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Lack of mitochondrial and nuclear-encoded subunits of complex I and alteration of the respiratory chain in *Nicotiana sylvestris* mitochondrial deletion mutants

(cytoplasmic male sterility/mitochondria/NADH:ubiquinone oxidoreductase/alternative oxidase/38-kDa subunit)

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ABSTRACT **We previously have shown that *Nicotiana sylvestris* cytoplasmic male sterile (CMS) mutants I and II present large mtDNA deletions and that the NAD7 subunit of complex I (the main dehydrogenase of the mitochondrial respiratory chain) is absent in CMS I. Here, we show that, despite a large difference in size in the mtDNA deletion, CMS I and II display similar alterations. Both have an impaired development from germination to flowering, with partial male sterility that becomes complete under low light. Besides NAD7, two other complex I subunits are missing (NAD9 and the nucleus-encoded, 38-kDa subunit), identified on two-dimensional patterns of mitochondrial proteins. Mitochondria isolated from CMS leaves showed altered respiration. Although their succinate oxidation through complex II was close to that of the wild type, oxidation of glycine, a priority substrate of plant mitochondria, was significantly reduced. The remaining activity was much less sensitive to rotenone, indicating the breakdown of Complex I activity. Oxidation of exogenous NADH (coupled to proton gradient generation and partly sensitive to rotenone) was strongly increased. These results suggest respiratory compensation mechanisms involving additional NADH dehydrogenases to complex I. Finally, the capacity of the cyanide-resistant alternative oxidase pathway was enhanced in CMS, and higher amounts of enzyme were evidenced by immunodetection.**

The genome of mitochondria encodes only a small number of their proteins (1). The mtDNA-coded polypeptides include subunits of complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3), the "first" proton-translocating, and therefore energy-transducing, complex of the respiratory chain. Complex I consists of 41 subunits in mammals (2) and more than 30 in *Neurospora crassa* (3), seven of which are the product of mitochondrial genes *nad1*, *2*, *3*, *4*, *4L*, *5*, and *6* (4). The mitochondrial genome in plants is larger than in other eukaryotes, and additional *nad9* (5) and *nad7* (6, 7) genes were identified recently. Several mutations of complex I mtDNA were characterized: in fungi (8), in humans, where they cause degenerative diseases (2), and in plants, namely nonchromosomal stripe (NCS) mutants of maize (9) and cytoplasmic male sterile (CMS) mutants I and II of *Nicotiana sylvestris* (10). CMS mtDNAs were generated by amplification/deletion processes involving two recombination events: rec1 in CMS I and II and rec2 in CMS I (11). The last two exons of *nad7* gene are deleted in CMS I (12), and the whole sequence is deleted in CMS II (Fig. 1). Lack of *nad7* expression was confirmed for CMS I by Northern and Western analyses (12) and could be related to the absence of a ≈40 kDa polypeptide in mitochondrial *in organello* protein synthesis (10)

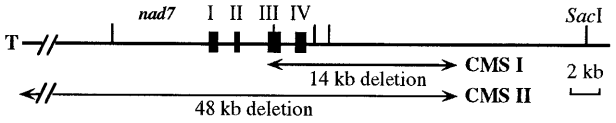

FIG. 1. Deletions in the *nad7* region of mtDNA in *N. sylvestris* CMS mutants (see text). CMS I lacks exons III and IV; CMS II lacks the entire gene.

We present here an integrated study of the developmental, molecular, and functional alterations associated with mitochondrial deletions in CMS I and II mutants of *N. sylvestris*.

MATERIALS AND METHODS

Plant Material. The *N. sylvestris* parental wild type (T) is a fertile botanical line of the Institut des Tabacs (SEITA, Bergerac, France). CMS I and II plants were derived from protoplast cultures and were maintained by backcrossing with T as the male. The plants were grown in a greenhouse under 16 h of fluorescent light at a day/night temperature of 24/17°C. For histology and fertility tests, plants were maintained in controlled chambers for 2 weeks, i.e., longer than the 10 days required for male gametogenesis; conditions were 9 or 16 h of lighting, each at 300 or 30 μmol of photosynthetically active radiation (PAR) photons $m^{-2} \cdot s^{-1}$. Cross-sections of anthers embedded in paraffin were stained with toluidine blue, and fresh pollen was stained with Alexander dye (13).

Western Blots and SDS/PAGE of Mitochondrial Proteins from Leaves or Pollen. For one-dimensional electrophoresis (14), a 10-μg protein was used. They were stained with 0.1% (mass/vol) Coomassie brilliant blue R-250 or were transferred for Western blotting (15) with antisera against *Neurospora crassa*, a 49-kDa subunit of complex I (16), wheat NAD9 (5) subunits of complex I, *Sauromatum guttatum* alternative oxidase (AOX) (17), or potato formate dehydrogenase (18)

Abbreviations: NCS, nonchromosomal stripe; AOX, alternative oxidase; CMS, cytoplasmic male sterility; PAR, incident photosynthetic active radiations (400–700 nm); T, wild parental type.

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applied at 10^{-3} dilution (10^{-2} for AOX). For two-dimensional SDS/PAGE, 100- or 10- to 20-μg proteins were used for Coomassie blue or silver nitrate (19) staining, respectively. All electrophoreses and Western blots were repeated at least five times.

Protein Microsequence Determination. Spots corresponding to the 38-kDa polypeptide were eluted from 40 dried Coomassie blue two-dimensional gels of fertile pollen mitochondrial proteins, rehydrated in distilled water, and submitted to N-terminal or internal sequence determination after digestion by endoprotease lysine C (14).

Purification of Leaf Mitochondria for Respiration Experiments. To obtain well coupled mitochondria, the standard protocol (20) was modified by raising the mannitol concentration to 0.6 M. All operations were carried out at 4°C. After grinding a few mature leaves, the filtered homogenate was centrifuged, and the crude mitochondrial pellet was purified on a self-forming 32% (vol/vol) Percoll gradient. Proteins were determined with BSA as standard (21, 22).

Respiration Measurements. Oxygen uptake at 25°C was measured with a Clark electrode (Rank Brothers, Cambridge, U.K.). The chamber contained 2–3 ml of the assay medium (0.6 M mannitol/20–30 mM KCl/5 mM $MgCl_2$/0.2 mM ATP/10 mM K phosphate, pH 7.2). A typical sequence of additions was: mitochondria (50- to 150-μg of protein ml^{-1} final); substrate (10 mM glycine, 10 mM succinate, or 1 mM NADH); 50–80 μM ADP; 2 μM carbonylcyanide *m*-chlorophenlyhydrazone or carbonylcyanide *p*-trifluoro-methoxyphenylhydrazone as uncoupler; 50 μM rotenone as complex I inhibitor; 0.2 mM KCN as cytochrome oxidase inhibitor; and 50 μM *n*-propylgallate or 1 mM salicylhydroxamic acid as AOX inhibitors. Minute injections were made to avoid dilution and solvent effects. About 2 min were allowed after each addition to reach steady state. The potential difference across the mitochondrial inner membrane [the major (23) component of the electrochemical proton gradient in plant mitochondria] was estimated with a tetraphenylphosphonium$^+$-sensitive electrode (24) inserted into the suspension.

RESULTS

CMS I and II Plants Present the Same Developmental Anomalies. CMS and T plants were compared throughout their life cycle (Table 1). Both mutants developed slowly (Fig. 2*A*), and, at the adult stage, their vegetative and floral organs were small. No seeds were produced during winter, neither spontaneously nor with manual pollination. Cross-sections indicated that, although the anther inner cell layer (tapetum) looked normal, only low amounts of degenerating microspores were present (Fig. 2 *B* and *C*). During summer, a few capsules were formed but with low seed mass. The proportion of viable pollen at anthesis varied between plants and flowers from a same plant but averaged 60% (Fig. 2 *H* and *I*).

Table 1. Phenotypical characteristics of the T line and CMS mutants of *N. sylvestris*

	T		CMS I and II	
Greenhouse conditions	Summer	Winter	Summer	Winter
Leaf length, cm	19		7	
Flowering time, days	102		165	
Stem height, cm	125		72	
Corolla length, mm	89		78	
Viable pollen, %	95	95	60	0
Capsule number, per plant	120	60	50	0
Capsule (free SP), mg	100	90	20	0
Capsule (manual SP), mg	110	95	60	0

Organ dimensions and flowering time correspond to plants (12 per genotype) grown in a statistical assay during spring and flowering during summer. Variance ratio *F* tests: CMS I and II values are not different at the 1% level and are pooled here; differences between T and CMS are highly significant. Leaf length: length of the largest leaf measured on 2-month-old plants at the rosette stage; SP, self-pollination.

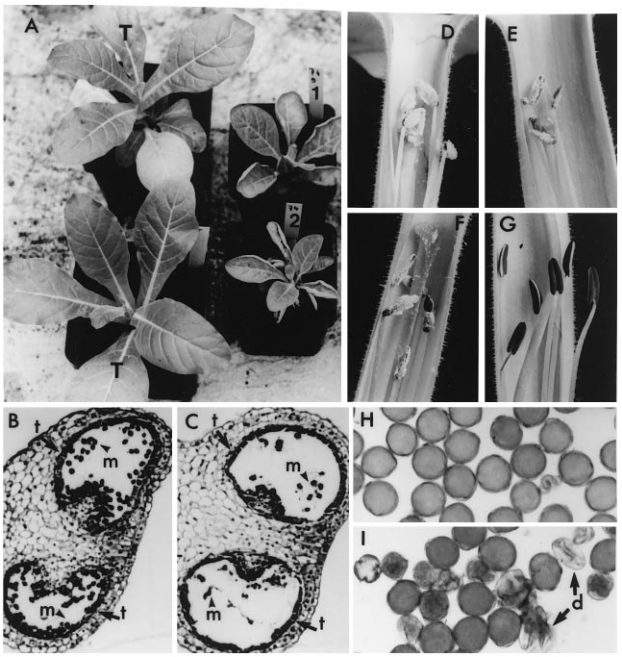

FIG. 2. Morphological abnormalities and conditional male sterility in CMS genotypes. (*A*) Two-month old plants grown in the greenhouse under 16 h of artificial light; 1, CMS I; 2, CMS II. (*B* and *C*) Cross-section of T (*B*) or CMS I (*C*) half-anther at the microspore stage, collected during winter in the greenhouse; t, tapetum; m, microspores. (*D–G*) Anthesis in T (*D* and *E*) and CMS II (*F* and *G*) flowers; plants were maintained for 3 weeks in controlled chambers with 16 h of light at 300 μmol of PAR photons $m^{-2}{\cdot}s^{-1}$ (*D* and *F*) or 9 h at 30 μmol of PAR photons $m^{-2}{\cdot}s^{-1}$ (*E* and *G*). (*H* and *I*) T (*H*) and CMS I (*I*) pollen collected at anthesis in summer and stained with Alexander dye; viable pollen is round and dark purple, dead pollen (d) is irregular and light green.

To better determine the environmental parameters of male sterility, assays were performed in controlled chambers (Fig. 2 *D–G*). CMS flowers shed pollen with both photoperiods at 300 μmol of PAR photons $m^{-2}{\cdot}s^{-1}$ but only with the 16-h photoperiod at 30 μmol of PAR photons $m^{-2}{\cdot}s^{-1}$. T plants were normally fertile in all conditions. Temperature had no effect over the range 17–30°C (not shown).

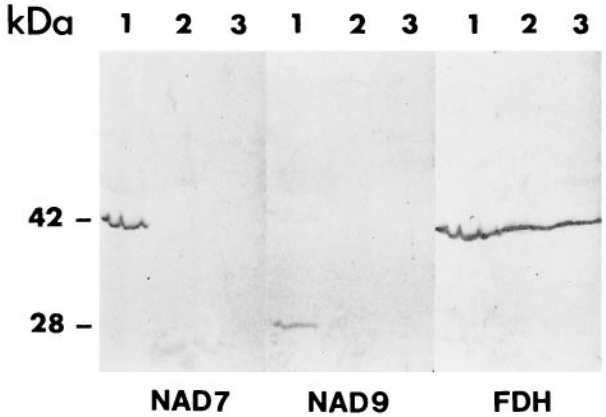

FIG. 3. Western blots of T and CMS mitochondrial proteins of complex I. Immunodetection was performed with antisera against the *N. crassa* 49-kDa subunit (NAD7) and the wheat NAD9. Potato formate dehydrogenase (FDH) served as the control. Lanes: 1, T; 2, CMS I; and 3, CMS II.

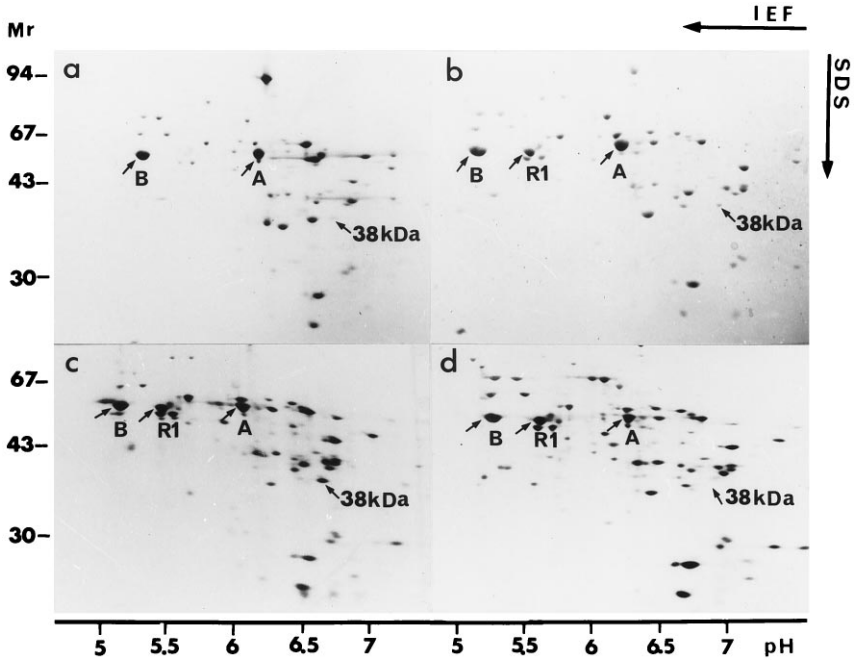

FIG. 4. Two-dimensional SDS/PAGE of mitochondrial proteins from fertile T and CMS plants. (*a* and *b*) Coomassie blue staining of T leaf (*a*) and T pollen (*b*). (*c* and *d*) Silver nitrate staining of T (*c*) and CMS I (*d*) mature pollen collected during summer in the greenhouse. A and B, α and β subunits, respectively, of mitochondrial ATPase. R1, additional β subunit specific to pollen (14).

CMS Mitochondria Are Deficient in NAD7, NAD9, and a Nucleus-Encoded, 38-kDa Subunit of Complex I. According to Western blots with antiserum against the *N. crassa* 49-kDa subunit, the equivalent *nad7* product (42 kDa) was missing in CMS I (12). We extended this observation to CMS II (Fig. 3). This result was expected from the deletion of all four *nad7* exons in this mutant. Surprisingly, the 28-kDa signal seen on T Western blots with wheat NAD9 antiserum was invisible on CMS blots (Fig. 3).

To look for other possible complex I defects, two-dimensional SDS/PAGE patterns of mitochondrial proteins from leaf and mature pollen were compared (Fig. 4). After silver staining, a 38-kDa spot, barely detectable with Coomassie blue (Fig. 4 *a* and *b*), was clearly visible on T patterns of leaf (not shown) and pollen (Fig. 4*c*), but it was missing in CMS I (Fig. 4*d*) and II (not shown).

The 38-kDa polypeptide was more abundant in pollen than in leaves (Fig. 4 *a* and *b*), allowing collection of sufficient amount for microsequencing. It showed significant homology with the 39- to 40-kDa complex I subunit, nucleus-encoded in all species thus far studied (25–28), especially with the related species *S. tuberosum* (Fig. 5). A nuclear location for the encoding gene in *N. sylvestris* could be inferred from two facts: (*i*) the N-terminal amino acid of mature protein is not a methionine, suggesting posttranslational processing, possibly involved in mitochondrial addressing; and (*ii*) the polypeptide does not correspond to a labeled spot on two-dimensional patterns of *in organello* synthesized T mitochondrial polypeptides (14).

N-terminal	1	10	20	24
38 kDa *N. s.*	A S N V A T S G G G H L A R K G X G G R V X V E			
38 kDa *S. t.*	A S N L A T G G A G P L I R K G T G G R S S V S			
39 kDa *V. f.*	V S T I A T - G V G H L V R T G			
40 kDa *N. c.*	I S D V T I T R T G K P I I R N Q G G R S S L G			
39 kDa *B. t.*	L H H A V I P N - G K - - - - - - G G R S S V S			
Internal	67 70	79 300	310	314
38 kDa *N. s.*	K L M G D L G Q I V P M K K D A L T F E D L G L A P H K			
40 kDa *N. c.*	K V T G D L G K V V M I E P E A K T F K D L G I E P A D			
39 kDa *B. t.*	R P M G D L G Q I I F M D P H L P G L E D L G V E A T P			

FIG. 5. Sequence homologies of the *N. sylvestris* 38-kDa polypeptide with complex I subunits of other species (25–28). *N. s.*, *Nicotiana sylvestris*; *S. t.*, *Solanum tuberosum*; *V. f.*, *Vicia faba*; *N. c.*, *Neurospora crassa*; *B. t.*, *Box taurus*. Numbering refers to *N. crassa* mature protein. X, not determined. Homologous regions are boxed.

In Isolated CMS Leaf Mitochondria, Oxygen Uptake with Glycine Is Dramatically Reduced, but Cyanide-Resistant Respiration Is Enhanced. The respiratory chain, with several alternative routes in plants (29–33), is schematized (Fig. 6). In addition to complex I (Fig. 6, [a]) inhibited by rotenone, at least three other NADH dehydrogenases have been described on inner (Fig. 6, [b]) and outer (Fig. 6, [c]) faces of internal membrane and on the outer face of external membrane (Fig. 6, [d]). Dehydrogenases channel electrons to ubiquinone, and [a]–[d] dehydrogenases potentially direct them to cytochrome oxidase. A second branching point occurs at ubiquinone, where electrons may pass either through the main cytochrome pathway or through AOX, which is insensitive to cyanide. Its involvement in the CMS trait has been suggested for various species (34–38).

Complex I activity was estimated by oxidation of glycine, a priority substrate in green tissues (39, 40). NADH, oxidized at high rates by the various dehydrogenases (29, 31–33), and succinate, oxidized by complex II, were also used. Inhibition by rotenone enabled evaluation of the contribution of complex I to glycine and NADH oxidation.

Fig. 7 shows how, after stabilization after substrate injection, a first addition of ADP induced a phosphorylating "state 3" that shifted to "state 4" when ATP hydrolysis balances synthesis. This is visualized by acceleration, then slowing down of respiration, which result from the fall and then rise of the

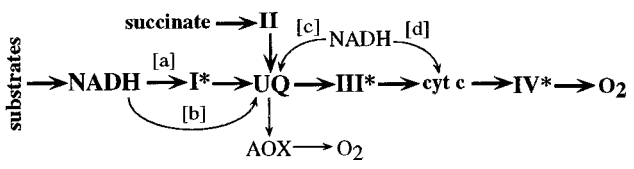

FIG. 6. Scheme of plant respiratory chain. Bold, main chain; I–IV, respiratory complexes; *, coupled proton translocation; UQ, ubiquinone; [a]–[d], NADH dehydrogenation pathways. Arrows symbolize electron transfer.

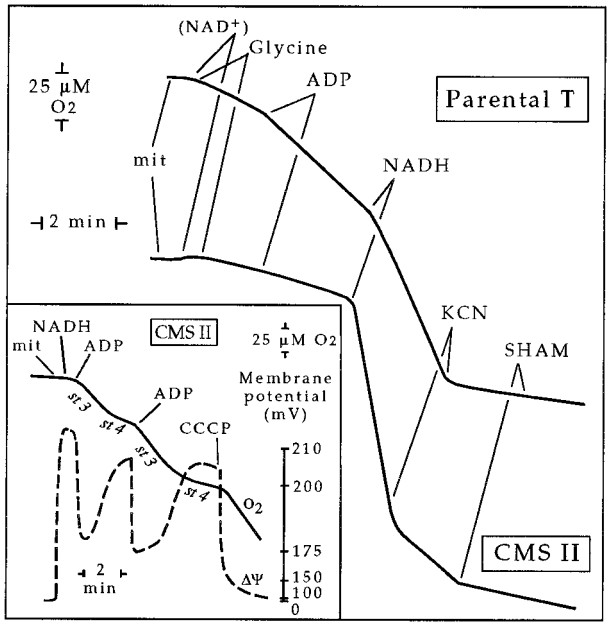

FIG. 7. Recorder traces of respiration measured with purified mitochondria from parental T and mutant CMS II leaves (same with CMS I). Mitochondrial proteins: 150 μg in 3 ml of the assay medium [4 mM glycine/500 μM ADP (main traces) or 75 then 120 μM (*Inset*)/0.7 mM NADH/0.2 mM KCN/1 mM salicylhydroxamic acid (SHAM)]. (*Inset*) O_2 uptake (solid line) and transmembrane potential $\Delta\Psi$ (dashed line, with tetraphenylphosphonium$^+$ electrode). st 3, state 3; st 4, state 4.

proton gradient (Fig. 7, *Inset*). When an uncoupler was added, the proton gradient was fully dissipated, and the chain functioned at its maximum rate. To avoid variable control of the redox chain by variable proton gradient, uncoupled rates are considered here.

Table 2 summarizes the results. Succinate respiration of CMS and T mitochondria were close to each other. In contrast, there was a dramatic (two–thirds) decrease in both mutants with glycine. In addition, although in T mitochondria almost half of glycine oxidation passed through complex I, judging from rotenone inhibition, the CMS residual activity was much less (≈20%) sensitive to it.

At variance with glycine, NADH respiration was 2- to 3-fold higher in mutants than in T; it was always well coupled (Fig. 7, *Inset*), and some inhibition by rotenone was observed, suggesting partial contribution of complex I. The high rates of NADH oxidation indicated that respiration with glycine or succinate was likely limited by the initial dehydrogenation step, not by the redox chain.

Surprisingly, the cyanide-resistant oxygen uptake, low (succinate) or undetectable (glycine, NADH) in isolated mitochondria of fertile plants, was significantly higher in mutants, with succinate (up to 35% of total respiration in CMS I) and even glycine (Table 2).

We evaluated AOX protein expression by immunodetection. Antibodies raised in *Sauromatum guttatum* (17) recognize AOX from other species, and the corresponding cDNA sequences has been cloned (41). In several nonthermogenic plants, a dimeric enzyme (36-kDa monomer) was associated with AOX activity. Recently, a multigene family for the AOX was characterized in soybean (42). In *N. sylvestris*, the weak 36-kDa signal seen on Western blots of T leaf mitochondrial proteins, using the AOX antiserum, was noticeably increased in both CMS (Fig. 8).

DISCUSSION

Until now, only two phenotypes have been associated with mtDNA reorganization in higher plants: (*i*) the widely distributed cytoplasmic male sterility trait that generally involves only male gametophytic development (43, 44), and (*ii*) the NCS variegated trait in maize (45). The *N. sylvestris* CMS I and II plants represent a new class of mitochondrial mutant.

CMS I and II Display Alterations in Vegetative and Reproductive Organs That Are Stably Transmitted Through Sexual and Somatic Generation. Some fertility could be restored by raising light, suggesting interactions between mitochondrial and chloroplast metabolism, which have been seen already for the maize NCS2 mutant (46). However, CMS I and II do not exhibit leaf variegation as NCS. This should result from the near homoplasmy of their genomes; indeed, the parental mtDNA fragments can only be detected by PCR amplification experiments (ref. 12 and unpublished work). No differences in fragment stoichiometry were found in leaf, bud, or pollen. On the contrary, NCS mutants are heteroplasmic; parental fragments can be detected by Southern hybridization, and plants give rise to both mutant and normal offsprings (9). In CMS, no phenotypic or molecular segregation was seen over eight sexual generations or after protoplast culture, suggesting that every CMS cell possesses a similar mtDNA organization. Heteroplasmy of NCS was proposed to account for their viability. In contrast, *N. sylvestris* CMS deletions, although associated with severe dysfunction, are compatible with plant survival.

CMS Mitochondria Are Deficient in Mitochondria and Nucleus-Encoded Complex I Subunits Other than NAD7. Even though the *nad9* gene was not deleted and its transcription was unchanged (unpublished work), no significant amount of NAD9 was detected by Western blotting (Fig. 3). Furthermore, the nucleus-encoded, 38-kDa subunit was undetectable by two-dimensional SDS/PAGE of CMS mitochondrial proteins. The analyzed CMS I and II plants contained a near isogenic T nuclear genome because they have been maintained by backcrossing with the parental line. Therefore, the absence, or reduced level, of two complex I subunits other than NAD7 results from some change in the mitochondrial genome. Two mechanisms may intervene: altered control of gene expression or defects in complex I assembly with rapid proteolysis of the nonintegrated subunits. Both situations have been found in lower eukaryotes. Down-regulation of nuclear genes by the mitochondrial genome has been described in yeast "petite" mitochondrial deletion mutants (47), and assembly of complex I is impaired in the *nuo21* disruption mutant of *N. crassa* (48). In this fungus, two assembly intermediates are formed: (*i*) a small, extrinsic subcomplex facing the matrix that consists of 13

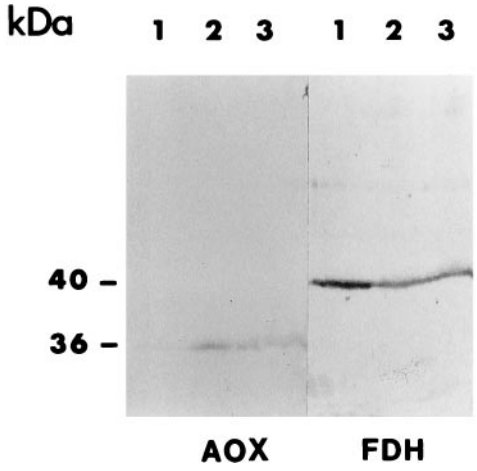

FIG. 8. Western blots of T and CMS AOX. Immunodetection with antisera against *S. guttatum* AOX and potato formate dehydrogenase (FDH) serving as control. Lanes: 1, T; 2, CMS I; 3, CMS II. The T lane was overloaded to detect the weak AOX signal (20 μg of protein instead of 10 μg).

nuclear-encoded subunits, including proteins of 49, 38 and 30 kDa; and (*ii*) a large, hydrophobic membraneous subcomplex that comprises all mitochondrial and the remaining nucleus-encoded subunits (16). An analogous structure was found in bovine heart mitochondria (2). In plants, the 49- and 30-kDa subunits are coded by mitochondrial genes *nad7* and *nad9* and, if the binary structure is as described above, *N. sylvestris* NAD7, NAD9, and 38-kDa subunits would belong to the extrinsic subcomplex. The purification of complex I from potato (25) and broad bean (26) mitochondria was recently reported, and its isolation from *N. sylvestris* T and CMS would help to clarify the role of missing subunits.

Complex I Activity Decreases While Functioning of Other NADH Dehydrogenases Increases in CMS. NAD7 is highly conserved among eukaryotes and prokaryotes, suggesting an important function (31). Thus, whatever the exact composition of complex I in CMS, its absence should be very deleterious. Indeed, glycine oxidation by purified CMS mitochondria dropped to less than one–third that of T controls (Table 2). A deficiency in glycine metabolism is unlikely because all implicated genes are nuclear-encoded, and, as we have stated already, CMS plants possess a near isogenic parental nuclear genome. A complex I dysfunction was also suggested in maize NCS2, deleted for *nad4–nad7* sequences (9). The respiration remaining in CMS mitochondria with glycine (Table 2) may have two origins. Either defective complex I still functions, albeit slowly, or it is relayed by additional NADH dehydrogenase(s). The slight rotenone inhibition of this residual activity suggests that defective complex I partially functions, but probably most electrons pass through additional dehydrogenases.

Unlike succinate oxidation through complex II, which is comparable to T control, oxidation of exogenous NADH by CMS mitochondria is significantly faster. Moreover, it generates an important proton gradient (Fig. 7, *Inset*), involving complexes III and IV (via sites [c] and [d], Fig. 6). This suggests a partial compensation of complex I deficiency for respiration and phosphorylation. An analogous situation was described for the *nuo51* mutant of *Aspergillus niger* that lacks functional complex I but maintains its respiration thanks to an alternative NADH pathway (49).

The mechanism of oxidation of cytosolic NADH by mitochondria in light is not well elucidated, and it has been proposed that, in some plant species, NADPH may be the source of redox equivalents (50). Consequently, a working hypothesis could be that the partial fertility of CMS mutants in normal light compared with low (Table 1; Fig. 2 *D-G*) is due to a sufficiently fast turnover of the photosynthetic chain to provide some extra ATP and NAD(P)H to cytosol and mitochondria, via the translocation of phosphorylated and reduced metabolites.

Table 2. Respiration activities of the T line and CMS mutants of *N. sylvestris*

	nmol O_2 min^{-1}·mg $prot.^{-1}$		
	T	CMS I	CMS II
Succinate 10 mM, *n*	9	8	9
No addition	218 ± 41	251 ± 80	299 ± 114
+KCN	18 ± 10	88 ± 58	69 ± 55
Glycine 10 mM, *n*	12	8	7
No addition	115 ± 58	37 ± 11	32 ± 18
+Rotenone	64 ± 24	30 ± 6	26 ± 9
+KCN	0 ± 0	4 ± 5	5 ± 4
NADH 1 mM, *n*	6	5	5
No addition	312 ± 101	986 ± 335	604 ± 266
+Rotenone	231 ± 90	682 ± 314	469 ± 305
+KCN	1 ± 1	11 ± 14	8 ± 13

Uncoupled rates (±SD for the shown number of independent mitochondrial preparations) are corrected for the background that may exist after inhibition of cytochrome and AOX with 200 μM KCN and 50 μM nPG, respectively. *F* tests of variance ratios applied to T and CMS lines (no significant differences between CMS I and CMS II) show: (*i*) uninhibited rates significantly different at ≤1% level (5% for CMS II with NADH), except not significant with succinate; (*ii*) rates with rotenone significantly different at ≤1% level (10% for CMS II with NADH); and (*iii*) rates with KCN significantly different at ≤2.5% level, except not significant with NADH.

The Capacity of the AOX Pathway Is Increased in CMS Mitochondria. In the T line, a significant cyanide-resistant respiration was found only with succinate. In CMS mutants, it became detectable with glycine and was strongly enhanced with succinate, where it could reach 30% of total respiration (Table 2). Succinate is a ready source of pyruvate, so this is in agreement with the observation that AOX is activated by pyruvate (51). The present measurements are minimal estimates of AOX capacity because the best values are reached only by combining pyruvate and dithiothreitol (52, 53). In our CMS mutants, the larger capacity of the AOX route was associated also with more enzyme (Fig. 8), showing that it was not only due to regulatory effect. A similar correlation between activity and quantity of AOX was reported in tobacco cell cultures (54). Nevertheless, it is not known whether AOX capacity *in vitro* correlates with its engagement *in vivo* or whether the AOX competes more effectively with the cytochrome chain in CMS than in T. Isotope discrimination of oxygen uptake by both pathways would be a promising approach to this problem (55).

At variance with complexes III and IV, the AOX does not translocate protons and therefore is energetically futile (53). The role of the alternative pathway in nonthermogenic tissues is enigmatical. One possible function is to maintain the Krebs cycle if the cytochrome chain is defective. Overexpression of AOX was found in *N. crassa* chloramphenicol-poisoned cells (16) and in mitochondrial mutants (56) but not in the *nuo21* complex I nuclear disruption mutant, in which the cytochrome pathway is unaffected (48). Unexpectedly, AOX activity is increased in *N. sylvestris* CMS although the majority of respiration still involves cytochrome oxidase (strong KCN effect; Table 2). However, in mitochondria from aged root slices of red beet, a correlation was noted (57) between enhanced AOX and NADH dehydrogenases activities, as in CMS (Table 2). It remains to be established if the increases in AOX capacity (Table 2) and protein content (Fig. 8) in CMS mutants have a physiological role. Our results clearly distinguish *N. sylvestris* mutants from other CMS mutants; the AOX pathway in the latter was unchanged or, at most, slightly inhibited when compared with fertile plants (34–38).

Conclusion. Although this cannot be strictly proven because of the absence of nuclear restorer genes, the sterility of *N. sylvestris* CMS I and II probably does not arise from the expression of abnormal chimeric mitochondrial genes, as in other CMS plants (44, 45), but rather from the deletion of coding sequences. Our recent analyses suggest that the only gene that is missing in these CMS mutants is *nad7*. Whatever the exact relationships between molecular and phenotypic alterations, male sterility in *N. sylvestris* CMS is clearly associated with respiratory dysfunction, a hypothesis long suggested (58) but scarcely supported by experimental data (59). This novel class of mutants should therefore be invaluable for studying the structure and functioning of complex I and the interactions between the different respiratory pathways in higher plants.

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