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L-Galactono-1,4-lactone Dehydrogenase Is Required for the Accumulation of Plant Respiratory Complex I^{*S}

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Mitochondrial NADH-ubiquinone oxidoreductase (complex I) is the largest enzyme of the oxidative phosphorylation system, with subunits located at the matrix and membrane domains. In plants, holocomplex I is composed of more than 40 subunits, 9 of which are encoded by the mitochondrial genome (NAD subunits). In *Nicotiana sylvestris*, a minor 800-kDa subcomplex containing subunits of both domains and displaying NADH dehydrogenase activity is detectable. The NMS1 mutant lacking the membrane arm NAD4 subunit and the CMSII mutant lacking the peripheral NAD7 subunit are both devoid of the holoenzyme. In contrast to CMSII, the 800-kDa subcomplex is present in NMS1 mitochondria, indicating that it could represent an assembly intermediate lacking the distal part of the membrane arm. L-galactono-1,4-lactone dehydrogenase (GLDH), the last enzyme in the plant ascorbate biosynthesis pathway, is associated with the 800-kDa subcomplex but not with the holocomplex. To investigate possible relationships between GLDH and complex I assembly, we characterized an *Arabidopsis thaliana* *gldh* insertion mutant. Homozygous *gldh* mutant plants were not viable in the absence of ascorbate supplementation. Analysis of crude membrane extracts by blue native and two-dimensional SDS-PAGE showed that complex I accumulation was strongly prevented in leaves and roots of *Atgldh* plants, whereas other respiratory complexes were found in normal amounts. Our results demonstrate the role of plant GLDH in both ascorbate biosynthesis and complex I accumulation.

The respiratory chain includes five enzymatic complexes embedded in the inner mitochondrial (mt)² membrane, ensuring oxidative phosphorylation. In a number of organisms, complexes I, III, and IV have been shown to be associated in a supercomplex called “respirasome” (1). The implications of this structural organization in terms of electron transport efficiency and/or complex assembly/stability are still under discussion (2,

3). Although only marginal amounts of “respirasomes” seem to be present in plant mitochondria, stable mt supercomplexes containing complex I (CI) and dimeric complex III have been described in several species (4–6), including tobacco (7).

In most eukaryotes, complex I (NADH:ubiquinone oxidoreductase; EC 1.6.5.3) catalyzes the oxidation of NADH and couples the transfer of electrons to ubiquinone with the translocation of protons from the matrix compartment to the intermembrane space. Eukaryotic complex I (CI) is around 1,000 kDa in size and includes more than 40 subunits (8, 9), of which several (10) are mt-encoded (named NAD in plants). Besides the subunits directly involved in NADH and ubiquinone binding and in electron transfer (Fe-S cluster bearing subunits) (11), the function of most CI subunits is still unknown. They are distributed in two large domains, a matrix domain and a membrane domain containing highly hydrophobic polypeptides which, in animal and fungi, include all NAD subunits (12). Although it shares more than 30 conserved subunits with all mt CI (13), the plant enzyme displays several peculiarities. First, the hydrophobic arm is unusually long and possesses specific protuberances (14). Second, two subunits of the peripheral arm, NAD7 and NAD9, are mt-encoded. Third, the plant complexes have been reported to contain specific subunits, among which are three to five γ -carbonic anhydrase (γ -CA) or γ -CA-like isoforms (15, 16) and the L-galactono-1,4-lactone dehydrogenase (GLDH), which catalyzes the last step in the plant ascorbate synthesis pathway (17, 18). Whereas γ -CA(like) isoforms have been demonstrated to be integral proteins of the CI membrane arm (16), the exact localization of GLDH is still unclear, and the protein has been proposed to be associated with a minor low molecular mass complex in *Arabidopsis* cell cultures (19).

As for other plant organelle enzymes involved in electron transport chains that include subunits of dual origin, CI assembly is a multiple-step intricate process with many levels of regulation and requires protein assembly factors. Models of CI assembly have recently emerged in fungi, mammals, and the algal *Chlamydomonas* (20), but little information is currently available in land plants, because of the scarcity of respiratory mutants.

In this study, we give new insights on the composition of CI assembly intermediates in plants, and we re-evaluate the association of GLDH with this complex by genetic and biochemical approaches using *Nicotiana sylvestris* and *Arabidopsis thaliana* mutants. The *N. sylvestris* CMSII mtDNA mutant, devoid of the mt *nad7* gene (21), and the nuclear NMS1 mutant, impaired in the processing of the mt *nad4* transcript (22), lack significant CI activity (23, 24). Both holo-CI and a minor complex, around 800 kDa in size, were previously shown by blue native analyses to be

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^S The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. 1.

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² The abbreviations used are: mt, mitochondrial; CI, complex I; GLDH, L-galactono-1,4-lactone dehydrogenase; γ -CA, γ -carbonic anhydrase; BN, blue native; PS, photosystem; LHCII, light-harvesting chlorophyll complex; *b₆f*, cytochrome *b₆f*; CIII, mitochondrial complex III; MOPS, 4-morpholinepropanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; BisTris, 2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)propane-1,3-diol; WT, wild type; NBT, nitro blue tetrazolium.

absent in CMSII mt membranes (7). Here we show that although holo-CI is similarly lacking in NMS1, the 800-kDa complex is present, indicating that it could be an assembly intermediate. GLDH was bound to the subcomplex only. To further evaluate possible relationships between GLDH and CI content, we characterized an *Arabidopsis* mutant interrupted in the *GLDH* gene. Homozygous *Atgldh* plants developed only when supplemented with ascorbate, and they were impaired in CI content, whereas other respiratory complexes were in normal amounts. These results show that GLDH expression is necessary for CI accumulation in plants.

EXPERIMENTAL PROCEDURES

N. sylvestris plants were grown in soil, in a greenhouse under a 16-h photoperiod, at a day/night temperature regime of 23/17 °C, and under natural illumination supplemented with artificial lighting when necessary to maintain a minimum of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ at the leaf surface.

Arabidopsis seeds of the SALK_060087 line carrying a T-DNA insertion in the At3g47930 gene were obtained from the Nottingham *Arabidopsis* stock center (25). Seeds were sterilized and sown under aseptic conditions on agar plates containing Gamborg B5 salt and vitamins, supplemented with 0.5% sucrose. The plated seeds were kept at 4 °C for 5 days, and transferred to an illuminated, temperature-controlled growth chamber. Plants were grown at 20 °C under a 16-h day/8-h night photoperiod of white light ($\sim 80 \mu\text{mol m}^{-2} \text{s}^{-1}$).

RNA Isolation and Reverse Transcription-PCR Analysis—Total RNA was prepared, using the TRIzol reagent (Invitrogen), following the manufacturer's instructions. RNA (1 μg) was treated with RQ1 RNase-free DNase (Promega, Madison, WI) and reverse-transcribed using random hexamers and SUPERScriptTM III first-strand kit (Invitrogen) following the manufacturer's recommendations. The expression levels of At3g47930 (*GLDH*) and At3g13920 (*TIF4A1*) were determined, using the following primers: GLDH-5.2, TCGAAGTAAGAGCCCCATTAG; GLDH-3.2, TAAGCAGTGGTGGAGAC-TGG; TIF4A-5, AGTCTCCCTGGTTATCAACTTC; and TIF4A-3, CGACTTCTTTTCTCCCTCAC.

Preparation of Mitochondria of *N. sylvestris* Leaves—Mitochondria were purified from 100 g of homogenized leaves by differential centrifugation and gradient centrifugation using two layers of 26 and 46% Percoll in 0.5 M mannitol, 10 mM MOPS-KOH, pH 7.2, 0.1% bovine serum albumin (7). Mitochondria were washed in 0.5 M mannitol, 10 mM MOPS-KOH, pH 7.2, and aliquots were frozen in liquid nitrogen and stored at -80°C when not used immediately.

Preparation of Crude Membrane Extracts from *A. thaliana* Leaves and Roots—Two-hundred mg of plant material were harvested and briefly pound at 0 °C, in a conical glass homogenizer in 2 ml of 75 mM MOPS-KOH, pH 7.6, 0.6 M sucrose, 4 mM EDTA, 0.2% polyvinylpyrrolidone 40, 8 mM cysteine, 0.2% bovine serum albumin (26). The lysate was filtrated across one layer of miracloth and centrifuged first at $1300 \times g$ for 4 min and the supernatant at $22,000 \times g$ for 20 min. The resultant sediment, which contained most of the thylakoid and mt membranes, was resuspended in 200 μl of 10 mM MOPS-KOH, pH 7.2, 0.3 M sucrose.

Electrophoretic Analyses—*N. sylvestris* mt suspensions (200 μg) were washed in water and resuspended in 50 μl of 10% sucrose, 50 mM BisTris, pH 7, 0.5 M aminocaproic acid, 1 mM EDTA, 0.5 μM Pefabloc SC. mt membranes were solubilized by addition of dodecyl maltoside (to 1%) or digitonin (to 2%). After centrifugation the supernatant was supplemented by blue G-250 (detergent/blue G-250 ratio of 8).

Crude *Arabidopsis* membranes suspensions were washed with 600 μl of water, sedimented at $22,000 \times g$ for 20 min, and resuspended in 150 μl of 50 mM BisTris/HCl, pH 7, 0.5 M 6-aminohexanoic acid, 1 mM EDTA, 0.3 M sucrose, and 0.5 mM Pefabloc SC. After addition of 30 μl of 10% β -dodecyl maltoside or 60 μl of 10% digitonin at 4 °C for 15–30 min, respectively, samples were centrifuged at $22,000 \times g$ for 8 min. The supernatants were supplemented with 8 or 16 μl of 5% Coomassie Blue G-250 (prepared in 0.020 M BisTris/HCl, 0.5 M 6-aminohexanoic acid) and an aliquot equivalent to 12 mg (leaves) or 20 mg (roots) of fresh weight material were loaded to each lane of a BN gel.

Protein complexes from *N. sylvestris* mt membranes or from *Arabidopsis* crude membrane extracts were separated by BN-PAGE using 4–13 or 3.6–12% gradient acrylamide gels in Bis-Tris buffer as described previously (7). Blue native gels (thickness 1.5 mm) were made as described previously (27, 28) with minor modifications. For analysis of crude membrane extracts, the gel buffer consisted of 0.05 M BisTris, 0.5 M 6-aminohexanoic acid, pH 7, 0.01% detergent and the cathode buffer contained 0.005% Blue G-250 and 0.01% detergent.

Two-dimensional BN/SDS electrophoresis was performed as described previously (7) using 9 or 10–18% polyacrylamide gels. After electrophoresis the proteins were silver-stained (29).

Determination of In-gel Enzymatic Activities—After BN-PAGE, the NADH dehydrogenase activity of CI was revealed by incubation of the gel in the presence of 1 mM nitro blue tetrazolium (NBT) and 0.2 mM NADH in 0.05 M MOPS, pH 7.6 (29), after washing for 20 min in 0.1 M MOPS, pH 7.6. The cytochrome oxidase activity was revealed after washing the gel in 50 mM sodium phosphate, pH 7.4, by incubation in the same buffer but containing 0.22 M saccharose, 0.5 mg/ml bovine heart cytochrome c, 0.5 mg/ml 3,3'-diaminobenzidine, and 24 units/ml catalase (30).

Immunodetection Analyses—Gels were first electroblotted onto polyvinylidene difluoride membranes using a semi-dry method in 50 mM Tricine, 15 mM BisTris at 0.8 mA/cm² for 1.5 h. Membranes were incubated with antibodies against galactono-1,4-lactone dehydrogenase (31) or against the C-terminal part of *Arabidopsis* γ -carbonic anhydrase (At1g47260) (15). Secondary antibodies were coupled to horseradish peroxidase, and detection was with nitro blue tetrazolium (Sigma) (BN-PAGE) or with ECL (Amersham Biosciences) (two-dimensional BN/SDS-PAGE).

RESULTS

Presence of a Discrete 800-kDa CI Subcomplex Containing GLDH in the *N. sylvestris* NMS1 Mutant—As reported previously for CMSII (7), no signal around 1,000 kDa, corresponding to the CI molecular mass, could be detected in BN-PAGE profiles of dodecyl maltoside-solubilized NMS1 mt membranes (Fig. 1A), whereas complexes III (around 500 kDa) and V

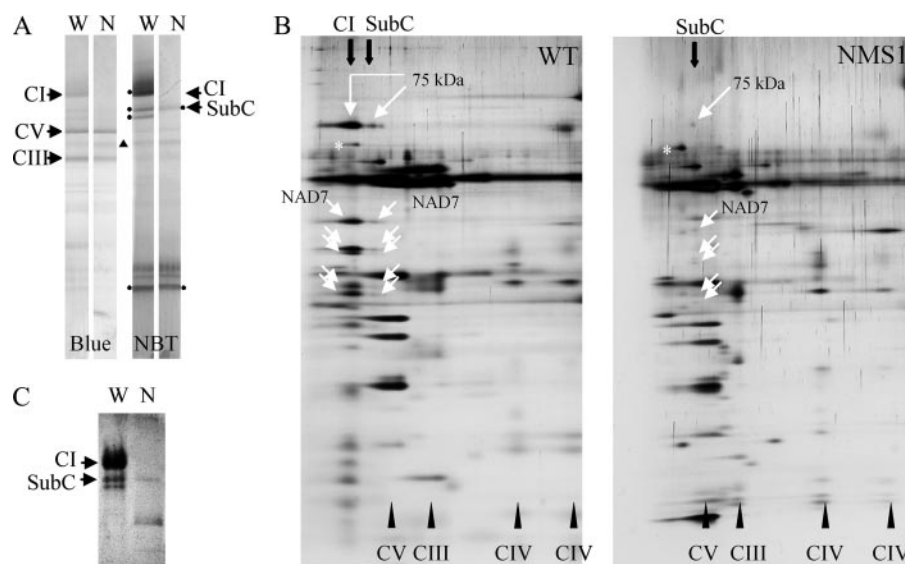


FIGURE 1. Characterization of the mt complexes in CI-deficient *N. sylvestris* mutants. *A*, resolution by BN-PAGE of respiratory complexes from WT (W) and NMS1 (N) leaves. Mitochondria were purified from leaves of NMS1 and WT, and dodecyl maltoside-solubilized proteins of mt membranes were resolved on 4/13% acrylamide gels. *Blue*, Coomassie Blue-stained complexes after electrophoresis. *NBT*, in-gel dehydrogenase activity in the presence of NADH and NBT. *Black circles* indicate the position of the signal, and a *black triangle* indicates the position of a photosystem I complex because of the very low contamination by thylakoid membranes. *B*, two-dimensional BN/SDS-PAGE analysis of mt membranes of NMS1 and WT. Proteins from dodecyl maltoside-solubilized membranes were first separated on 4/13% gradient acrylamide gel. Strips of this gel were incubated for 20 min in 0.0625 M Tris-HCl, 15% glycerol, 2% SDS and analyzed on 9/18% acrylamide SDS gel. *CI*, *CIII*, *CIV*, and *CV* denote complexes I, III, IV, and V, respectively. *SubC*, 800-kDa subcomplex. Some subunits of CI and 800-kDa subcomplex are indicated by *white arrows*. The spots (indicated by a *white **) present in all preparations was identified by mass spectrometry (data not shown) as the 70-kDa subunit of a vacuolar H^+ -ATPase. *C*, immunodetection of γ -CA subunit in mt membranes of NMS1 and WT. Proteins from dodecyl maltoside-solubilized membranes of NMS1 (N) and WT (W) were separated on 4/13% gradient acrylamide gels by BN-PAGE. After blotting to a polyvinylidene difluoride membrane, the anti- γ -CA immune signal was detected by nitro blue tetrazolium kit (Sigma).

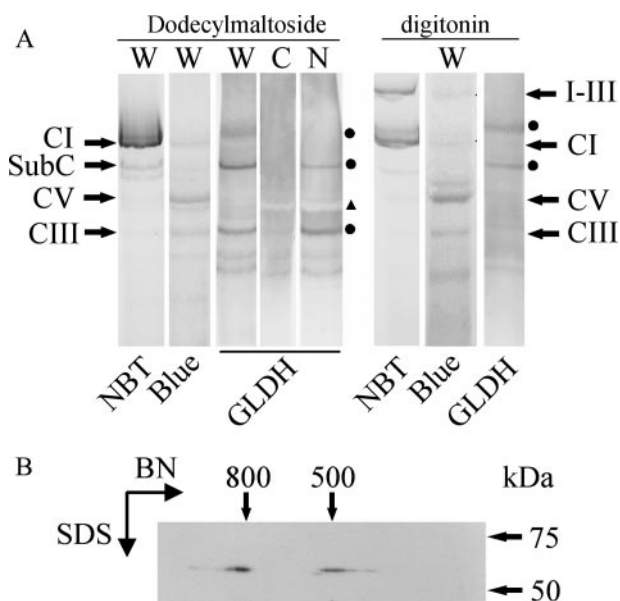


FIGURE 2. Immunodetection of galactono-1,4-lactone dehydrogenase in mt membranes of CI-deficient mutants and WT plants. *A*, immune signal was detected after BN-PAGE separation of dodecyl maltoside or digitonin-solubilized mt proteins extracted from leaves of WT (W), CMSII (C), or NMS1 (N) on a 3.6/12% acrylamide gel. *SubC*, 800-kDa subcomplex. *NBT*, in-gel NADH/NBT-stained WT proteins. *Blue*, aspect of the WT blot after protein transfer allowing localization of the main complexes. *GLDH*, immune detection of an anti-GLDH signal on WT, CMSII, or NMS1 blots. The *black circle* indicates the position of the immune signals, and the *black triangle* indicates the position of contaminating PSI. *B*, localization of GLDH polypeptides after two-dimensional (BN/SDS-PAGE) resolution of dodecyl maltoside-solubilized WT mt membranes.

(around 600 kDa) were readily visible. Similarly, the major NADH/NBT signal revealing NADH dehydrogenase activity was lacking in NMS1 (Fig. 1A). However, a weak NADH/NBT signal around 800 kDa was present in NMS1 as in the WT (Fig. 1A), suggesting the presence of a minor CI subcomplex in both lines. The polypeptide composition of the mt complexes was resolved by two-dimensional BN/SDS-PAGE (Fig. 1B). In the WT, the 1,000- and 800-kDa CI complexes shared many subunits, including the previously characterized 75-kDa subunit, belonging to the NADH dehydrogenase module (8) as well as NAD7 (7). In NMS1, these polypeptides were associated with the 800-kDa subcomplex only (Fig. 1B), in good agreement with its NADH dehydrogenase activity (Fig. 1A).

The composition of the subcomplex was further investigated by immunological studies. Using anti- γ -CA antibodies, a major signal at 1,000 kDa was observed in the WT profile only. In addition, a weak signal was observed in both WT and

NMS1 profiles at around 800 kDa (Fig. 1C), indicating that the subcomplex contains a part of the membrane arm. In contrast, no signal was seen at the level of holo-CI in the WT profiles using antibodies raised against GLDH (Fig. 2A). Likewise no signal was detected either at the level of CI or at the level of the supercomplex I–III when mt membrane proteins were solubilized by digitonin (Fig. 2A), a treatment that preserves CI/CIII association (5, 7). However, a clear signal was observed below CI in WT and NMS1 only, at the same mobility as the 800-kDa subcomplex characterized by in-gel NADH/NBT staining. A second signal was detected around 500 kDa (Fig. 2A). In addition, a diffuse band above the position of CI appeared only in WT profiles, although some discrete bands were detectable below the 500-kDa band in all profiles. Two-dimensional BN/SDS-PAGE showed that both 800- and 500-kDa immunosignals were associated with a polypeptide of about 60 kDa (Fig. 2B), in good agreement with the GLDH molecular mass (32). Whether the 500-kDa signal represents a still unidentified low molecular weight CI subcomplex, devoid of the NADH-oxidizing module, remains to be determined. These results show that GLDH is not associated with holo-CI in *N. sylvestris* but to a subcomplex accumulating both in the WT and in the NAD4-deficient NMS1 mutant, but not in the NAD7-deficient CMSII mutant.

Complex I Accumulation Is Impaired in an *A. thaliana* *glh* Mutant—A possible implication of GLDH in CI formation was further investigated by examining the accumulation of respiratory complexes in an *A. thaliana* *glh* insertion mutant. The

Arabidopsis nuclear genome contains a single *GLDH* gene (At3g47930) (33), and the SALK_060087 line was identified as a putative mutant containing a T-DNA insertion within this gene. SALK_060087 seeds were germinated *in vitro* on gelose

medium, and roughly a quarter (22.2%) of them (as expected from a single and recessive mutation) exhibited a delayed germination, developed chlorotic cotyledons (Fig. 3A), and ultimately died at the cotyledon stage. However, all the chlorotic seedlings could be rescued by 10 mM ascorbate supplementation (Fig. 3B). PCR amplification using *gl dh* internal and T-DNA left border primers showed these plants to be indeed homozygous for the T-DNA insertion in the last intron of the *gl dh* gene (supplemental Fig. 1, A and B). In the offspring of heterozygous *gl dh/GLDH* plants, ascorbate-dependent plantlets were selected and checked for the genotype and *GLDH* expression. No *GLDH* transcript could be detected by semi-quantitative reverse transcription-PCR (supplemental Fig. 1C). Taken together, these results indicate that these plantlets are homozygous *gl dh* mutants, deficient in ascorbate synthesis.

A protocol was developed to determine the composition of mt complexes from minute amounts of material without previous mt purification (see under "Experimental Procedures"). Dodecyl maltoside- or digitonin-solubilized proteins from leaf or root crude membrane extracts were resolved by BN-PAGE and BN/SDS-PAGE. In one-dimensional BN-profiles of leaves,

main bands represented complexes originating from thylakoid membranes, which were identifiable from their constitutive spots appearing in the second dimension (Fig. 4, A and B), in agreement with the previous reports of proteomic investigation of plant photosystems (34, 35). The presence of mt CI was clearly revealed by in-gel NADH/NBT staining of the BN profiles from dodecyl maltoside-solubilized proteins of Col-0 leaf membrane extracts (Fig. 4A), whereas no significant NADH/NBT staining at 1,000 kDa could be detected in *gl dh* leaf extracts. Similarly, NADH/NBT staining was seen at the levels of CI (1,000 kDa) and supercomplex I–III (1,500 kDa) in the profiles of digitonin-solubilized proteins from Col-0 leaf membrane extract (Fig. 4B) but not in profiles of *gl dh* extract. In contrast, cytochrome oxidase in-gel staining gave identical responses in Col-0 and *gl dh* mutant (Fig. 4B).

The two-dimensional BN/SDS-PAGE profiles from dodecyl maltoside-solubilized proteins of Col-0 and *gl dh* leaf extracts were comparable, and similar amounts of spots originating from the mt complexes III and V on both sides of PSI were detected (Fig. 4C). The extracts contained several major or minor complexes having their mobility close to that of CI, as PSII-LHCII, or vacuo-

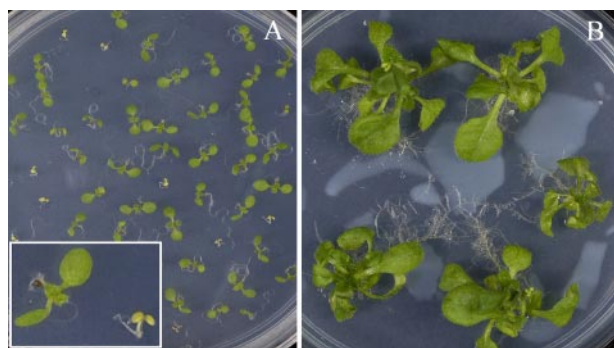


FIGURE 3. Aspect of *gl dh* plants grown on *in vitro* gelose medium. A, heterozygous and *gl dh* homozygous plants following germination on gelose medium without ascorbate supplementation. Inset, enlargement showing the difference in size and pigmentation between homozygous and heterozygous plantlets. B, homozygous *gl dh* plants after 6 weeks of growth on a medium supplemented with ascorbic acid.

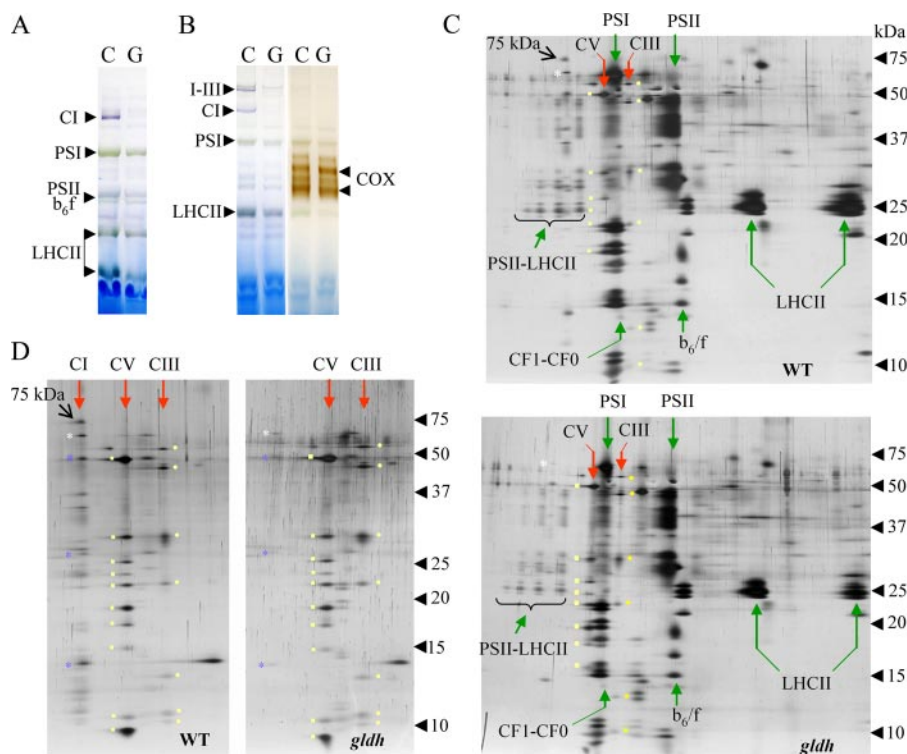


FIGURE 4. Characterization by electrophoreses of the respiratory complexes in the *gl dh* mutant. Proteins of crude membrane extracted from leaves or roots of *gl dh* mutant (G) or Col-0 (C) plants were solubilized with dodecyl maltoside or digitonin and resolved by BN-PAGE or BN/SDS-PAGE. A, in-gel NADH/NBT staining following resolution by BN/PAGE of dodecyl maltoside-solubilized proteins from membranes prepared from *gl dh* and Col-0 leaves. B, in-gel CI (left) and cytochrome oxidase (right) staining following resolution by BN/PAGE of digitonin-solubilized proteins from membranes extracted from *gl dh* and Col-0 leaves. C, resolution of polypeptides of dodecyl maltoside-solubilized proteins from membranes extracted from *gl dh* and Col-0 leaves by two-dimensional BN/SDS-PAGE. D, resolution of polypeptides of dodecyl maltoside-solubilized proteins from membranes extracted from *gl dh* and Col-0 roots by two-dimensional BN/SDS-PAGE. Main complexes of thylakoid and mt membranes are indicated on the profiles. CI denotes mt complex I; I–III, supercomplex I, III₂; PSI, photosystem I; PSII, photosystem II; *b₆f*, cytochrome *b₆f*; LHCII, light-harvesting chlorophyll complex; COX, cytochrome oxidase; CV, mt complex V; CIII, mt complex III, CF1-CF₀, thylakoid ATP synthetase. The 75-kDa subunit of CI was indicated on the second dimensional pattern. Subunits from complex V are marked by yellow squares at left and complex III subunits by yellow circles at right on the second dimensional pattern. The white * indicates the 70-kDa subunit of the vacuolar ATPase. The blue * indicate other putative subunits of vacuolar ATPase.

lar ATPase, a known contaminant of *N. sylvestris* (Fig. 1, B and C) or *Neurospora crassa* mt preparations (36). Thus only the 75-kDa spot seen above the PSII-LHCII trace represents an unambiguous marker for CI. It was seen in the Col-0 but not in the *gldh* two-dimensional profiles (Fig. 4C) in good agreement with the data of NADH/NBT stain obtained following the first dimension.

The results obtained from leaf extracts were confirmed by analyses of root extracts. Spots originated from dodecyl maltoside-solubilized proteins of Col-0, and *gldh* mt membranes represent main spots on two-dimensional profiles (Fig. 4D) fitting well with the profiles obtained from mt preparations of *N. sylvestris* leaves (Fig. 1). Comparable amounts of spots derived from complexes III and V were observed in the profiles of both lines, whereas spots derived from CI were lacking in the *gldh* mutant. Because this deficiency could not be explained by a decrease of mt membranes in the sample as shown by the comparable levels of complexes III, IV, and V, these results indicated that CI is lacking in *gldh* plants.

DISCUSSION

As in other organisms, the elucidation of complex I assembly in plants depends on the availability of mutants of either peripheral or membrane domains. Here we bring new insights to this process using available *N. sylvestris* mutants, the CMSII mutant lacking the peripheral NAD7 subunit (21) and the NMS1 mutant lacking the membrane NAD4 subunit (22), as well as a newly characterized *A. thaliana gldh* mutant. *Atgldh* seedlings did not develop beyond the cotyledon stage in the absence of ascorbate supplementation, confirming that GLDH activity is an obligatory step in the guanosine diphosphate mannose pathway for ascorbate biosynthesis in plants (17, 18). A strict ascorbate requirement has already been shown for the development of *Arabidopsis* mutants impaired in the synthesis of GDP-L-galactose phosphorylase, an enzyme involved upstream in the ascorbate biosynthesis pathway in plants (37). However, if ascorbate supplementation to *gldh* plants allowed greening and development, their growth rate remained very low. Reduced growth rates were also observed in antisense *gldh* tomato plants (38).

Holo-CI could not be detected in leaf mt membranes of the *N. sylvestris* NMS1 mutant lacking the membrane NAD4 subunit, as is the case in the CMSII mutant devoid of the peripheral NAD7 subunit (7). However, in contrast to CMSII, a minor 800-kDa form previously observed in the WT (7) was detected in NMS1. This subcomplex contains subunits characteristic of the peripheral domain (e.g. NAD7 and the 75-kDa component of the dehydrogenase module) and displays in-gel NADH dehydrogenase activity (Fig. 1). The presence of this subcomplex in NMS1 mitochondria despite the lack of holo-CI indicates that it is probably an assembly intermediate and not a degradation product. Existence of a subcomplex comprising matricial subunits has been previously reported in the maize NAD4-deficient NCS2 mutant (39). However, the presence in the *N. sylvestris* subcomplex of the γ -CA subunit, an integral membrane subunit of plant CI (16), shows that it also contains a part of the membrane arm. In contrast to γ -CA, GLDH, the enzyme catalyzing the last step in ascorbate synthesis (17), was only associ-

ated with an 800-kDa subcomplex and not with the holo-CI in *N. sylvestris* mitochondria. Ascorbate synthesis carried out by the sub-CI-bound GLDH form of *N. sylvestris* leaves is not essential, as previously suggested for the low molecular mass CI of *Arabidopsis* cell cultures (40), because ascorbate levels are not markedly altered in the CMSII mutant lacking this subcomplex (41, 42). Interestingly, GLDH was also associated with a 500-kDa mt complex (Fig. 2), but whether it corresponds to a CI assembly intermediate remains to be shown.

Taken together, these results support the following model for the CI assembly process in plants: the peripheral arm including the N and Q modules (8) would bind to a large membrane segment, resulting in an assembly intermediate around 800 kDa that includes GLDH. The formation of mature CI would require the elimination of GLDH and the addition of a set of subunits, including NAD4, the analogous subunit (NUOM) being located at the tip of the membrane arm in *Escherichia coli* (43). Similarly, the 700-kDa subcomplex that accumulates in a *Chlamydomonas reinhardtii* NAD4-deficient mutant includes subunits of both the matrix and the membrane arm, but it is devoid of ND5, located in the membrane arm tip (44). This 700-kDa subcomplex in the *C. reinhardtii* ND5-deficient mutant is deprived of a set of subunits located at the distal domain of the CI membrane arm (45). Lack of the membrane arm distal segment does not prevent the integration of γ -CA into the CI subcomplex of either *N. sylvestris* NAD4-deficient (this work) or *C. reinhardtii* NAD5-deficient mutants (45). The subcomplexes accumulating in *Chlamydomonas* have been proposed to be assembly intermediates (44), and the analysis of *N. sylvestris* mutants strongly supports common points between the late steps of plant and *C. reinhardtii* CI assembly processes.

Alternatively, it can be hypothesized that fixation of GLDH to the assembly intermediate impairs further attachment of the distal membrane domain, and therefore the holoenzyme can only derive from a GLDH-free subcomplex and the presence of NAD4. In this hypothesis, the GLDH-associated subcomplex would not be a productive intermediate. However, such a model is not supported by the study of the *Atgldh* insertion mutant, which shows that the absence of GLDH expression inhibits the accumulation of the holoenzyme. Indeed, complex I and its associated NADH dehydrogenase activity were lacking in *Atgldh* leaf and root membranes, although the other respiratory complexes were unaffected. Lack of complex I in the *gldh* mutant demonstrates that GLDH is involved in the process leading to the formation of CI, although the exact mechanism remains to be elucidated. GLDH may be involved in the synthesis/stability of individual subunits and/or assembly intermediates. To date, no plant CI assembly factors have been characterized. In fungi and mammals, several protein factors associated with high molecular weight CI subcomplexes but not with the holoenzyme have been shown to be directly involved in the CI assembly process. In *N. crassa*, CIA30 and CIA84, two proteins that do not belong to the mature holoenzyme, were shown to be associated with a large membrane segment accumulating in the nuo21 mutant unable to assemble holo-CI (46). Disruption of *CIA* genes resulted in the inability to form the membrane segment. Recent studies of human CI

assembly have led to the characterization of discrete subassembly complexes (most of them in the range of 800 and 450–500 kDa) and the identification of at least three different assembly factors, B17.2L (47, 48), NDUFAF1, a CIA30 homologue (49, 50), and Ecsit (51). The deficiency of each of these three factors leads to a severe reduction in human CI levels and activity. Although further biochemical studies are needed to elucidate the kinetic steps of the CI assembly process, our results demonstrate the dual function of GLDH in plants, *i.e.* ascorbate synthesis and holocomplex I accumulation.

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REFERENCES

- Schägger, H. (2002) *Biochim. Biophys. Acta* **1555**, 154–159
- Genova, M. L., Baracca, A., Biondi, A., Casalena, G., Faccioli, M., Falasca, A. I., Formigini, G., Sgarbi, G., Solaini, G., and Lenaz, G. (2008) *Biochim. Biophys. Acta* **1777**, 740–746
- Schägger, H., de Coo, R., Bauer, M. F., Hofmann, S., Godinot, C., and Brandt, U. (2004) *J. Biol. Chem.* **279**, 36349–36353
- Eubel, H., Heinemeyer, J., and Braun, H. P. (2004) *Plant Physiol.* **134**, 1450–1459
- Eubel, H., Jansch, L., and Braun, H. P. (2003) *Plant Physiol.* **133**, 274–286
- Krause, F., Reifschneider, N. H., Vocke, D., Seelert, H., Rexroth, S., and Dencher, N. A. (2004) *J. Biol. Chem.* **279**, 48369–48375
- Pineau, B., Mathieu, C., Gerard-Hirne, C., De Paepe, R., and Chétrit, P. (2005) *J. Biol. Chem.* **280**, 25994–26001
- Brandt, U. (2006) *Annu. Rev. Biochem.* **75**, 69–92
- Carroll, J., Fearnley, I. M., Shannon, R. J., Hirst, J., and Walker, J. E. (2003) *Mol. Cell Proteomics* **2**, 117–126
- Adams, K. L., and Palmer, J. D. (2003) *Mol. Phylogenet. Evol.* **29**, 380–395
- Hirst, J., Carroll, J., Fearnley, I. M., Shannon, R. J., and Walker, J. E. (2003) *Biochim. Biophys. Acta* **1604**, 135–150
- Walker, J. E. (1992) *Q. Rev. Biophys.* **25**, 253–324
- Gabaldon, T., Rainey, D., and Huynen, M. A. (2005) *J. Mol. Biol.* **348**, 857–870
- Dudkina, N. V., Eubel, H., Keegstra, W., Boekema, E. J., and Braun, H. P. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102**, 3225–3229
- Perales, M., Eubel, H., Heinemeyer, J., Colaneri, A., Zabaleta, E., and Braun, H. P. (2005) *J. Mol. Biol.* **350**, 263–277
- Sunderhaus, S., Dudkina, N. V., Jansch, L., Klodmann, J., Heinemeyer, J., Perales, M., Zabaleta, E., Boekema, E. J., and Braun, H. P. (2006) *J. Biol. Chem.* **281**, 6482–6488
- Smirnov, N., Conklin, P. L., and Loewus, F. A. (2001) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52**, 437–467
- Wheeler, G. L., Jones, M. A., and Smirnov, N. (1998) *Nature* **393**, 365–369
- Heazlewood, J. L., Howell, K. A., and Millar, A. H. (2003) *Biochim. Biophys. Acta* **1604**, 159–169
- Vogel, R. O., Smeitink, J. A., and Nijtmans, L. G. (2007) *Biochim. Biophys. Acta* **1767**, 1215–1227
- Pla, M., Mathieu, C., De Paepe, R., Chétrit, P., and Vedel, F. (1995) *Mol. Gen. Genet.* **248**, 79–88
- Brangeon, J., Sabar, M., Gutierrez, S., Combettes, B., Bove, J., Gendy, C., Chétrit, P., Des Francs-Small, C. C., Pla, M., Vedel, F., and De Paepe, R. (2000) *Plant J.* **21**, 269–280
- Gutierrez, S., Sabar, M., Lelandais, C., Chétrit, P., Diolez, P., Degand, H., Boutry, M., Vedel, F., de Kouchkovsky, Y., and De Paepe, R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 3436–3441
- Sabar, M., De Paepe, R., and de Kouchkovsky, Y. (2000) *Plant Physiol.* **124**, 1239–1250
- Alonso, J. M., Stepanova, A. N., Leisse, T. J., Kim, C. J., Chen, H., Shinn, P., Stevenson, D. K., Zimmerman, J., Barajas, P., Cheuk, R., Gadrinab, C., Heller, C., Jeske, A., Koesema, E., Meyers, C. C., Parker, H., Prednis, L., Ansari, Y., Choy, N., Deen, H., Geralt, M., Hazari, N., Hom, E., Karnes, M., Mulholland, C., Ndubaku, R., Schmidt, I., Guzman, P., Aguilar-Henonin, L., Schmid, M., Weigel, D., Carter, D. E., Marchand, T., Risseuw, E., Brogden, D., Zeko, A., Crosby, W. L., Berry, C., and Ecker, J. R. (2003) *Science* **301**, 653–657
- Bartoli, C. G., Yu, J., Gomez, F., Fernandez, L., McIntosh, L., and Foyer, C. H. (2006) *J. Exp. Bot.* **57**, 1621–1631
- Schägger, H. (2001) *Methods Cell Biol.* **65**, 231–244
- Wittig, I., Braun, H. P., and Schagger, H. (2006) *Nat. Protoc.* **1**, 418–428
- Blum, H., Beier, H., and Gross, H. J. (1987) *Electrophoresis* **8**, 93–99
- Zerbetto, E., Vergani, L., and Dabbeni-Sala, F. (1997) *Electrophoresis* **18**, 2059–2064
- Bartoli, C. G., Gomez, F., Gergoff, G., Guimet, J. J., and Puntarulo, S. (2005) *J. Exp. Bot.* **56**, 1269–1276
- Bartoli, C. G., Pastori, G. M., and Foyer, C. H. (2000) *Plant Physiol.* **123**, 335–344
- Leferink, N. G., van den Berg, W. A., and van Berkel, W. J. (2008) *FEBS J.* **275**, 713–726
- Ciambella, C., Roepstorff, P., Aro, E. M., and Zolla, L. (2005) *Proteomics* **5**, 746–757
- Heinemeyer, J., Eubel, H., Wehmhoner, D., Jansch, L., and Braun, H. P. (2004) *Phytochemistry* **65**, 1683–1692
- Marques, I., Dencher, N. A., Videira, A., and Krause, F. (2007) *Eukaryot. Cell* **6**, 2391–2405
- Dowdle, J., Ishikawa, T., Gatzek, S., Rolinski, S., and Smirnov, N. (2007) *Plant J.* **52**, 673–689
- Alhaghdow, M., Mounet, F., Gilbert, L., Nunes-Nesi, A., Garcia, V., Just, D., Petit, J., Beauvoit, B., Fernie, A. R., Rothan, C., and Baldet, P. (2007) *Plant Physiol.* **145**, 1408–1422
- Karpova, O. V., and Newton, K. J. (1999) *Plant J.* **17**, 511–521
- Millar, A. H., Mittova, V., Kiddle, G., Heazlewood, J. L., Bartoli, C. G., Theodoulou, F. L., and Foyer, C. H. (2003) *Plant Physiol.* **133**, 443–447
- Dutilleul, C., Garmier, M., Noctor, G., Mathieu, C., Chétrit, P., Foyer, C. H., and de Paepe, R. (2003) *Plant Cell* **15**, 1212–1226
- Vidal, G., Ribas-Carbo, M., Garmier, M., Dubertret, G., Rasmusson, A. G., Mathieu, C., Foyer, C. H., and De Paepe, R. (2007) *Plant Cell* **19**, 640–655
- Baranova, E. A., Holt, P. J., and Sazanov, L. A. (2007) *J. Mol. Biol.* **366**, 140–154
- Cardol, P., Matagne, R. F., and Remacle, C. (2002) *J. Mol. Biol.* **319**, 1211–1221
- Cardol, P., Boutaffala, L., Memmi, S., Devreese, B., Matagne, R. F., and Remacle, C. (2008) *Biochim. Biophys. Acta* **1777**, 388–396
- Küffner, R., Rohr, A., Schmiede, A., Krull, C., and Schulte, U. (1998) *J. Mol. Biol.* **283**, 409–417
- Lazarou, M., McKenzie, M., Ohtake, A., Thorburn, D. R., and Ryan, M. T. (2007) *Mol. Cell Biol.* **27**, 4228–4237
- Ogilvie, I., Kennaway, N. G., and Shoubridge, E. A. (2005) *J. Clin. Invest.* **115**, 2784–2792
- Dunning, C. J., McKenzie, M., Sugiana, C., Lazarou, M., Silke, J., Connelly, A., Fletcher, J. M., Kirby, D. M., Thorburn, D. R., and Ryan, M. T. (2007) *EMBO J.* **26**, 3227–3237
- Vogel, R. O., Janssen, R. J., Ugalde, C., Groenestein, M., Huijbens, R. J., Visch, H. J., van den Heuvel, L. P., Willems, P. H., Zeviani, M., Smeitink, J. A., and Nijtmans, L. G. (2005) *FEBS J.* **272**, 5317–5326
- Vogel, R. O., Janssen, R. J., van den Brand, M. A., Dieteren, C. E., Verkaart, S., Koopman, W. J., Willems, P. H., Pluk, W., van den Heuvel, L. P., Smeitink, J. A., and Nijtmans, L. G. (2007) *Genes Dev.* **21**, 615–624