NADH-ubiquinone oxidoreductase (Complex I, EC 1.6.5.3) is the largest complex of the mitochondrial respiratory chain. In eukaryotes, it is composed of more than 40 subunits that are encoded by both the nuclear and mitochondrial genomes. Plant Complex I differs from the enzyme described in other eukaryotes, most notably due to the large number of plant-specific subunits in the membrane arm of the complex. The elucidation of the assembly pathway of Complex I has been a long-standing research aim in cellular biochemistry. We report the study of Arabidopsis mutants in Complex I subunits using a combination of Blue-Native PAGE and immunodetection to identify stable subcomplexes containing Complex I components, along with mass spectrometry analysis of Complex I components in membrane fractions and two-dimensional diagonal Tricine SDS-PAGE to study the composition of the largest subcomplex. Four subcomplexes of the membrane arm of Complex I with apparent molecular masses of 200, 400, 450, and 650 kDa were observed. We propose a working model for the assembly of the membrane arm of Complex I in plants and assign putative roles during the assembly process for two of the subunits studied.

NADH-ubiquinone oxidoreductase (Complex I, EC 1.6.5.3) is the main entry point for electrons in the mitochondrial respiratory chain. It is the largest and most complicated complex of the respiratory chain. In higher eukaryotes, it is composed of more than 40 subunits. It has a dual genetic origin: 5 to 9 subunits are encoded by the mitochondrial genome, and the others are encoded by the nuclear genome. This complex consists of two parts forming an L-shaped structure: the membrane arm forms the base of the L and is embedded within the inner mitochondrial membrane, while the matrix arm forms the side of the L, bounded at one end of the membrane arm and perpendicularly protruding into the soluble matrix space. The matrix arm contains all the Fe-S clusters and the NADH oxidizing activity, while the membrane arm contains the ubiquinone-binding site. The detailed composition, mechanistic analysis of function, and elucidation of the assembly pathway of Complex I have been long-standing areas of research in a range of different species.

The localization of the different subunits within the two arms of mitochondrial Complex I has been extensively investigated in the bovine enzyme. This purified Complex I has been fragmented into three parts: \( \lambda \), corresponding to the matrix arm, \( \beta \), corresponding to the membrane arm, and \( \alpha \), the matrix arm and some membrane subunits that allow the anchoring of the matrix arm in the inner membrane (1). Complex I assembly has been most intensively investigated using mutants of genes encoding Complex I subunits in Neurospora crassa (2) and human (3, 4). Some mutants lacking one Complex I subunit do not contain a fully assembled Complex I but accumulate stable subcomplexes of Complex I. The formation of stable assembly subcomplexes can be identified using antibodies raised against specific Complex I subunits (2–4). Other approaches used to study Complex I assembly have involved following the reassembly of Complex I after treatment with mitochondrial translation inhibitors (5), use of a tagged subunit to determine the entry point of mitochondrial encoded subunits (6), or monitoring Complex I assembly by radiolabeling techniques (7).

Taken together, all these data show that the assembly of Complex I follows an evolutionarily conserved sequence (for review see Ref. 8). The matrix arm is assembled independently in the mitochondrial matrix. The assembly of the membrane arm is initiated within the inner membrane. The assembled matrix arm is grafted onto this pre-complex, and finally the membrane arm is expanded to terminate the process. In Escherichia coli, a similar assembly pathway has been deduced from a combination of genetic studies (9, 10).

To date, eight assembly factors, AIF (11), CIA30 (12), Ecsit (13), B17.2L (14), C6ORF66 (15), C8ORF38 (16), C20ORF7 (17), and C3ORF60 (18) have been described for human Complex I. The function of these factors during Complex I assembly has not been precisely elucidated, but a model for the sequence of human Complex I assembly, including these assembly fac-
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tors, has been proposed (19). Moreover, several of these assembly factors have been associated with roles other than Complex I biogenesis, indicating a possible link between Complex I activity and other cellular processes (20). Other proteins described as Complex I assembly factors are proteins involved in the assembly of the iron-sulfur proteins such as Ind1 (21, 22), suggesting that the assembly of Complex I without Fe-S centers is not possible.

In plants, mitochondrial Complex I differs from the enzyme described in non-photosynthetic eukaryotes. The analysis of Arabidopsis Complex I indicates that >30% of the subunits are specific to plants (23–27). Electron microscopy images of purified Complex I show the presence of an additional domain facing the matrix (24). Taken together these data suggest that plant Complex I has evolved function(s) linked to the specific roles of mitochondria in plants. A recent study has shed the first light onto the internal architecture of Complex I in plants. Arabidopsis Complex I was purified and broken down into smaller fragments using low concentrations of detergent. The composition of the different fragments was determined by mass spectrometry (MS), and a model for Complex I disassembly was established (26). The comparison of these data with the internal architecture of bovine Complex I (1) shows that the composition of the matrix arm is very similar between plants and mammals. On the other end, the membrane arm of plant Complex I differs from the one of mammals. The plant-specific subunits were all found in fragments of the membrane arm (26). This behavior could be predicted for some plant-specific subunits by hydrophobicity analysis and had been described for the carbonic anhydrase-like proteins in Complex I for some time (24). Because the membrane arm of plant Complex I is different from non-photosynthetic eukaryotes Complex I, efforts have to be made to understand its organization, function(s), and assembly.

Complex I assembly in plants is not currently understood. Combining studies of maize, tobacco, or Arabidopsis mutants, a model for Complex I assembly in plant has been proposed (8). However, this model was based on the very little direct experimental data on the internal organization of Complex I available at that time. Moreover, the number of mutants defective in Complex I described in higher plants is much smaller than the number of Complex I mutants available in mammals and fungi. In the model plant Arabidopsis, only a few mutants in nuclear encoded Complex I subunits have been described (28–30). Recently, the study of genes involved in the expression of the mitochondrial genome has allowed the identification of new mutants impaired in Complex I (31–33). Other mutants affected in the expression of mitochondrial Complex I genes have also recently been described, but the effect on Complex I abundance or activity has not yet been shown (34–38). These mutants provide vital tools for the analysis of Complex I assembly.

Little is known regarding factors involved in the assembly of Complex I in plants. The only potential Complex I assembly factor described so far in plants is the L-galactonolactone dehydrogenase (GLDH) in Arabidopsis (39). This enzyme is involved in ascorbate synthesis and has been identified in a smaller version of Complex I but not in the mature complex (23). The actual role of GLDH in Complex I assembly is unknown. Altogether, this suggests a variation in Complex I assembly pathway between the animal and plant kingdoms. A forward genetic screen has been designed in Chlamydomonas to identify new assembly factors but to date only mutants in the PSDW subunit have been characterized (40).

In this report we have studied Arabidopsis mutants in Complex I subunits using Blue-Native PAGE to identify Complex I subcomplexes. The analysis of these subcomplexes provides new information on the composition and assembly of the membrane arm of Arabidopsis Complex I.

EXPERIMENTAL PROCEDURES

Plant Material and Culture Conditions—The T-DNA insertion lines SALK_072274 (cal2), SALK_019040 (39kDa), and SALK_113376 (b12) were obtained from the seed stock center of the Arabidopsis Biological Resources Center (Ohio State University, available on-line). Arabidopsis seeds were surface-sterilized in a solution containing 70% (v/v) ethanol and 0.1% (v/v) Tween 20 for 5 min, washed first in 70% (v/v) ethanol, and then in 100% (v/v) ethanol, dried, and plated on 0.5 × Gamborg B5 medium containing 1% (v/v) sucrose and 0.7% (w/v) agar. Seeds were cold-stratified at 4 °C for 2 days and placed in a growth cabinet under a long-day photoperiod (16 h of light at 22 °C and 8 h of dark at 18 °C). Two-week-old seedlings were transferred on soil and grown in a growth chamber under a short-day photoperiod (8 h of light at 22 °C and 16 h of dark at 18 °C).

Isolation of Mitochondria—Mitochondria were extracted from the aerial part of short-day-grown plants according to Meyer et al. (28).

One-dimensional SDS-PAGE and Transfer—Mitochondrial proteins (20 µg) were solubilized in sample buffer (2% SDS, 125 mM Tris-HCl, 10% glycerol, 10% β-mercaptoethanol, 0.002% bromphenol blue, pH 6.8) and heated at 95 °C for 5 min. The samples were loaded on 12% (w/v) polyacrylamide, 0.1% (w/v) SDS gels. The separated proteins were transferred onto a PVDF membrane (Immobilon-P, Millipore) in transfer buffer (58 mM Tris, 39 mM glycine, 0.0375% SDS (w/v), 20% (v/v) methanol) using a semi-dry blotter ( Hoeffer).

BN-PAGE in Gel Activity and Transfer—Blue-Native PAGE (BN-PAGE) was performed according to the method described by Jänisch et al. (41). Mitochondrial proteins (100 µg) were solubilized with dodecylmaltoside (1% (w/v) final) in 75 µL of Aca buffer (750 mM aminocaproic acid, 0.5 mM EDTA, 50 mM Tris-HCl, pH 7.0) and incubated 20 min at 4 °C. The samples were centrifuged 10 min at 20,000 × g, and Serva Blue G (0.2% (w/v) final) was added to the supernatant. The samples were loaded onto a 4.5–16% (w/v) gradient gel. In-gel nitro blue tetrazolium (NBT) reduction activity staining was performed according to Zerbetto et al. (42). The gel was washed three times for 5 min with distilled water and incubated in the reaction medium (0.14 mM NADH, 1.22 mM NBT, 0.1 mM Tris-HCl, pH 7.4). When the dark-blue stain was visible, the reaction was stopped by transferring the gel to 40% (v/v) methanol/10% (v/v) acetic acid. For the transfer onto the PVDF membrane (Immobilon-P, Millipore), the BN gels were washed in cathode buffer (50 mM Tricine, 15 mM Bis-Tris, pH 7.0) after migration. The complexes were then transferred under native conditions in cathode buffer
for 16 h at 4 °C. After transfer, the membranes were stained for 5 min in staining buffer (30% (v/v) methanol, 7% (v/v) acetic acid, 0.005% (w/v) Coomassie Blue R250). The background was reduced by washing the membranes in 30% (v/v) methanol/7% (v/v) acetic acid. Native molecular weight markers (66–669 kDa, GE Healthcare) were used to estimate the size of complexes and subcomplexes.

**Protein Overexpression and Production of Antibodies**—cDNAs encoding for the C-terminal part of the 18-kDa subunit (Glu37–Asn154), the C-terminal part of MWFE (Met22–Ser65), and NDU9 (Met1–Asp110) were cloned in the Gateway entry vector pDONR207 (Invitrogen) and subsequently transferred to the destination vector, pDEST15 (Invitrogen), which allows the N-terminal fusion of GST. These chimeric proteins were over-expressed in *E. coli* BL21 pLysS cells and purified on GST-Sepharose. The purified proteins were injected into independent rabbits (4 doses of 250 μg per protein). The rabbits were bled after the third and fourth injections, and the sera were used unpurified in immunodetection experiments.

**Immunodetection**—Membranes were incubated with the primary antibodies at the dilution indicated in Table 1. Secondary antibodies linked to horseradish peroxidase were used, and the signals were detected by chemiluminescence (GE Healthcare). The images were recorded using a Luminescent Image Analyzer (LAS 100, Fuji, Japan).

**Fractionation of Mitochondria and Digestion**—Total mitochondria (500 μg) were freeze/thawed (liquid nitrogen/room temperature) three times and then centrifuged at 20,000 × g for 15 min at 4 °C to separate the soluble fraction (supernatant) from the membrane fraction (pellet). 50 μg of each fraction was acetone-precipitated for 2 h at −80 °C, centrifuged at 20,000 × g for 20 min at 4 °C, and the pellets were air-dried. Each pellet was resuspended in 88 μl of 50 mM NH₄HCO₃. After adding 1 μl of 45 mM DTT, the samples were incubated 15 min at 50 °C. Then 1 μl of 0.1 M iodoacetamide was added, and the sample was further incubated for 15 min at 25 °C before the addition of 10 μl of trypsin 0.1 μg/μl. The samples were incubated at 37 °C for 16 h, and the reaction was stopped by adding 5 μl of 10% (v/v) TFA. The samples were dried down before the MS analysis.

**Mass Spectrometry**—Samples were analyzed on a 6510 Q-TOF mass spectrometer (Agilent Technologies) with an HPLC Chip Cube source. The chip consisted of a 40-nl enrichment column (Zorbax 300SB-C18 5 μm) and a 150-mm separation column (Zorbax 300SB-C18 5 μm) driven by the Agilent Technologies 1100 series nano/capillary liquid chromatography system. Both systems were controlled by using MassHunter Workstation Data Acquisition for Q-TOF (version B.02.00, Build 1128.5, Patches 1–3, Agilent Technologies) software, and then the same sample was run again. Following the second run a new list of excluded peptides was added to the previous list, and the new list was loaded for the third run. Resulting mzdata.xml files were concatenated into a single mzdata.xml file using mzdataCombinator v1.0.6 (The University of Western Australia Centre of Excellence for Computational Systems Biology, available online) and were then searched against an in-house Arabidopsis database comprising ATH1.pep (release 9) from The Arabidopsis Information Resource (TAIR) and the mitochondrial and plastid protein sets (TAIR). This sequence database contained a total of 33,621 protein sequences (representing 13,487,170 residues). Searches were conducted using the Mascot search engine version 2.2 (Matrix Science) utilizing error tolerances of ±100 ppm for MS and ±0.6 Da for MS/MS. “Max Missed Cleavages” was set to 1, the Oxidation (M), Carbamidomethyl (C), variable modifications, and the Instrument were set to ESL-Q-TOF, and the Peptide charges were set at 2+ and 3+. Results were filtered using “Standard scoring.” “Max. number of hits” was set to AUTO, “Significance threshold” was set at p < 0.05, and the “ions score cut-off” was 32. The false discovery rate, calculated by automatically searching the data against a decoy database, for these datasets, using an ion score cut-off of 32, was 3.75%. Several smaller peptides with an ion score of 27–32 were included following manual inspection of spectra. Peak lists used in searches are available through the ProteomeCommons Tranche Project “Composition and Assembly Plant Complex I through Analysis of Subcomplexes in Arabidopsis” at www.proteomecommons.org.

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The data associated with this manuscript may be downloaded from ProteomeCommons.org Tranche (http://www.proteomecommons.org) using the following hash: LBj3sPNyKv6XzPm6w2R54UpkCjDzv9mtXbD8dovdBs+LTSceyTkcwWoKqSsY2KZUM1j91J98f59boZta77oAAAAAADyg==. The hash may be used to prove exactly what files were published as part of this manuscript’s data set, and the hash may also be used to check that the data has not changed since publication.
As migration, the gels were fixed in 40% (v/v) ethanol/10% (v/v) acetic acid for 2 h. The gels were then stained with the Flamingo Fluorescent gel stain (Bio-Rad) for 3 h. The background was reduced by washing the gels in 0.1% (v/v) Tween 20 for 10 min. After rinsing the gels in distilled water, they were scanned using a Typhoon Tryo scanner (GE Healthcare) using the settings for SYPRO Ruby stain detection.

**Table 1**

| Subunit | Calculated molecular mass | Antibodies type | Dilution used | Reference |
|---------|---------------------------|-----------------|---------------|-----------|
| MWFE    | 8                         | Polyclonal      | 1/1000        | This work |
| Nad6    | 23                        | Polyclonal      | 1/1000        | (33)      |
| Nad9    | 23                        | Polyclonal      | 1/20000       | (49)      |
| CA2     | 31                        | Polyclonal      | 1/10000       | (29)      |
| 18 kDa  | 18                        | Polyclonal      | 1/2000        | (28)      |
| NDU9    | 9                         | Polyclonal      | 1/1000        | This work |
| PSST    | 20                        | Polyclonal      | 1/5000        | Zickermann et al., unpublished |

**Figure 1.** Characterization of the antibodies used in this study. Each of the antibodies is named after the subunit it recognizes, A, Western blots on SDS-PAGE of wild-type mitochondrial fractions. The molecular weights are indicated on the left. B, BN-PAGE separation of mitochondrial complexes from WT sample. The position of Complexes I (CI) and III (CIII) of the respiratory chain and of the ATP synthase (holoenzyme (CV) and F1 part (F1)) were revealed by Coomassie staining of the gel (lane 1) and are indicated on the left. The presence of Complex I was revealed by NADH-dependent NBT reduction staining (lane 2). Western blot analysis was carried out with antibodies directed against the subunits MWFE (lane 3), Nad6 (lane 4), Nad9 (lane 5), CA2 (lane 6), 18kDa (lane 7), NDU9 (lane 8), and PSST (lane 9). Note that the antisera against the 18 kDa and PSST subunits caused nonspecific detection of the abundant ATP synthase (Complexes V and F1).

**Figure 2.** Characterization of the Complex I mutants used in this study. Mitochondrial fractions were separated on two BN gels. The first gel shows staining for NBT reduction activity (A), and the second shows staining with colloidal Coomassie (B). The size in kilodaltons of Complex I (1000), Complex V (600), and Complex III (480) are used as molecular weight marker and are indicated on the left. The asterisk indicates the presence of Rubisco in some fractions.
RESULTS

Characterization of Complex I Antibodies—We generated antibodies against three Complex I subunits (18kDa, MWFE, and NDU9) and obtained antibodies raised against CA2, Nad9, Nad6, and the PSST subunit (see Table 1 for summary). In Western blot experiments on wild-type mitochondrial fractions, all the antibodies we used recognized a protein with a molecular weight corresponding to the calculated value (Fig. 1A and Table 1). We then separated mitochondrial protein complexes from wild-type plants on BN-PAGE, transferred the complexes under native conditions to PVDF membranes, and probed them with the antibodies against Complex I subunits. All the antibodies used in this study are able to recognize the Complex I proteins from the native complex (Fig. 1B), indicating that all of them could be used to search for subcomplexes of Complex I from BN-PAGE.

Collection and Isolation of Complex I Mutants—In many species, cells that do not express one Complex I subunit are unable to form a fully assembled Complex I but accumulate subcomplexes of Complex I. To investigate whether this is the case in plant cells, we collected reported mutants deficient in at least one Complex I subunit. These mutants are ndufs4 (At5g67590), ndufa1 (At3g08610), ca2 (At1g47260), and css1 (At1g30010). The ndufs4 and ndufa1 mutants lack the 18kDa and MWFE subunits, respectively (28). These two mutants will be called 18kDa and mwfe in this report to be consistent with the nomenclature used in other studies of Complex I composition in plants. ca2 lacks a plant-specific subunit of Complex I (29). css1 is a mutant that is affected in the expression of nad4 (34). We isolated Arabidopsis lines lacking Complex I subunits CAL2, 39kDa, or B12 due to insertional inactivation of cal2 (At3g48680), 39kDa (At2g20360), and b12 (At2g02510), respectively. These mutant lines were isolated from T-DNA insertion lines SALK_072274 (cal2), SALK_019040 (39kDa), and SALK_113376 (b12) (supplemental Fig. S1A). Homozygous mutants did not show any visible growth phenotype when grown under standard conditions (supplemental Fig. S1B).

Complex I Levels in the Mutants—Mitochondrial fractions were extracted from wild-type and mutant plants. In this study we loaded whole mitochondrial protein extract on the BN gels to detect any potential subcomplex whether soluble or associated with the membrane fraction. A typical BN gel was stained for NADH-dependent NBT reduction activity and is shown in Fig. 2A. In the wild-type lane, Complex I was strongly stained at ~1000 kDa, and an additional band was detected at ~800 kDa. This band very likely corresponds to the smaller version of Complex I that has previously been described in plant mitochondria (23). Some low intensity NBT reduction staining was also seen at 300–400 kDa in wild type and is of unknown origin, and some intense staining was seen at ~50 kDa, which we have
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previously identified as lipoamide dehydrogenase (28). These two staining patterns remained unchanged in the mutant analysis. The activity staining revealed different patterns of staining among the mutants. 

850 kDa was also detected in the wild-type sample. The intensity of this band was increased in 

ca2 and css1. Interestingly, no additional in-gel-staining activities were visible in the mutants, suggesting that no detectable subcomplexes have maintained an NBT reduction activity.

Similar BN gels were run, and the complexes were either transferred to a PVDF membrane or stained with Coomassie Blue (Fig. 2B). Complex I abundance varied from non-detectable levels (18kDa) to wild-type-like levels (b12 and cal2). No new complexes, absent from the wild-type sample, were detected by Coomassie staining in any of the mutants. Altogether, these data indicate that some Complex I subunits are more critical than others for Complex I assembly and activity.

Identification of Subcomplexes—To identify potential subcomplexes or partially assembled Complex I, we decided to use an immunological approach in line with reports in other species. We reasoned that, although subcomplexes might exist at very low abundance in wild type, they were likely to accumulate to detectable levels in mutants that blocked the full assembly pathway. We probed PVDF membranes of mitochondrial complexes separated by BN-PAGE with several antibodies specific for different Complex I subunits. We reproduced each immunodetection several times using a different membrane for each replicate experiment to ensure the patterns observed are reproducible; representative images are given in Fig. 3 (a representative immunodetection obtained with each class of antibodies is presented in supplemental Fig. S2). Using antibodies directed against the subunits of either MWFE, NDU9, or PSST, an additional band was only detected in the 18kDa and 39kDa mutants (Fig. 3A). This band corresponds to a complex of ~650 kDa and was also detected in both mutants with the antibodies raised against Nad6 (Fig. 3B). The Nad6 antibodies also reacted with a 450-kDa complex in 18kDa and 39kDa and another complex of 400 kDa in 18kDa, 39kDa, mwfe, and css1 (Fig. 3B). The antibodies against 18kDa did not recognize any other complexes that were not present in the wild-type lane (supplemental Fig. S2). The antibodies against Nad9 did not recognize any specific subcomplex related to Complex I. The apparent faint detection of a 400-kDa subcomplex in 39kDa (Fig. 3C) was not reproducible, and when it was detected, it was also present in the wild-type samples on images obtained after longer exposure (supplemental Fig. S3).

Finally, the antibodies against CA2 recognized the 650-kDa complex in 18kDa and 39kDa but also the 450- and 400-kDa complexes in 18kDa, 39kDa, mwfe, and css1 mutants (Fig. 3D). Additionally, a 200–kDa complex is detected in 18kDa, mwfe, and css1 (Fig. 3D). This complex was also detected once in a mitochondrial protein extract from the 39kDa mutant. Thus, overall, the set of antibodies used in this study allowed us to identify four subcomplexes of Complex I, with apparent molecular masses of 200, 400, 450, and 650 kDa. Although it cannot be concluded with certainty that subcomplexes of a given size observed in different mutants are exactly identical in composition, it seems unlikely that their composition in different mutants would be altered by more than a few subunits. Such a size difference would probably not be detectable on BN gels.

To confirm that the subcomplexes identified were not the result of nonspecific reactions of the antibodies, we further investigated the composition of these subcomplexes in the 18kDa mutant (because it contained all subcomplexes identified) using two-dimensional electrophoresis. Mitochondrial complexes from 18kDa plants were analyzed using BN-PAGE in the first dimension and SDS-PAGE in the second dimension (Fig. 4A). After transfer of the proteins on a PVDF membrane, a Western blot analysis was performed using the five antibodies that detect a subcomplex in this mutant (Fig. 4B). The signals from Complex I subunits corresponding to the size of subcomplexes in the BN-PAGE dimension, are found specifically and at the correct molecular mass in the SDS-PAGE dimension. This
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To specifically analyze the composition of the most common subcomplex detected in the analysis of the BN-PAGE with antibodies most likely represent specific reactions of those antibodies to Complex I subunits.

Analysis of the Composition of Subcomplexes—To complement the immunological approach, we designed a proteomic strategy to look for the presence of Complex I subunits in soluble and membrane fractions from wild-type and 18kDa plants. The fractions were analyzed by non-gel complex mixture LC-MS/MS, and the presence of Complex I subunits was monitored in the background of the mitochondrial soluble and membrane proteome. In wild-type mitochondria, 18 Complex I subunits were detected in the membrane fraction through identification of 34 unique peptides using a total of 73 distinct MS/MS spectra. Only one Complex I protein, B13 subunit, was represented by one peptide in a single MS/MS spectra in the soluble fraction (Table 2). This indicates that the fractionation performed was mild enough to avoid Complex I disruption. In 18kDa, 8 of these 18 Complex I subunits were detected, all 8 were found in the membrane fraction, while 2 were also present in the soluble fraction (Nad7 and Nad9). Thus 18kDa retained the presence of the mitochondrial-encoded subunits and all the plant-specific CA-like proteins but lacked identification of 10 subunits, notably the Fe-S and FAD binding subunits: 75kDa, 51kDa, and 24kDa. Although this approach is not adapted for the analysis of individual subcomplex compositions, it is consistent with membrane-located subcomplexes being present, and thus this list of membrane-retained subunits can be considered as putative components of the assembly subcomplexes of the membrane arm of Complex I.

To specifically analyze the composition of the most commonly observed and the largest of the subcomplexes, the 650-kDa complex on BN-PAGE. We excised the band corresponding to Complex I in wild type and the portion of the gel located at 650 kDa in 18kDa, separated the proteins in the two samples on two-dimensional Tricine gels (25), and stained with a high sensitivity fluorescent dye (Flamingo, Bio-Rad). The fluorescent dye improved the visual detection limit for the low abundance subunits, and we overlaid the two images to compare the protein profiles (Fig. 5). Because the intensity of most spots was too low for MS analysis, we annotated the spots detected by comparison with the reference profiles obtained for respiratory complexes (25). Almost all subunits of Complex V are found in the 650-kDa sample (green spots in Fig. 5). This was expected as the 650-kDa subcomplex is located on BN-PAGE just above Complex V (~600 kDa in Arabidopsis), the most abundant complex of the membrane fraction. Sixteen spots present in Complex I are absent from the 650-kDa subcomplex (red spots in Fig. 5), and 17 spots are present in both samples. This analysis and comparison to identifications in Ref. 25 allowed us to estimate which Complex I subunits were present in the subcomplex and which were absent (Table 3). Most of the subunits detected in the membrane fraction by the MS approach were deduced to be present in the 650-kDa subcomplex on the analysis of the two-dimensional Tricine gel.

**DISCUSSION**

In this study we investigated the assembly of Complex I in mutants deficient for Complex I subunits. We identified Complex I subcomplexes in several mutants investigated. The b12 and cal2 mutants present a wild-type-like profile with normal levels of Complex I and no subcomplexes. The plant ortholog of the B12 subunit was only discovered recently in our earlier study of Complex I composition in Arabidopsis (25). Bioinformatic analysis indicated that two genes (At2g02510 and At1g14450) encode this protein, with 84% similarity between the two gene products (27). Thus the mutation in At2g02510 reported in this study is most likely not eliminating the B12 subunit from the mitochondrial fraction. CAL2 is part of a family of five carbonic anhydrase/carbonic anhydrase-like subunits. Another member of this family could compensate for its absence in the cal2 mutant background as the genes encoding

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**TABLE 2**

Analysis by complex mixture LC-MS/MS of the soluble and membrane fractions of 18kDa and wild-type mitochondria

| AGI Subunit | Soluble WT | Membrane WT | Soluble 18kDa | Membrane 18kDa |
|-------------|------------|-------------|---------------|----------------|
|            | Score Pept | Score Pept  | Score Pept    | Score Pept     |
| At1g47260   | CA2        | 172 (4)     | 179 (5)       |
| At2g20360   | 39kDa      | 165 (3)     | 127 (2)       |
| At5g37510   | 75kDa      | 165 (4)     | 127 (2)       |
| At5g8530    | 51kDa      | 120 (2)     | 79 (2)        |
| At1g00070   | Nad9       | 104 (1)     | 77 (2)        |
| At5g47890   | B8         | 101 (2)     | 101 (2)       |
| At5g03100   | DAP13      | 75 (2)      | 144 (2)       |
| At5g20640   | B13        | 67 (3)      | 137 (2)       |
| At2g27730   | 16kDa      | 60 (2)      | 115 (3)       |
| At1g66510   | CA3        | 59 (1)      | 59 (1)        |
| At1g00510   | Nad7       | 59 (2)      | 115 (3)       |
| At4g02580   | 24kDa      | 58 (2)      | 58 (2)        |
| At5g63150   | CAL1       | 53 (2)      | 53 (2)        |
| At3g48680   | CAL2       | 53 (2)      | 53 (2)        |
| At3g18410   | PDSW       | 45 (1)      | 45 (1)        |
| At1g14450   | B12        | 37 (1)      | 37 (1)        |
| At4g20150   | Ndu9       | 37 (1)      | 37 (1)        |
| At1g00285   | Ndu2       | 37 (1)      | 37 (1)        |
| At2g31490   | Ndu8       | 34 (2)      | 60 (1)        |

* AGI, Arabidopsis Gene Identifier. Score: peptide scores were assigned by Mascot. Pept: number of peptide ions identified that match the protein sequence with number of distinct MS/MS spectra in parentheses.
the different carbonic anhydrases could represent functionally redundant isoforms (27). Despite these issues, other mutants provided evidence for the presence/absence of subunits in a membrane subcomplex of Complex I, and we propose a new assembly model for this membrane arm in plants based on this empirical dataset.

Identification of Subcomplexes

In our work we identified subcomplexes of similar size in different mutants. We have shown by Western blots that a few subunits are common in these subcomplexes. We cannot exclude that a subcomplex of a given size present in different mutants might represent different assembly intermediates, but this is unlikely as the presence of one subunit in two subcomplexes of similar size has not been described in any investigation of the assembly of Complex I to our knowledge.

Assembly of the Membrane Arm—Because almost all Complex I subunits identified in the MS analysis of 18kDa mitochondria are present in the membrane fraction, the subcomplexes identified in this study most likely derive from the membrane arm. This is consistent with what has been observed in other species, namely that both arms of Complex I assemble independently (8, 19). The present work gives insights into the assembly and composition of the membrane arm of Arabidopsis Complex I. CA2 was detected in all subcomplexes indicating that it was most likely incorporated in the early stage of Complex I assembly. This was also confirmed by the non-detection of subcomplexes in ca2. Nad6 was detected in all subcomplexes except in the one of 200 kDa. This is consistent with Nad6 being inserted in the growing Complex I during the transition from the 200-kDa to the 400-kDa subcomplex. The mwfe and css1 mutants contain the 200-kDa and 400-kDa subcomplexes but not the 450-kDa subcomplex, suggesting that the MWFE and Nad4 subunits are not essential for the formation of the 200-kDa and 400-kDa subcomplexes but that the transition from the 400-kDa to the 450-kDa subcomplex requires these two subunits. As the anti-MWFE antibodies did not detect the 450-kDa subcomplex, this subunit is probably not found in this subcomplex but is important for the integration of other subunits (including Nad4) in the 400-kDa subcomplex. Interestingly, MWFE is subsequently associated with the growing Complex I,

FIGURE 5. Two-dimensional Tricine analysis of the 650kDa subcomplex in 18kDa. The portion of the gel containing the 650-kDa in 18kDa was excised, and the subunits were separated on a two-dimensional Tricine gel. A similar gel was run at the same time using Complex I from wild-type sample. After staining, the gels were scanned. The image obtained for Complex I is presented in A, and the image for the 650-kDa subcomplex is in B. The images were converted in false colors (green for the 650-kDa subcomplex and red for Complex I) and overlaid (C). 42 visible spots were identified and labeled as only in Complex I (red), only in the 650-kDa subcomplex (green), and in both samples (yellow). The proteins likely to be present in these spots are given in Table 3 based on previous literature reports (25).
because it is present in the 650-kDa subcomplex. The role of MWFE would thus be consistent with its use to allow the assembly of a subset of mitochondria-encoded subunits. A similar role for MWFE has been proposed in mammals in a conditional assembly system (43). Three subunits (NDU9, PSST, and MWFE) were only detected in the 650-kDa subcomplex and were absent from the smaller subcomplexes. They are proposed to be incorporated into the membrane arm in the later stage of its assembly.

Most of the subunits found in the membrane fraction of 18kDa were identified in subcomplexes of the membrane arm obtained after fractionation of the purified Complex I (26). Our analysis found CA2 to be part of the core module of the membrane arm. In their disassembly study, Klodmann et al. (26) concluded that the carbonic anhydrase module is lost from the membrane arm in the early phase of the disassembly of the membrane arm (transition from 550 to 450 kDa). This indicates that we were most likely observing assembly subcomplexes and not subcomplexes resulting from a disassembly of Complex I.

In plants an additional complex related to Complex I has been described (23). This smaller version of Complex I (Fig. 2A, 800 kDa) is low abundant and contains an additional subunit, the GLDH, the last enzyme in the ascorbate synthesis pathway (23). The exact composition of this complex is not known, but it contains subunits from the matrix and membrane arms (23) and has a NBT reduction activity (39). This complex has been proposed to be an assembly complex lacking part of the membrane arm (39). In a GLDH mutant, no Complex I is detected, suggesting a role of GLDH during Complex I assembly (39). Because no immunodetections were carried out to observe subcomplexes in this mutant, it cannot be concluded yet at which step of Complex I assembly GLDH might be essential.

### TABLE 3

Analysis of proteins present in the two-dimensional Tricine gel separation of WT and 18kDa samples

| Spot | AGI       | Subunit | CI WT | sub650 18kDa | CV 18kDa |
|------|-----------|---------|-------|-------------|----------|
| 1    | At5g37510 | 75 kDa  | +     |             |          |
| 2    | AtMg01190 | ATP1    |       |             |          |
| 3    | At5g08670/At5g08680/At5g08690 | ATP2    |       |             |          |
| 4    | AtMg00060/AtMg00513/AtMg00665 | Nad5    | +     |             |          |
| 5    | AtMg00510 | Nad7    |       | +           |          |
| 6    | At2g20360 | 39 kDa  |       |             |          |
| 7    | AtMg00285/AtMg01320 | Nad2    | +     |             |          |
| 8    | AtMg00580 | Nad4    |       | +           |          |
| 9    | At4g28510 | Prohibitin1 |       |             |          |
| 10   | At5g07770 | Prohibitin3 |       |             |          |
| 11   | At1g32760 | CA2     |       |             |          |
| 12   | At5g36310 | CAL1    | +     | +           |          |
| 13   | At1g19580 | CA1     |       | +           |          |
| 14   | At2g21870 | ATP-FAD |       |             | +        |
| 15   | At4g02580 | 24 kDa  | +     |             |          |
| 16   | AtMg00510 | Nad7    |       | +           |          |
| 17   | At1g79010/At1g16700 | 23 kDa  | +     |             |          |
| 18   | At3g52300 | ATP7    | +     |             |          |
| 19   | At5g25940 | ATM1    | +     |             | +        |
| 20   | AtMg00640 | ATP4    | +     |             |          |
| 21   | At4g47030 | ATP16   | +     |             |          |
| 22   | At3g111770 | 20 kDa  | +     |             |          |
| 23   | AtMg00140/AtMg00830 | ATP6    |       |             |          |
| 24   | AtMg00270 | Nad6    | +     |             |          |
| 25   | At5g7590 | 18 kDa  | +     |             |          |
| 26   | At2g33220/At1g04630 | B16.6  |       |             |          |
| 27   | At3g03100 | DAP13   |       |             | +        |
| 28   | At3g18140/At1g49140 | PDSW    | +     |             |          |
| 29   | At2g27730 | 16 kDa  | +     |             |          |
| 30   | At1g67530 | 11 kDa  | +     |             |          |
| 31   | At4g12260 | B14     | +     | +           |          |
| 32   | At3g63100/At5g18800 | PGIV    | +     | +           |          |
| 33   | At5g00990 | Nad3    | +     | +           |          |
| 34   | At4g00858 | NDU10   | +     |             |          |
| 35   | At5g37570 | ASH1    | +     |             |          |
| 36   | At2g42310/At3g57785 | NDU12  |       | +           |          |
| 37   | At5g7890 | B8      | +     |             |          |
| 38   | At1g51650 | ATP15   | +     |             |          |
| 39   | At2g02510/At1g16700 | B12    | +     |             |          |
| 40   | At4g20150 | NDU9    | +     |             |          |
| 41   | At1g62000 | AGGG    | +     |             |          |
| 42   | At4g30010 | ATP17   | +     |             |          |
| 43   | At3g98610 | MWFE    | +     | +           |          |
| 44   | AtMg01080 | ATP9    | +     | +           |          |
In ca2 and css1 the 850-kDa band carrying a NBT reduction activity increased compared with wild-type samples (Fig. 2A). A similar band has been described in the maize NCS2 mutant (44) and the tobacco NMS1 mutant (39). The later two mutants are impaired in Nad4, a subunit localized in the distal part of the membrane arm of the complex (45). The presence of this band in wild-type samples at very low levels suggests that it is a stable assembly intermediate, a stable degradation product, or a stable miss-assembly product. Because the abundance of this complex was increased in the absence of a subunit from the distal part (Nad4) or the central part (CA2), this complex most likely corresponds to Complex I lacking the distal part of the membrane arm.

The absence of detection of any subcomplexes in ca2 indicated that either none of the subunits tested could assemble into a stable subcomplex when CA2 was missing or the only stable intermediate detectable is the 850-kDa subcomplex.
because it was more abundant in ca2. Taken together our data suggest that, in the presence of CA2, the membrane arm of Complex I can assemble into a 650-kDa complex, and we propose a working model for its assembly in Fig. 6. Because we were not able to detect any subcomplex containing a subunit from the matrix arm, we are unable to determine at which step the matrix arm is anchored onto the membrane arm. More data using other antibodies and mutants, or using alternative strategies are needed to investigate the assembly of the matrix arm and to characterize the assembly pathway of Complex I.

Composition of the Two Arms—Combining the two-dimensional Tricine and the MS approaches, the location of each subunit within the two arms of the Arabidopsis Complex I can be estimated. A summary of these locations is presented in Table 4, together with the data obtained by Klodmann et al. (26, 27) and a prediction of transmembrane domains using HMMTOP (46). Ten subunits are consistently predicted to be in the matrix arm. The membrane arm is composed of at least 29 subunits, including 9 subunits with no predicted transmembrane domain; these subunits are considered to be peripheral proteins. Only nine subunits could not be allocated to either arm because of the absence of data or contradictory results. A more intensive analysis of the organization of Complex I is needed to resolve these issues.

This analysis emphasizes the uniqueness of plant Complex I. Indeed most of the plant-specific subunits are assigned to the membrane arm of this complex, and all of them were identified in the membrane arm by at least one experiment. This suggests that the potential additional functions of Complex I in plants are not linked to the NADH oxidation and the electron transfer to ubiquinone but could be associated with the proton transport activity of the membrane arm. Interestingly, a role in a proton-driven carbonate exporter has been postulated for the carbonic anhydrase subunits of Complex I (47), but proof for this hypothesis also awaits further data.

Roles of the 18 kDa and 39 kDa Subunits—Our study included two mutants of subunits predicted to be localized in the matrix arm (18kDa and 39kDa). In 18kDa the transition from the 650-kDa membrane subcomplex to the fully assembled Complex I did not appear to occur from our data. The 18kDa subunit does not possess any conserved motif that would be evidence of a known function. Possible roles for this subunit that could be proposed include its participation in the assembly of the matrix arm or a role in the anchoring of the matrix arm on the membrane arm. In 39kDa, a similar subcomplex pattern to the 18kDa mutant was observed, however, a much larger portion of Complex I can be assembled. These observations led to the hypothesis that, although the 39kDa subunit may be in the matrix arm, it may not be essential for the interaction between the matrix and membrane arms. This is consistent with the presence of this subunit in the fragment a but not in the fragment b of bovine Complex I (1). Interestingly, neither of these subunits are found in any subcomplexes when purified Arabidopsis Complex I is fragmented (26), suggesting that both have loose/peripheral association with Complex I that is readily lost during mild detergent dissociation. As a consequence, based on the available data, we suggest a chaperone-like/assembly factor role for the 18kDa and the 39kDa subunits in Complex I assembly.

Fate of Non-assembled Subunits—This study also indicates that Complex I subunits not assembled to a membrane-localized complex are extremely difficult to detect in mitochondrial fractions. Indeed, no nuclear-encoded subunits were found in the soluble fraction of 18kDa mitochondria. Interestingly, the only Complex I subunits found in the soluble fraction were the mitochondrial encoded Nad7 and Nad9 that are known to play a structural role in the matrix arm of Thermus thermophilus Complex I (48). These subunits are also found in the membrane fraction, but Nad9 could not be detected in the 650-kDa subcomplex. More importantly, we were unable to detect any assembly intermediate of the matrix arm as soluble proteins in the 18kDa mutant. We cannot exclude that such intermediates were lost during sample preparation or somehow masked from detection in the mutant, but it was remarkable that we were unable to detect any peptides from the Fe-S subunits in the soluble fraction of 18kDa. Also, in our previous study of 18kDa we observed a strong reduction in these Complex I subunits in mitochondrial fractions on two-dimensional gels but no regulation of the expression of nuclear genes encoding Complex I subunits (28). We propose that a degradation machinery or translational control machinery is present in this mutant to deplete unassembled Complex I subunits from the mitochondrial matrix. Such post-translational regulation would be consistent with the notion that the NADH oxidation activity of the matrix arm alone is toxic for the mitochondria if it is not coupled to the quinone reduction.

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