Novel Nuclear-encoded Subunits of the Chloroplast NAD(P)H Dehydrogenase Complex*§

Received for publication, July 16, 2008, and in revised form, October 28, 2008 Published, JBC Papers in Press, October 28, 2008, DOI 10.1074/jbc.M805404200

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The NAD(P)H dehydrogenase (NDH) complex functions in photosystem I cyclic electron transfer in higher plant chloroplasts and is crucial for plant responses to environmental stress. Chloroplast NDH complex is a close relative to cyanobacterial NDH-1L complex, and all fifteen subunits so far identified in NDH-1L have homologs in the chloroplast NDH complex. Here we report on the identification of two nuclear-encoded proteins NDH48 and NDH45 in higher plant chloroplasts and show their intimate association with the NDH complex. These two membrane proteins are shown to interact with each other and with the NDH complex enriched in stroma thylakoids. Moreover, the deficiency of either the NDH45 protein or the NDH48 protein in respective mutant plants leads to severe defects in both the accumulation and the function of the NDH complex. The NDH48 and NDH45 proteins are not components of the hydrophilic connecting domain of the NDH complex but are strongly attached to the hydrophilic membrane domain. We conclude that NDH48 and NDH45 are novel nuclear-encoded subunits of the chloroplast NDH complex and crucial both for the stable structure and function of the NDH complex.

Multisubunit protein complexes embedded in the thylakoid membrane, the photosystem (PS)I and PSII complexes, the cytochrome (cyt) b6f complex, and the ATP synthase, are fundamental prerequisites for light harvesting and solar energy conversion into chemical form. Yet there are other thylakoid multiprotein complexes that are essential for plant sustenance under changing environmental conditions. In higher plant chloroplasts, the NAD(P)H dehydrogenase (NDH) complex functions in PSI cyclic electron flow and chlororespiration (1, 2). The expression and activity of the NDH complex in plant chloroplasts are highly regulated, and this complex seems to be crucial for acclimation of the photosynthetic apparatus to environmental cues (3). Under stress conditions like heat and drought, the NDH complex diverts electrons to cyclic electron flow around PSI to avoid over-reduction of the acceptor side of the PSI complex and formation of reactive oxygen species (ROS), thus eventually protecting the photosynthetic apparatus against oxidative stress (3).

The NDH complex in chloroplasts closely resembles the cyanobacterial NDH-1L complex (4). Fifteen subunits of the NDH-1L complex have so far been identified, comprising 7 NDH subunits (NDHA-G) in the hydrophobic domain embedded in the thylakoid membrane and 8 subunits in the peripheral hydrophilic domain (NDHH-O) (5). The same subunits as in cyanobacterial NDH-1L have been found in chloroplast NDH complex (6–8), most of them being encoded by the chloroplast genome and only three (NDHM, NDHN, and NDHO) by the nuclear genome. The catalytically active subunits (homologs of NuoE, NuoF, and NuoG in Escherichia coli) that constitute the separate electron input device, if present, remain unknown both in cyanobacteria and chloroplasts.

Recently, several Arabidopsis thaliana (referred hereafter Arabidopsis) mutants with a defective NDH complex have been identified, based on chlorophyll (Chl) fluorescence characteristics typical for plants lacking NDH activity (9) and on the analysis of the epitope-tagged NDH complexes (7). By these methods, several auxiliary proteins that participate in different phases of biogenesis of the NDH complex, from gene transcription to the stability of the complex have been characterized (7, 9–14). Here we have identified the novel proteins At1g15980 and At1g64770 in Arabidopsis chloroplasts by a proteomic approach and shown their intimate association with the NDH complex and hence designated them as NDH48 and NDH45, respectively.

EXPERIMENTAL PROCEDURES

Plant Material and Growth Conditions—Arabidopsis ecotype Columbia plants were used as a wild type (WT). SALK_111363 (15) line possessing a T-DNA insert within At1g64770 gene was screened by immunoblotting (for antibody, see below). The antisense ndh48 mutant plants (as-ndh48) were generated by amplifying 311 nt from the coding region of At1g15980 gene from WT cDNA by PCR using forward primer 5′-GGGGACAAAGTTTGTACAAAGAAGCAG-
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GCTGGCTTCTCATTCTGGTTACC and reverse primer 5′-GGGGACCACCTTTGACAAAGAAGCTGGTC-
AAGCTGACGGTGTAACCCCTTGG. The PCR product was cloned into pDONR201 vector (Gateway® Cloning,
Invitrogen), and thereafter in the antisense orientation into
pK2WG7 vector under the control of the CaMV 35S promoter.
The vector was transformed by electroporation into the
Agrobacterium tumefaciens strain C58 (16). Arabidopsis plants
were transformed by the floral dip method (17), and seeds har-
vested from the transformed plants were germinated on MS
plates containing 2% sucrose and 50 mg liter−1 kanamycin sul-
fate. Arabidopsis mutants lacking the NDHO protein (SALK_068922) encoded by the At1g74880 gene, and plants
defective in nucleus-encoded factor, CRR2, essential for proper
expression of the NDHB gene were used as controls (7, 9).

Plants were grown under a photon flux density of 150 μmol
of photons m−2 s−1 in 16-h light regime at 23 °C. Experiments
were performed with 3-week-old full-size plants if not other-
wise stated.

Statistical Analyses—The numerical data were subjected to
statistical analysis by Student’s t test with statistical significance
at the p values <0.001.

Southern Blotting—Southern blotting of 2 μg of HindIII-di-
gested WT and SALK_111363 genomic DNA was made according to the standard procedures (18). A 665-bp probe was
PCR-amplified by SALK_111363 genomic DNA using T-DNA
specific primers 5′-GCTTTGAAGACGTGGTTGGAACG
and 5′-CGCAGCACTCTCAAGACG.

Northern Blotting—Total RNA was isolated with TRIzol
(Invitrogen) according to the Tri reagent total RNA isolation
protocol (Sigma), and Northern blotting was performed
according to a standard procedure (18).

Isolation, Separation, and Detection of Proteins—Thylakoid
membranes for immunoblotting were isolated as described in
Ref. 19. Intact chloroplasts were isolated as in Ref. 20, broken up
with shock buffer (50 mM Hepes-KOH pH 7.6, 5 mM sorbitol, 5
mM MgCl2) followed by separation of the soluble stroma and
thylakoids by centrifugation at 18,000 × g, 4 °C for 20 min.
Thylakoid subfractionation into grana and stroma membranes
was performed as in Ref. 20. Detaching of the peripheral thyla-
koid proteins with 2M NaBr was performed as described in Ref.
21. The digestion of isolated thylakoid membranes with trypsin
(10 μg/ml) was performed for 20 min on ice. Chl was deter-
mined according to Ref. 22 and Chl per leaf area as described in
Ref. 23.

The polypeptides were separated with SDS-PAGE (15% poly-
acrylamide, 6 M urea) (24), and after electrophoresis the pro-
teins were electroblotted to a polyvinylidene difluoride mem-
brane (Millipore, Watford, Herts, UK). Gels were loaded
depending on the linearity of the antibody used (3 μg of Chl: NDH45, NDH48, NDHH, Cytf; 0.5 μg of Chl: D1, CP43, PsbO;
0.2 μg of Chl: Atpβ, PsAB, RbcL). Blue native (BN)-PAGE and
two-dimensional BN/SDS-PAGE were performed as described in
Ref. 25. Immunoblotting using chemiluminescence detection
was performed according to standard procedures. NDH48
and NDH45 antibodies were produced by immunizing rabbits
with synthetic peptides LTERGK qTYLENK, VYPDHH-
WPEPAEYT and ESSGWFGSEEGKPG, NTPPSKFET-
IDQGR, respectively (Eurogentec, Seraing, Belgium). Quant-
ification of different proteins was carried out with Fluor
ChemTM 8000 image analyzer (Alpha Innotech Corporation,
San Leandro, CA).

In Gel Trypsin Digestion and Sample Preparation for Mass
Spectrometry—In gel trypsin digestion and sample preparation
for mass spectrometry were performed as described in Ref. 26.
MALDI-TOF (matrix-assisted laser desorption-ionization
time-of-flight) analysis was performed in a reflectron mode on
a Voyager-DE PRO mass spectrometer (Applied Biosystems).

Measurements of Photosynthetic Parameters—The maximal
photophysical efficiency of PSII was determined as Fv/Fm
using a Hansatech Plant Efficiency Analyzer (King’s Lynn, UK)
after 30 min of dark incubation. The redox state of P700 was
measured with a PAM fluorometer (PAM-101/102/103, Walz)
equipped with an ED-P700-DW-E unit by monitoring absorb-
ance changes at 810 nm and using 860 nm as a reference. Leaves
were kept in the dark for 3 min prior to the measurement. The
P700 was oxidized by far-red light from a photodiode (FR-102,
Walz) for 30 s, and the subsequent re-reduction of P700 in the
dark was monitored. Post-illumination increase in Chl fluores-
cence, the F0 “rise”, was monitored as described in Ref. 27.

NAD(P)H Oxidation Assays—Thylakoids were isolated via
intact chloroplasts (see above), and the oxidation of NAD(P)H
was measured at 25 °C in the presence of either duroquinone or
ferricyanide as described in Refs. 2, 28.

H2O2 Detection by 3,3′-Diaminobenzidine (DAB) Method—
H2O2 accumulation in leaves was detected with the DAB
method as described in Ref. 29. The DAB solution was incor-
porated into detached 14-day-old plants overnight in darkness,
and the accumulation of H2O2 was studied after exposure of
plants to growth light conditions.

RESULTS

Novel Thylakoid Proteins Encoded by At1g15980 and
At1g64770 Genes—A proteome analysis of ribosome-nas-
cent chain complexes (associated with fragments of stroma
thylakoids) from Arabidopsis chloroplasts revealed several
novel proteins of unknown function (20). They may repre-
sent novel subunits of protein complexes or play a role of
auxiliary proteins in the assembly and/or function of the
thylakoid membrane protein complexes. Here we have addressed the functional role of two nuclear-encoded, NDH-
related proteins (see below), NDH48 (At1g15980) with an
apparent molecular mass of 48 kDa and NDH45 (At1g64770)
of 45 kDa. Both of these proteins possess the chloroplast
targeting signal in the N terminus of the amino acid
sequence. Homology searches (NCBI Blast) revealed that the
NDH48 and NDH45 proteins are conserved among higher plants,
with amino acid identities up to 62 and 52% with corresponding proteins in Oryza sativa, respectively. In con-
text, cyanobacteria entirely lack the close homologs of the
NDH48 and NDH45 proteins (data not shown).

Generation of the Mutant Lines—A reverse genetics
approach was taken to characterize the functional roles of the
NDH48 and NDH45 proteins. The SALK_111363 mutant line
with a T-DNA insert within the At1g64770 gene was ordered
from the SALK mutant collection. According to the immuno-
**Characterization of the ndh45 and as-ndh48 mutant plants**

**A**, immunoblot of the thylakoid membrane proteins isolated from WT, ndh45, and as-ndh48 plants expressing either 10% (as-ndh48;1) or 20% (as-ndh48;2) of the NDH48 protein compared with that present in the WT plants. **B**, Southern blot of the HindIII-digested WT and SALK_111363 DNA probed with a radiolabeled T-DNA fragment indicating only one T-DNA insert in ndh45 plants. **C**, immunoblot from the soluble stroma (S) and thylakoid (T) fractions of isolated chloroplasts. Immunoblotting was performed with antibodies against NDH45, NDH48, RbcL (Rubisco large subunit), and D1 proteins. Protein gels were loaded on Chl basis, the amounts depending on the linearity of the antibody used (see “Experimental Procedures”).

**FIGURE 2. Interaction of NDH45 and NDH48 with the thylakoid membrane and with each other.** A, immunoblot from the thylakoid membranes isolated from WT plants and washed with 2 m NaBr to detach the peripheral thylakoid membrane proteins. Antibodies against the NDH45, NDH48, NDHH, PsbO, and Atpβ were used. Thylakoid-bound proteins (T) and the proteins released by the high salt treatment (R) are shown. B, immunoblot of the NDH45, NDH48, and PsbO proteins from WT thylakoids exposed to mild trypsin digestion. C, Northern blot demonstrating the transcript levels of the NDH45 and NDH48 genes in the WT and ndh45 plants. 18S ribosomal RNA was used as a control. Protein gels were loaded on Chl basis, the amounts depending on the linearity of the antibody used (see “Experimental Procedures”).

**TABLE 1**

Characteristics of the photosynthetic apparatus in ndh45 and as-ndh48 plants

|                  | WT     | ndh45  | as-ndh48 |
|------------------|--------|--------|----------|
| Chl/leaf area    | 24.4 ± 1.8 | 24.9 ± 1.9 | 25.3 ± 1.3 |
| Chl a/b          | 3.7 ± 0.0 | 3.7 ± 0.1 | 3.6 ± 0.1 |
| Photochemical efficiency | 0.82 ± 0.07 | 0.82 ± 0.07 | 0.83 ± 0.07 |

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treatment were rapidly and efficiently degraded after release (Fig. 2A).

To determine the membrane topology of the NDH45 and NDH48 proteins more precisely, thylakoids isolated from the WT plants were subjected to mild digestion with trypsin, allowing the degradation of only the stroma-exposed parts of the thylakoid membrane proteins. Whereas the NDH48 protein was partially degraded by the protease, the NDH45 protein remained intact, similarly to the luminal control protein, PsbO (Fig. 2B). However, the NDH45 protein seems not to locate within the luminal compartment, because a harsh trypsin treatment that degraded the PsbO protein had hardly any effect on the NDH45 protein (data not shown).

Interestingly, the deficiency of either the NDH45 or the NDH48 protein in the two mutant plants resulted in a total loss or strong down-regulation of the other protein, respectively, from the thylakoid membrane (Fig. 1A). Thus, the accumulation of the NDH45 and NDH48 proteins seemed to be mutually co-regulated. The deficiency of the NDH48 protein from ndh45 thylakoids was more pronounced than the deficiency of NDH45 in the as-ndh48 thylakoids, which is most likely due to a small amount of NDH48 protein left in the as-ndh48 plants. To address the mutual stabilization of the NDH48 and NDH45 proteins, it was examined whether the lack of the NDH48 protein in ndh45 thylakoids results from defects in transcription of the NDH48 gene. Northern blot analysis demonstrated that the amount of the NDH48 mRNA was almost the same in WT and ndh45 plants (Fig. 2C), indicating that the loss of the NDH48 protein in ndh45 thylakoids is dependent on post-transcriptional regulation.

**FIGURE 3.** Localization of the NDH48 and NDH45 proteins in thylakoids isolated from WT Arabidopsis. A, immunoblot from thylakoid membranes fractionated into stroma (ST) and grana (GT) thylakoids by digitonin and subsequently separated by denaturing SDS-PAGE. Gels were loaded on Chl basis, the amounts depending on the linearity of the antibody used (see “Experimental Procedures”). Immunoblots using antibodies against NDH48, NDH45, CP43, and PsbA are shown. B, non-denaturing BN-PAGE of intact thylakoids (T) and the stroma (ST) and grana (GT) thylakoid fractions. Unstained gel (left panel) and immunoblot were performed with antibody against the NDH45 protein (right panel). C, silver-stained two-dimensional BN/SDS-PAGE of stroma thylakoids (upper panel) and pieces of corresponding immunoblots using protein-specific antibodies (lower panel). Upper panel, proteins were identified with MALDI-TOF (identified proteins are listed in Table 2). Subunits of PSI in the supercomplex of ≈1000 kDa are indicated by arrows (31).
Further study of the protein complexes from intact thylakoids as well as from isolated grana and stroma membranes by BN-PAGE, enabling the separation of the protein complexes according to their size, revealed the presence of NDH45 (Fig. 3B) and NDH48 (data not shown) proteins in a large supercomplex of ≈1000 kDa, with only minor fraction of these proteins in a smaller protein complex of ≈800 kDa. For analysis of the protein composition of the ≈1000-kDa protein complex harboring the NDH45 and NDH48 proteins in the thylakoid membrane, the stroma thylakoids were subjected to two-dimensional BN/SDS-PAGE and subsequent identification of the protein subunits by immunoblotting and mass spectrometry (Fig. 3C and Table 2). Besides NDH45 and NDH48, the NDHH, NDHN, and NDHK proteins were found in the same complex as well as the PPL2 protein (At2g39470), essential for accumulation and activity of the NDH complex (30). In addition, the CYP20-2 protein (At5g13120) was present in the complex, and also the PSI reaction center protein PsAB was identified by immunoblotting (Fig. 3C). Not only PsAB but also several other PSI proteins were present in the supercomplex, with identification based on our previous work (31). The composition of the ≈800-kDa protein complex, in turn, remained enigmatic because according to immunoblotting, it did not contain PsAB or NDHH proteins (Fig. 3C), indicating that the PSI complex as well as the hydrophilic subunits of the NDH complex might not be present in this complex.

### TABLE 2

| Spot | Protein | AGI-code | MW* | Peptides | Cover | Score |
|------|---------|----------|-----|----------|-------|-------|
| 1    | NDH48   | At1g15980| 51.3| 12       | 27    | 151   |
| 2    | NDH45   | At1g64770| 38.3| 9        | 22    | 141   |
| 3    | NADH dehydrogenase subunit H (NDHH) | Atcg01110 | 45.6 | 14 | 30 | 185 |
| 4    | NADH dehydrogenase subunit K (NDHK) | Atcg00430 | 25.6 | 5 | 15 | 78 |
| 5    | Peptidylprolyl isomerase (CYP20-2) | At5g13120 | 28.5 | 6 | 25 | 85 |
| 6    | PsbP domain protein (PPL2) | At2g39470 | 27.2 | 7 | 29 | 89 |
| 7    | PsbP domain protein (PPL2) | At2g39470 | 27.0 | 9 | 38 | 128 |
| 8    | NDH48 dehydrogenase subunit N (NDHN) | At5g58260 | 23.7 | 7 | 36 | 112 |

* Molecular mass is predicted from the amino acid sequence.

**Further study of the protein complexes from intact thylakoids as well as from isolated grana and stroma membranes by BN-PAGE, enabling the separation of the protein complexes according to their size, revealed the presence of NDH45 (Fig. 3B) and NDH48 (data not shown) proteins in a large supercomplex of ≈1000 kDa, with only minor fraction of these proteins in a smaller protein complex of ≈800 kDa. For analysis of the protein composition of the ≈1000-kDa protein complex harboring the NDH45 and NDH48 proteins in the thylakoid membrane, the stroma thylakoids were subjected to two-dimensional BN/SDS-PAGE and subsequent identification of the protein subunits by immunoblotting and mass spectrometry (Fig. 3C and Table 2). Besides NDH45 and NDH48, the NDHH, NDHN, and NDHK proteins were found in the same complex as well as the PPL2 protein (At2g39470), essential for accumulation and activity of the NDH complex (30). In addition, the CYP20-2 protein (At5g13120) was present in the complex, and also the PSI reaction center protein PsAB was identified by immunoblotting (Fig. 3C). Not only PsAB but also several other PSI proteins were present in the supercomplex, with identification based on our previous work (31). The composition of the ≈800-kDa protein complex, in turn, remained enigmatic because according to immunoblotting, it did not contain PsAB or NDHH proteins (Fig. 3C), indicating that the PSI complex as well as the hydrophilic subunits of the NDH complex might not be present in this complex.

**ndh45 and as-ndh48 Mutants Show Impaired Function of the NDH Complex**—Functional characterization of the thylakoid membrane protein complexes did not reveal any malfunction of PSII measured as photochemical efficiency of PSII (F<sub>v</sub>/F<sub>m</sub> and Table 1). Functional modification, however, was recorded in the re-reduction rate of P<sub>700</sub> in darkness, which was slowed down both in ndh45 (t<sub>1/2</sub> = 1.76 s) and as-ndh48 (t<sub>1/2</sub> = 1.85 s) mutants compared with WT (t<sub>1/2</sub> = 1.27 s) (Fig. 4A). This might be related to slower cyclic electron transfer around PSI in the mutant plants, thus indirectly suggesting a possible functional flaw in the NDH complex. To confirm the malfunction of the NDH complex in the ndh45 and as-ndh48 plants, the post-illumination rise in Chl fluorescence (32), which is dependent on NDH-mediated reduction of plastoquinone pool in darkness (33), was measured. In this experiment, two earlier characterized Arabidopsis NDH mutant lines, ndho and cr2-2, were used as controls (7, 9). The reduced level of F<sub>v</sub>“rise” after light to dark transition in as-ndh48 mutant plants and especially in ndh45 compared with WT provided direct evidence of a lack or impaired function of the NDH complex (Fig. 4B). As expected, also the ndho and cr2-2 plants showed malfunction of the NDH complex (Fig. 4B). In addition, we measured the rate of NAD(P)H oxidation using thylakoids isolated from WT, ndh45, or ndho chloroplasts. In these experiments either ferricyanide or duroquinone was used as an electron acceptor. However, no statistically significant difference was found between WT, ndh45, and ndho thylakoids in the rate of oxidation of NADH or NADPH (data not shown).

**ndh45 Plants Accumulate H<sub>2</sub>O<sub>2</sub> in Dark-Light Transition**—NDH-mediated cyclic electron flow is important in adjusting the photosynthetic electron flow and in preventing the accumulation of ROS upon sudden changes in environmental condition. Accordingly, the production of ROS in WT, ndh45, and ndho plants was tested after transfer of 14-day-old plants from darkness to growth light conditions for 4 min and for 1 h. The DAB staining revealed a higher accumulation of H<sub>2</sub>O<sub>2</sub> in ndh45 and ndho mutant leaves compared with WT, both immediately after the lights were turned on following the diurnal dark period and still after 1 h in light (Fig. 5), which implies higher oxidative stress in mutant plants.
Accumulation and Attachment of NDH45 and NDH48 Proteins to the Thylakoid Membrane Are Distorted in crr2-2 Mutant Plants but Not in the ndho Mutant Plants—To study the role of the NDH48 and NDH45 proteins in the structure/assembly of the NDH complex, we estimated the amounts of the NDH48 and NDH45 proteins in thylakoids isolated from the ndho and from the crr2-2 mutants. ndho mutant lacks the hydrophilic subunits of the NDH connecting domain (7), whereas the accumulation of both the hydrophobic NDH8 subunit and the hydrophilic NDHH is disturbed in the crr2-2 mutant plants (9). The NDH45 and NDH48 proteins were present in thylakoids of the ndho mutant in amounts similar to those in WT while the crr2-2 thylakoids contained clearly reduced amounts of the NDH45 and NDH48 proteins (Fig. 6A). This indicates that the NDH48 and NDH45 proteins do accumulate normally in the thylakoid membrane in the absence of the hydrophilic subunits of the NDH complex. Accumulation, however, is diminished when both the hydrophobic and hydrophilic subunits of the NDH complex are present in reduced amounts.

The location of the NDH45 and NDH48 proteins in thylakoid protein complexes of the ndho and crr2-2 mutant plants was studied further by BN-PAGE. In WT and in ndho thylakoids the NDH45 and NDH48 proteins were found either in the \( \geq 1000 \)-kDa complex or in the \( \leq 800 \)-kDa complex (Fig. 6B), of which the \( \geq 1000 \)-kDa supercomplex represents the NDH complex together with the PSI complex (Fig. 3C). It should be noted, however, that in ndho thylakoids, the \( \geq 1000 \)-kDa supercomplex was found to be slightly smaller because of the lack of the hydrophilic subunits of the NDH complex (Fig. 6B). In contrast, in crr2-2 thylakoids the amount of the NDH supercomplex as well as the NDH45 and NDH48 proteins was clearly reduced compared with WT, indicating that the hydrophilic subunits of the NDH complex are required for stable attachment of NDH45 and NDH48 (Fig. 6B). Notably, some NDH45 and NDH48 proteins were found in the \( \leq 800 \)-kDa protein complex in the crr2-2 thylakoids (Fig. 6B).

To study further the interaction of the NDH45 and NDH48 proteins with the NDH complex, the thylakoids isolated from the WT, ndho, and crr2-2 plants were mildly digested with trypsin and subsequently subjected to SDS-PAGE and immunoblotting with NDH45 and NDH48 antibodies. Interestingly, while NDH48 was only partially digested by the proteases in the WT thylakoids, the protein was more susceptible to digestion in the ndho thylakoids and fully degraded in the crr2-2 thylakoids (Fig. 6C). This clearly demonstrated that the hydrophilic domain of the NDH complex protects the NDH48 protein from proteolytic degradation. On the contrary, the NDH45 protein was protected against proteolysis in WT and in both NDH mutants studied (Fig. 6C).

DISCUSSION

The structure and subunit composition of the NDH complex of plant chloroplasts is poorly known compared with other thylakoid multiprotein complexes, the PSI, PSII, and cyt b6f complexes as well as the ATP synthase (34). Contrary to these major photosynthetic protein complexes, no structural data are available from chloroplast NDH complex, and only low resolution single particle electron microscopy images of cyanobacterial NDH-1 complexes are available (35, 36). Although the NDH complex is not a component of the main linear photosynthetic electron transfer pathway, it plays a critical role in cyclic electron flow around PSI (3) together with the PGR5-dependent cyclic pathway (37). Cyclic electron transfer routes guarantee a proper production of ATP in relation to NADPH to fulfill the energy requirements of important metabolic processes. Indeed, the absence of one cyclic pathway is not lethal, but the elimination of both pathways prevents photoautotrophic growth (37). NDH-dependent cyclic electron flow has been shown to be of particular importance for plant survival under various environmental stress conditions (3).

Here we have identified two novel nuclear-encoded chloroplast proteins from Arabidopsis, NDH48 and NDH45, which are crucial for the assembly and function of the NDH complex. Both proteins locate exclusively in the thylakoid membrane.
(Fig. 1C), and the association of the NDH45 and NDH48 with the thylakoid membrane is fairly strong, because the majority of these proteins remain attached to the thylakoid membrane despite the high salt treatment (Fig. 2A). Moreover, the NDH48 protein is shown to locate on the stromal side of the thylakoid membrane, while the NDH45 protein is buried in the thylakoid membrane and not accessible to the protease treatment (Fig. 2B). This is surprising because the prediction programs (TMHMM, ARAMEMNON) expect both of the proteins to be hydrophilic. The expression of NDH45 and NDH48 is co-regulated, but it does not occur at the transcriptional level (Fig. 2C) but at the level of protein-protein interaction. Indeed, in the absence of NDH45, the NDH48 protein is largely missing from the thylakoid membrane, and vice versa (Fig. 1A), indicating that the protein–protein interaction of NDH45 and NDH48 has a stabilizing effect, and the lack of one protein does not allow stable membrane association of the other one, which instead is subjected to degradation (Fig. 2A).

Fractionation of the thylakoid membrane and separation of the multisubunit protein complexes revealed that both NDH45 and NDH48 are present in a supercomplex of \( \approx 1000 \) kDa located in the stroma lamellae of the thylakoid network (Fig. 3B). Most likely, the NDH45 and NDH48 proteins are attached to the thylakoid membrane by the NDH complex, because several NDH subunits were likewise identified in the supercomplex, together with the auxiliary protein PPL2, which previously has been shown to be associated with the NDH complex (Fig. 3C and Table 2) (30). The strongest support in favor of this hypothesis, however, comes from the analysis of \( \text{as-ndh48} \) and \( \text{ndh45} \) plants, which were specifically affected in the accumulation and function of the NDH complex (Table 1, Figs. 1A and 4). Residual activity of the NDH complex in \( \text{as-ndh48} \) plants indicates that the small amounts of NDH48, NDH45, and NDHH proteins (as well as other NDH subunits) present in these plants (Fig. 1A) are capable of sustaining small-scale activity of the complex. It is also interesting to note that the problems in NDH-mediated cyclic electron transfer in the absence of either the NDH45 or the NDHO protein are similarly reflected in the accumulation of \( \text{H}_2\text{O}_2 \) when the plants are suddenly exposed to light after the diurnal dark period (Fig. 5); the conditions where the carbon fixation reactions are not yet activated and cyclic electron flow is needed for dissipation of excess energy.

In agreement with our results, a recent study with maize bundle sheath chloroplasts, rich in the NDH complex, showed that the NDH48 and NDH45 homologs co-migrate in BN-PAGE with the NDH complex (38). Co-expression analysis also links the NDH48 protein to the same regulon with PPL2, NDHN, and NDHL (30). Besides NDH45, NDH48, and PPL2 proteins, yet another protein, CYP20-2 was identified in the same supercomplex by the two-dimensional BN/SDS-PAGE (Fig. 3C). The presence of CYP20-2 in the NDH supercomplex is surprising because this protein, thylakoid lumen cyclophilin of 20 kDa, has been reported to function as an auxiliary protein assisting the insertion of the light harvesting complex II (LHCII) to PSII and thus functioning in the formation of the PSII-LHCII supercomplexes (39). Our result, however, suggests that CYP20-2 protein probably has an alternative role with respect to the NDH complex and this interpretation is further supported by the co-migration of the homolog of CYP20-2 (TLP20) with the maize NDH complex in BN-PAGE analysis (38). Moreover, in the supercomplex of \( \approx 1000 \) kDa, several subunits of the PSI complex were identified, which is in line with a recent report showing that the chloroplast NDH complex interacts with the PSI complex in \textit{Arabidopsis} (40). The intimate co-operation of the NDH complex with PSI would certainly favor the cyclic electron flow around PSI and possibly explains why the genes encoding the activity subunits of the NDH complex are missing from plant and cyanobacterial genomes. In this context it is worth noting that when NAD(P)H was used as a substrate for the NDH complex no difference in activity could be recorded between WT and any of the \( \text{ndh} \) mutant thylakoids (including \( \text{ndho} \) which completely lacks the NDH complex) giving support to recent speculations about electron donors other than NAD(P)H for the chloroplast and cyanobacterial NDH complex.

Besides the major \( \approx 1000 \)-kDa complex, NDH45 and NDH48 were also present in a smaller supercomplex of \( \approx 800 \) kDa, which seems not to contain the subunits of the PSI complex or hydrophilic NDHH subunit of the NDH complex (Fig. 3C). The composition and role of this protein complex remains to be resolved but it might represent a subassembly step of the NDH supercomplex.

Although the final answer to the question whether the NDH45 and NDH48 proteins are intrinsic components of the NDH complex or whether they play an auxiliary function in the biogenesis, assembly and function of NDH is waiting for the high-resolution structural analysis of the NDH complex, the results presented here strongly support the first assumption. We show that the NDH45 and NDH48 proteins are not components of the hydrophilic connecting domain of the NDH complex but instead are attached to the hydrophobic membrane domain (Fig. 7). This interpretation is based on the fact that NDH45 and NDH48 form a complex and are stable in the absence of the NDHO protein, which is crucial for the assembly of the hydrophilic connecting domain of the NDH complex, whereas in the \( \text{cyp2-2} \) mutant both the hydrophobic membrane arm and also the NDH45 and NDH48 proteins are in shortage.

![FIGURE 7. Schematic view of the NDH complex and the subcomplex in stroma thylakoids (5T). A, a hypothetical scheme of the NDH complex emphasizing the novel subunits NDH45 and NDH48. Hydrophilic connecting domain is composed of NDH4-O subunits and the hydrophobic membrane domain of the NDHA-G subunits. The possible activity domain is not addressed. B, NDH subcomplex emphasizing a stable assembly of NDH45 and NDH48 with the membrane domain of the NDH complex in the absence of the hydrophilic connecting domain (\textit{ndho} plants). In the absence of the hydrophilic connecting domain, the NDH48 protein is susceptible to degradation from the stromal side of the thylakoid membrane as demonstrated in Fig. 6C.](image-url)
Novel NDH Subunits

(Fig. 6, A and B). On the other hand, the NDH45 and NDH48 proteins are crucial for accumulation of NDHH (Fig. 1A) and therefore probably also for the stable attachment of the hydrophilic connecting domain to the hydrophobic membrane domain of the NDH complex. The hydrophilic arm of the NDH complex seems to surround the NDH48 protein thus partially protecting it from digestion by proteases while the NDH45 protein is likely to be buried inside the thylakoid membrane below the NDH48 protein thus being protected from digestion by proteases when NDH48 is present.

It is interesting to note that the NDH45 and NDH48 proteins do not have homologs in cyanobacterial genomes and it may turn out that the chloroplastic NDH complex has acquired more nuclear-encoded subunits than have been envisaged so far (7, 38), and thus the chloroplastic NDH complex is likely to have more structural complexity than the cyanobacterial one.

Acknowledgments—We thank Salk Institute Genomic Analysis Laboratory for providing the sequence-indexed Arabidopsis T-DNA insertion mutants, Prof. Toshiharu Shikanai for providing the crr2-2 Arabidopsis mutant line, and the proteomics unit in the Turku Center of Biotechnology for maintenance of the mass spectrometry facility.

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