Targeting the NAD7 Subunit to Mitochondria Restores a Functional Complex I and a Wild Type Phenotype in the *Nicotiana sylvestris* CMS II Mutant Lacking *nad7*

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The mitochondrial DNA of the *Nicotiana sylvestris* CMSII mutant carries a 72-kb deletion comprising the single copy *nad7* gene that encodes the NAD7 subunit of the respiratory complex I (NADH-ubiquinone oxidoreductase). CMSII plants lack rotenone-sensitive complex I activity and are impaired in physiological and phenotypical traits. To check whether these changes directly result from the deletion of *nad7*, we constructed CMS transgenic plants (termed as CMSnad7) carrying an edited *nad7* cDNA fused to the CAMV35S promoter and to a mitochondrial targeting sequence. The *nad7* sequence was transcribed and translated and the NAD7 protein directed to mitochondria in CMSnad7 transgenic plants, which recovered both wild type morphology and growth features. Blue-native/SDS gel electrophoresis and enzymatic assays showed that, whereas fully assembled complex I was absent from CMSII mitochondria, a functional complex was present in CMSnad7 mitochondria. Furthermore, a supercomplex involving complex I and complex III was present in CMSnad7 as in the wild type. Taken together, these data demonstrate that lack of complex I in CMSII was indeed the direct consequence of the absence of *nad7*. Hence, NAD7 is a key element for complex assembly in plants. These results also show that allotypic expression from the nucleus can fully complement the lack of a mitochondrial-encoded complex I gene.

Complex I, which couples electron transfer from NADH to ubiquinone and proton translocation across the inner mitochondrial membrane (1–5), is a large multimolecular complex containing more than 40 subunits in mammals or plants (4–6). In all organisms investigated so far (mammals, fungi, bacteria), the assembly of these subunits determines a hydrophobic membrane domain and a matrix protruding peripheral arm (7). The reaction mechanism of this large complex is not well understood, but the peripheral arm is thought to bear all the FMN and iron-sulfur cluster cofactors (8). Bacteria possess in their cytoplasmic membranes a functionally similar complex composed of 14 subunits representing the catalytic core, all of which have homologues in eukaryotic complexes (9, 10). Although less information about complex I structure is available in plants than in mammals or fungi, (11, 12) it has been shown that, in addition to the 14 subunits of prokaryotic origin, a large number of *Arabidopsis* complex I subunits have counterparts in mammals (6).

In all eukaryotes, complex I is composed of subunits of both nuclear and mitochondrial ("nad") genes origin. However, the number of mitochondrial encoded complex I subunits varies among kingdoms. At least three *nad* genes, i.e. *nad1*, *nad4*, and *nad5*, are ubiquitously present in the mitochondrial DNA of eukaryotes with a functional respiratory chain involving complex I (13). Plant mitochondrial DNA encodes nine complex I subunits (14), which all have homologues in bacterial complex I. Of these, NAD7 and NAD9 are probably located in the matrix arm of the complex as are their homologues of the 49- and 30-kDa peptides of fungi or mammals, which are nuclear encoded (15, 16). The 49-kDa subunit (whose counterparts are named NuoD in bacteria, NAD7 in plants, NDUFS2 in humans), which belongs to the module connecting the NADH oxidizing moiety located in the peripheral arm to the membrane part, has been suggested to participate in ubiquinone reduction (16), although it is possibly not directly involved in the binding of an iron-sulfur cluster (17).

In humans, defects in components of the respiratory chain (which from complex I alterations) lead to a number of pathologies that mainly affect mitochondria-rich tissues and lead to encephalomyopathies, cardiomypathies, the Leber hereditary optic neuropathy, and other mitochondrial diseases (18–21). The severity of the symptoms is dependent on multiple factors with biochemical or metabolic thresholds (20).

Unlike animals, plants and most fungi have rotenone-insensitive enzymes called alternative NAD(P)H-ubiquinone oxidoreductases, which can oxidize NAD(P)H. In contrast to complex I (rotenone-sensitive NDH-1-type enzyme), these NDH-2-type enzymes do not catalyze coupled electron transport. Meanwhile, their functions are not yet well characterized (22–26). The mitochondrial membranes of the yeast Saccharomyces cerevisiae are devoid of complex I and only contain the alternative NDH-2 enzyme (27). The *S. cerevisiae* *NDH1* gene has been used successfully to transfect a complex I-deficient Chinese hamster cell line (28) and a human cell line (29), restoring respiration capacity. Therefore, plant and fungi mutants with a deficient complex I are potentially viable. Plants also contain a non-phosphorylating terminal oxidase (alternative oxidase) that has the capacity to transfer electrons from ubiquinol to oxygen, bypassing the proton translocating complexes, III and IV (30).

A complex I mutant has previously been described in the tobacco species *Nicotiana sylvestris*. This mutant, named CM-
SI1, exhibits reduced growth and abnormal morphological traits in leaves and flowers. We have shown that a recombination/amplification process resulted in a 72-kb deletion in the mutant mitochondrial genome (31, 32). A number of genes and orfs appeared to be deleted following this event or to exhibit rearranged promoter regions (33–35). Of the deleted sequences, only the nad7 gene is present as a single copy, whereas others such as nad3 or orf87 are duplicated in the mitochondrial genome. Further characterization of the CMSII complex I showed that, in addition to NAD7, other subunits, e.g. NAD9, NAD1, and the nuclear-encoded 23- and 38-kDa subunits were also missing (36, 37). As a result, CMSII plants are devoid of rotenone-sensitive respiration (complex I) but exhibit enhanced rotenone-insensitive NADH oxidation (NDH2-type dehydrogenases), resulting in increased global leaf respiration (38, 39).

Here, we examined the structure of complex I in CMSII plants to determine whether the observed defects were correlated only with the loss of nad7. For this purpose, we used a strategy consisting of allotypic expression of nad7, and targeting the gene product to CMSII mitochondria.

**EXPERIMENTAL PROCEDURES**

**Plant Material**—The fertile wild type (WT) N. sylvestris was provided by the Institut des Tabacs (Bergerac, France). The CMSII mutant was obtained from a spontaneous culture of N. sylvestris maintained in vitro with the wild type. For CMSII transformation, seeds were sterilized in 10% calcium hypochloride and germinated on agar supplemented with standard nutritive medium under a 16-h photoperiod at a day/night temperature of 23/17 °C.

**RNA Isolation and RT-PCR Amplification of the nad7 cDNA**—For total RNA isolation, leaf tissue pieces (100 mg) from young leaves were harvested in liquid nitrogen. RNAs were extracted by the TRIzol-chloroform procedure (Invitrogen). RT-PCR was performed as follows. Reverse transcription (RT) reaction was performed for 1 h at 42 °C in a mix containing 2 μg of total RNAs, 1 μl of random hexamer mix (100 μM; Invitrogen), 6 μl of 5× enzyme buffer, 2 μl of dNTP mix (10 mM), 2 μl of dithiothreitol (100 mM), 1 μl of RNasin (50 units), 2 μl of M-MLV reverse transcriptase (400 units; Invitrogen). Aliquots (5 μl) of the RT reaction were then used in standard PCR amplification (25 cycles) with the labeled PCR products (targeting sequence) were digested with NcoI.

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The abbreviations used are: WT, wild type; AOX, alternative oxidase; BiTris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxypropyl)propane-1,3-diol; BN, blue-native; MOPS, 3-(N-morpholino)propanesulfonic acid; Tes, 2-[2-hydroxy-1,1-bis(hydroxymethyl)amino]ethanesulfonic acid; TMBZ, tetramethyl-3,3',5,5'-benzidene; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)glycine; RT, reverse transcription; PVDF, polyvinylidene difluoride.
Potato formate dehydrogenase presequence

**Nicotiana sylvestris nad7** cDNA

| ATG | GCG | AGT | ACC | GCT | ATA | GCT | TAT | CAG | GCA | TCT | ACT | AGT | CTC | TGC | AGC |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 126/43 | 237/43 | 240/43 | 244/43 | 245/43 | 246/43 | 247/43 | 248/43 | 249/43 | 250/43 | 251/43 | 252/43 | 253/43 | 254/43 | 255/43 |

**FIG. 1.** Nucleotide sequence of the formate dehydrogenase presequence-nad7 construct. Nucleotide sequence and translation of the potato formate dehydrogenase mitochondrial presequence fused to the N. sylvestris nad7 cDNA (the edited sites are highlighted, leucine residues resulting from editing are in **bold**). The arrow indicates the cleavage site of the targeting sequence.

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Mass Spectrometry Analysis of Peptides—Spots collected from BN/SDS-PAGE were analyzed by peptide mass fingerprint of tryptic peptides in a matrix-assisted laser desorption ionization time-of-flight mass spectrometer (Voyager D E super STR; Applied Biosystems).

Enzymatic Assays—NADH or deamino-NADH-cytochrome c reductase activities were measured at 550 nm in an assay medium consisting of 0.3 M sucrose, 20 mM MOPS-KOH, pH 7.2, 2.5 mM MgCl₂, 0.5 mM EGTA, 0.002% bovine serum albumin (26), and 0.1 mM KCN. The assay was checked to take into account nonspecific variations of the absorption.

Kinetics were followed at 340 nm, and the evolution of the 400 nm absorption was periodically checked to take into account nonspecific variations of the absorption.

The molar absorption coefficient of NADH was 6.22 mM⁻¹ cm⁻¹.

Enzymatic Assays—NADH or deamino-NADH-dehydrogenase activities were measured in a mitochondrial-assisted laser desorption ionization time-of-flight mass spectrometer (Voyager D E super STR; Applied Biosystems).

RESULTS

Transformation of CMSII with an Edited nad7 cDNA, Plant Regeneration, and Expression of the Transgene in CMSnad7—Sequencing of the genomic region spanning nad7 in N. sylvestris showed that this gene consisted of four exons interrupted by three cis-spliced introns, resulting in a complex transcription pattern (33). Sequencing of the 1185-bp nad7 cDNA and comparison with the genomic sequence revealed the presence of 28 editing sites (Fig. 1). Fourteen codon changes resulted in leucine residues, of which eleven consisted of serine to leucine transitions. Differences between the protein sequence (Fig. 1, coordinates 38, 53, 213, 218, 228, 360) derived from nuclear and mitochondrial nad7 genes in several species (46) and in N. sylvestris showed that, following edition, the N. sylvestris nad7 mRNA sequence matched better to the liverwort, bovine, or Neurospera nuclear-encoded nad7 gene than with the N. sylvestris genomic sequence. This was in agreement with the hypothesis of Kobayashi et al. (46) that the nuclear nad7 genes in these species originated from mitochondria-encoded and -edited mRNA molecules.

A number of regenerating plantlets from transformed plants grown on kanamycin plates were transferred in a greenhouse. All plants exhibited a restored phenotype (Fig. 2) at flowering time as regards plant height, leaf and flower shape, and set seeds. They were fertile, although the 35S promoter is not expressed in pollen, suggesting that enough stable complex I assembled in mother pollen cells can function and allow fertile pollen production in mature anthers.

In the kanamycin-resistant plants, PCR experiments (Fig. 3A) showed the presence of the full-length cDNA using total DNAs as template (three are shown in Fig. 3, A–C). This was observed without segregation in the progeny of the primary transformants over five generations. To analyze their mitochondrial genome, we used a probe consisting of a cloned 5.25-kb SacI fragment present in the WT mitochondrial DNA but absent in CMSII (32). The probe was hybridized on Southern blots carrying control and transformed plant total DNAs restricted with SacI. Fig. 3B shows that regenerants A, B, and C shared a SacI recombinant fragment of 11.8 kb specific to the nucleus origin of the CMSII mitochondrial genome (Fig. 3B) whereas in the WT the transcribed pattern of the cis-spliced nad7 mitochondrial gene typically consisted of three transcripts 1.5, 1.7, and 2.3 kb in size (33).

To check for the presence of nad7 transcripts in the transgenic plants, blots carrying total RNAs were hybridized using the nad7 cDNA as a probe. As shown in Fig. 3C, all regenirant plants exhibited a 1.6-kb transcript corresponding to the transcription of the nad7 cDNA, whereas in the WT the transcription pattern of the cis-spliced nad7 mitochondrial gene was observed without segregation in the progeny of the primary transformants over five generations.

To determine whether the NAD7 polypeptide was produced from the nad7 cDNA sequence and was addressed to mitochondria, protein extracts from WT, CMSII, and CMSnad7 leaf mitochondria were analyzed by SDS-PAGE and probed with anti-NAD7 antibodies. As observed in Fig. 4A, no signal could be seen in the CMSII extract, whereas a cross-reacting product in the range of 43 kDa was present in both WT and CMSnad7 extracts, indicating that a mature nad7 polypeptide indeed accumulated in mitochondria of the transgenic line. Very com-
Assembly of Complex I in Transgenic CMSII Plants—To compare the composition of complex I in the WT, CMSII, and CMSnad7 lines, mitochondrial protein complexes were analyzed by BN-PAGE. The larger complexes, obtained after solubilization with dodecylmaltoside or digitonin, were separated on BN gels and their polypeptide composition further analyzed by SDS-PAGE. The polypeptide patterns obtained from dissociated complexes I, III, V, and from the two different migrating supercomplex, including complex I and complex III. The black circle indicates the position of complex I satellite band. D, immunodetection of NAD7 in the two-dimensional gels (see Fig. 6). Only the region of the immunological response in wild type (WT) or transgenic CMSII (T) gels is shown.

FIG. 4. Accumulation of NAD7 in mitochondrial membranes and in leaves of the wild type and the CMSII and CMSnad7 lines of N. sylvestris. A, mitochondria were purified from WT, CMSII, and CMSnad7 (Tr) leaves, and then membrane polypeptides (equivalent to 50 μg of mitochondrial proteins) were solubilized by SDS and resolved by SDS-PAGE. B, total extracts were prepared from leaf discs from the three lines of N. sylvestris, and then polypeptides (equivalent to 20-mm2 leaf area) were separated by SDS-PAGE. After electrophoresis, polypeptides were transferred on a PVDF membrane and probed with a serum raised against NAD7.
As observed in Fig. 5, A and B, a fully assembled complex I was completely absent from dodecylmaltoside-solubilized CMSII mitochondrial membranes. No signal was visible at either the expected complex I or satellite band locations (Fig. 5, A–C). Gel strips of the first dimension were analyzed by SDS-PAGE in the second dimension to resolve polypeptides (stained with silver nitrate) from WT (A, B), CMSII (C, D), and CMSnad7 (E, F); I, complex I; III, complex III; IV, complex IV (two forms); V, ATP synthetase; SC, supercomplex comprising complex I and complex III. White circles (left of complex I and the supercomplex) indicate the position of some complex I subunits. White stars (right of complex III or left of the supercomplex) indicate the position of some complex III subunits. The position of NAD7 was determined by immunodetection (see Fig. 5D) and mass spectrometry. The three spots analyzed by mass spectrometry are indicated by the three black arrows in panel E. The white arrow in panel C visualizes the position of complex I subunits in the WT.

As observed in Fig. 5, A and B, a fully assembled complex I was completely absent from dodecylmaltoside-solubilized CMSII mitochondrial membranes. No signal was visible at either the expected complex I or satellite band locations (Fig. 5, A–C). In the second dimension SDS gel, polypeptides were missing at the expected complex I position (Fig. 6C), and NAD7 was not detectable using the anti-NAD7 serum (Fig. 5D). The lower part of the BN gel gave no evidence of polypeptides that could be assigned to a partial complex I, strongly suggesting the total absence of complex I in CMSII. In contrast, complex I could be recovered from membranes of the CMSnad7 lines, following Coomassie blue staining (Fig. 5A), NADH and nitro blue tetrazolium staining (Fig. 5B), and cross-reaction with the anti-NAD7 serum (Fig. 5C). Second dimension polypeptide analysis (Fig. 6E) and anti-NAD7 polypeptide cross-reaction (Fig. 5D) gave similar results compared with the WT. In addition, following two-dimensional BN/SDS gel electrophoresis of dodecylmaltoside-solubilized CMSnad7 mitochondrial membranes, the assignment of three spots (the 76-kDa and NAD7 subunits of complex I and the mitochondrial processing peptidase of...
complex III, Fig. 6E, arrows) was confirmed by fingerprint mass spectrometry analyses.

When mitochondrial membranes from the WT were solubilized with digitonin, a band of lower mobility than that of complex I (Fig. 5A) was stained with NADH and nitro blue tetrazolium (Fig. 5B) and contained polypeptides associated with complexes I and III (Fig. 6B). This band could be assigned to a supercomplex involving complex I and a dimer of complex III as reported in bovine, Arabidopsis, potato, bean, and barley mitochondrial membranes (48–50). As expected, CMSII mitochondria were devoid of this supercomplex (Fig. 6D), which in contrast was present in the mitochondria of the restored CM-Snad7 (Fig. 6F). Polypeptides associated with the complex I satellite band of lower molecular mass including NAD7 were detectable in all the WT and transgenic patterns (Fig. 6, A, B, E, and F), but not in CMSII.

We thus concluded that in CMSSnad7 leaves expression of the nad7 cDNA and efficient transport of NAD7 to mitochondria allowed the proper assembly of this subunit with its partner subunits, giving rise to a bona fide complex I. Moreover, a supercomplex associating complexes I and III was stabilized in the mitochondrial membranes of the transgenic line as in the WT.

**Restoration of NADH Oxidation by Complex I in CMSSnad7 Mitochondrial Membranes**—The oxygen uptake in the presence of malate or NADH was maintained in isolated CMSII mitochondria (38). However, this oxidation was not sensitive to rotenone and was thus independent of complex I activity, involving most likely NDH-2-type alternative enzymes. We measured the rate of antimycin A-sensitive cytochrome c reduction in osmotically shocked mitochondria of the three lines using deamino-NADH as electron donor, a substrate for complex I that cannot be used by the alternative NADH dehydrogenases (51). As shown in Fig. 7, whereas the deamino-NADH-cytochrome c activity in WT membranes was fully sensitive to rotenone, addition of NADH restored a large capacity for cytochrome c reduction. As expected, the mitochondrial membranes from CMSII were not able to oxidize deamino-NADH for the reduction of cytochrome c and could oxidize NADH only. On the contrary, as indicated in Fig. 7, a high rate of cytochrome c reduction sensitive to rotenone was observed in the mitochondrial membranes of the CMSSnad7 in the presence of deamino-NADH. This confirms, first, the lack of complex I activity in mitochondrial membranes isolated from CMSII and, second, shows that the complex activity was restored in the mitochondrial membranes of CMSSnad7 plants. Similar conclusions were drawn from measurements of the rate of deamino-NADH oxidation in the presence of the acceptor decylubiquinone (not shown). Therefore, the assembly of complex I in the transgenic line was associated with restoration of its functional state.

**AOX Gene Expression**—In isolated mitochondrial membranes of CMSII, the cyanide-resistant respiration via the alternative oxidase (AOX) was highly enhanced as were the AOX transcript and protein amounts (36, 38, 39). As observed in Fig. 8A, AOX transcript accumulation was similar in both CMSSnad7 and WT. Fig. 8B shows that in the CMSII total extract, the antibodies raised against AOX gave strong signals likely corresponding to the monomeric and dimeric forms of AOX, in contrast with the weak response in the WT or CMSSnad7 plants. Thus, recovery of the complex I activity in CMSSnad7 was also associated with the reversion of the AOX expression pattern.

**DISCUSSION**

A large mitochondrial DNA deletion comprising the nad7 gene encoding the complex I NAD7 subunit was previously shown to be associated with slow development, reduced vegetative and floral organs, and male sterility in the N. sylvestris CMSII mutant (36). Respiratory measurements demonstrated the absence of complex I activity and activation of endogenous NDH-2 enzymes in CMSII (38). However, these alternative enzymes cannot fully replace complex I to maintain a healthy phenotype. In this work we reported the successful rescue of the CMSII defective phenotype by restoring the synthesis of NAD7 via nuclear transformation with the corresponding cDNA. Such a strategy was first performed for mitochondrial genes to replace subunit 8 of ATPase in the aop 1 mutant of yeast (52). More recently this was done with the ATP6 mitochondrial subunit in a human cell line deficient in ATP synthesis (53). In these experiments, as mitochondrial and universal genetic codes differ in mammals and fungi, the chimeric genes required some modifications in their original sequence as well as the linking of a mitochondrial targeting sequence. In contrast, in plant mitochondria, because of the editing process, this strategy does not require such sequence changes prior to the cDNA cloning.

**No Complex I Is Assembled in CMSII**—The data presented here demonstrated the complete absence of complex I in the mitochondrial CMSII mutant of N. sylvestris previously shown to lack several nuclear and mitochondrial-encoded subunits (35–37). Indeed, no complex I polypeptides could be detected in the mutant at their expected locations in two-dimensional BN/
Complex I Restoration in Nicotiana sylvestris

SDS-PAGE. Consistent with the total absence of complex I, we did not detect any high molecular mass complex having NADH dehydrogenase activity in CMSII mitochondrial membranes that were not able to oxidize deamino-NADH, a specific substrate for complex I (51). The partial complex I (including NAD7) observed in WT mitochondrial membranes (see satellite band in Figs. 5, A–C, and 6A) was also absent from CMSII. This subcomplex is likely to correspond to that described in the NAD4-deficient mutants of maize (54) or Chlamydomonas reinhardtii (55). Our results are consistent with the hypothesis that this subcomplex is a complex I intermediate devoid of most of the membrane arm (54).

The Lack of NAD7 Is Responsible for Complex I Misassembly—Nuclear expression of nad7 in CMSnad7 reestablished both complex I assembly and the wild type phenotype. These results demonstrate that the NAD7 subunit plays a key role in the complex I assembly in plants. The CMSII mutant compares well with the nuo49 (defective in the 49-kDa subunit) mutant of Neurospora crassa (27), which exhibits a total lack of peripheral arm of complex I, whereas in the nuo51 (defective in the 51 kDa harboring FMN and iron-sulfur cluster) an almost complete but not functional complex I is formed (27).

We previously proposed that loss of translational elements in the 5′-region of nad11A (the first trans-spliced exon of nad1) could affect the translation of the nad1 mRNA that is present in CMSII (35). However, restoration of a fully assembled and functional complex I in CMSn(ad7 shows that the lack of NAD1 is in fact related to that of NAD7, as is the case for the other subunits previously shown to be missing in CMSII mitochondria (36, 37). These observations strongly suggest that the missing NAD1 subunit is in fact synthesized in CMSII but rapidly degraded in the absence of assembly with its partner subunits, as this was shown to occur for MWFE (an integral membrane subunit of complex I) in a Chinese hamster fibroblast mutant (57). Alternatively, and less probably, the synthesis of NAD1 could require the presence of either the NAD7 subunit or a subcomplex including NAD7. A number of data suggest that NAD7 is in a tight interaction with the other subunits of the connecting module known as 30-kDa, PSST, and TYKY subunits in mammals for the constitution of the ubiquinone binding pocket (3, 8, 16, 56). NAD1 was proposed to contain also a binding site for ubiquinone (4) and could thus be close to the 49-kDa subunit in the complex. Our results suggest that NAD7 is directly involved not only in the peripheral arm assembly but also in the formation and/or stability of components of the membrane arm. Whether a residual membrane arm is assembled in CMSII remains to be investigated.

In N. crassa, complex I mutant analyses established as a general rule that both the peripheral and membrane arms of complex I are independently assembled before their connection (58, 59). However, it was reported that a defect in a membrane arm subunit could prevent the proper formation of the peripheral arm (60). In the dum20 mutant of C. reinhardtii, the loss of ND1 prevents the assembly of any complex (55). In mammals, the recent development of a conditional system for assembly of complex I gives evidence for the involvement of membrane arm subunits in the stability of the peripheral arm (61). Therefore, there is no consensus model for complex I assembly in the different organisms, and in human the assembly of subunits of the peripheral and membrane arms was recently proposed to depend on each other (62). Interestingly, a 310-kDa subcomplex involving the 49-kDa and NAD1 subunits was isolated from mitochondrial membranes of patients exhibiting complex I deficiencies. This subcomplex was proposed to represent an early intermediate in the assembly process of complex I (62).

Restoring the Organization of the Respiratory Chain in the CMSnad7—Numerous data, first obtained in yeast and mammalian mitochondria (63, 64), suggest that individual respiratory complexes are not randomly distributed in the inner mitochondrial membrane but rather are physically associated to form supercomplexes, including complexes I and III (the latter as a dimeric form) as core of the so-called “respirasome,” and various amounts of complex IV (49). Metabolic flux control analysis of NADH oxidation in bovine heart mitochondria supports the view that association of complexes I and III expresses a functional organization of the respiratory chain (65).

Recently, a similar structural organization in respiratory supercomplexes was found in higher plant non-green tissues; these supercomplexes did not include the non-proton-pumping alternative NDH-2 dehydrogenase and the alternative oxidase enzymes (48, 50). Here, we have shown that a supercomplex associating complex I and complex III can be recovered from mitochondrial membranes purified from N. sylvestris leaves. Their two-dimensional polypeptide patterns (BN/SDS-PAGE) were very close to those obtained from non-green tissues in several plant species (48, 50). In addition to functional consequences as direct substrate channeling between complexes, the physical association between complexes I and III could have a major effect on the stability of each of them (66, 67). As expected, no supercomplex was found in CMSII. However, this absence did not lead to a significant reduction in the amount of complex III. Thus, in plants, a total lack of complex I did not result in a defective assembly or instability of complex III, in agreement with the results obtained in mouse or human mutant cell lines (68, 66). However, this result is in contrast with the data concerning patients exhibiting alteration in the NDUF2 subunit (67). In CMSn(ad7 mitochondria, the presence of complex I was correlated with the formation of a supercomplex comprising complexes I and III as in the WT line, suggesting the recovery of a WT functional respirasome. The reversion of the alternative oxidase expression pattern observed in the CMSnad7 is another indication for the restoration of the wild type properties of the respiratory chain, because dysfunctions in the mitochondrial electron transport chain are sensed by the nucleus and result in the constitutive enhanced expression of the aox genes in maize (69) and Nicotiana (38, 39) mutants.

Transfer of NAD7 to Mitochondria—We showed here that nad7 is expressed in the transgenic CMSII and that a product with the same apparent molecular mass as the authentic NAD7 subunit is accumulated. This suggests that the formate dehydrogenase targeting peptide was correctly cleaved, likely by the mitochondrial processing peptidase. Most probably, the precursor protein, synthesized in the cytosol, was recognized by the TOM complex (translocone of the outer mitochondrial membrane) and imported into mitochondria in an unfolded form by the way of TIM23 translocase complex and the involvement of mitochondrial Hsp70 (70, 71). It can be assumed that the hydrophilic character of NAD7, which does not contain any hydrophobic transmembrane helix, favors its import to mitochondria. Following its total or partial transfer, the protein had to be refolded and assembled with its partner subunits. In a mutant of N. crassa lacking the subunit homologous to TYKY, the peripheral arm does not assemble (72). In this case, it was suggested that binding of the iron-sulfur cluster to this subunit is a necessary step for the assembly of the peripheral arm. NAD7 subunit is probably not involved directly in such a process, and the additional steps that are required by its synthesis outside the mitochondria in CMSn(ad7 did not alter the assembly of complex I.

Given the successful transfer and expression of nad7 in the
nucleus and the proper assembly of complex I in transgenic CMSII, one can wonder why nad7 is still located in the mitochondrion in plants whereas it is nuclear-borne in so many eukaryotes. Finally, the synthesis of NAD7 inside mitochondria does not appear as a prerequisite for the assembly of plant complex I.

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Addendum—A recent study supports the view that the protein complexes forming the mitochondrial respiratory chain are organized in respirasomes in spinach leaves (Krause, F., Reischneider, N. H., Vocke, D., Seelert H., Rexroth, S., and Dencher, N. A. (2004) J. Biol. Chem. 279, 48369–48375).

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Complex I Restoration in Nicotiana sylvestris 26001
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