dramatically disturb translation. (Li *et al.* 1988; De Paepe *et al.* 1990).

All the available evidence thus supports the hypothesis that the only unique gene that is deleted in CMSII is *nad7*. This is also likely to be the case for the CMSI mutant, of which the 15-kb deletion is part of the  $\delta$  region (Chétrit *et al.* 1992) and contains the last two

exons of *nad7* (P1a *et al.* 1995). However, lack of NAD9 and of the 38-kD nuclear-encoded subunit of complex I was observed in both CMS, probably due to a misassembly of the complex (Gutierrez *et al.* 1997b), and recent results suggested that the NAD1 polypeptide is also lacking (P. Chétrit and S. Gutierrez, unpublished results). In plants, as in other organisms, complex I is composed of more than 30 subunits, most of them nuclear encoded, and we do not know what subunits actually remain in either CMS mutant. It is possible that post-transcriptional and/or post-translational changes affecting other gene products, either nuclear or mitochondrial, may occur as a secondary effect of the initial mt mutation.

Whatever the exact composition of complex I in CMS mutants, its activity is dramatically decreased, if not completely absent, and this is likely to be the basis for the abnormal phenotype (Gutierrez *et al.* 1997b). Similar situations have been documented in fungi, where absence of the 51-kD subunit, the NADH-binding subunit (Fecke *et al.* 1994), or of the 21-kD subunit involved in complex I assembly (Nehls *et al.* 1992; Vieira da Silva *et al.* 1996), are compatible with cell survival, although associated with respiration defects and reduced growth. Complex I mt mutations are also well documented in humans, in which they are often associated with degenerative diseases (see reviews in Wallace 1993, 1994). Such mutations are usually heteroplasmic, the severity of the defect being correlated with the proportion of mutant mt genomes as compared to normal ones. However, some LHON (Leber's hereditary optic neuropathy) mutations approach the homoplasmic stage. The *N. sylvestris* CMS are the first near homoplasmic complex I mutants to be described in higher plants. The only other plant complex I mutants are the NCS mitochondrial mutants of maize, which are thought to survive only thanks to their heteroplasmic state (Newton *et al.* 1989). In CMSII as in CMSI, it is possible that very low amounts of normal complex I are formed, due to the presence of subliminal P molecules. Accordingly, presence of *nad7* transcripts has been evidenced by RT-PCR in CMSI (P1a *et al.* 1995). However, in our opinion, CMS survival could better be accounted for by the increased activity of the other NADH-dehydrogenase activities existing in plants (Gutierrez *et al.* 1997b). Such alternative respiration routes also exist in fungi and are probably involved in the survival of the *Neurospora* complex I mutants.

In summary, the deletion carried by the CMSII mtDNA, one of the largest described in higher plants in terms of genetic information, involves one single recombination event, in contrast to most other mt variants that often reveal complex and multiple rearrangements (Hanson and Folkerts 1992). Both *N. sylvestris* CMSI and CMSII mutants can be classified as loss-of-function mutants, in contrast to most other CMS that are thought to be gain-of-function mutants. Their



survival seems to be assured thanks to two properties shared by other higher plant species: at the molecular level, redundancy of mt genetic information, and at the physiological level, presence of alternative respiration routes able to compensate, at least partially, for defects in the main pathway. However, *N. sylvestris* CMS plants present severe developmental defects, namely those concerning reproductive characters, and it is very unlikely that such mutants could survive in natural conditions. Selection of unfavorable new equilibria of mt molecules following *in vitro* cultures remains a mystery, showing that we are far from even a limited understanding of how mt genomes are maintained or evolve, in coordination (or conflict) with their nuclear partners.

We thank P. H. Gouyon, I. Small, C. White, C. Small-Colas des Francs, B. Lejeune, and J. W. Tregear for helpful discussions and comments.

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Communicating editor: K. J. Newton