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Review

Structure and biogenesis of the chloroplast NAD(P)H dehydrogenase complex ☆

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ABSTRACT

Eleven genes (*ndhA-ndhK*) encoding proteins homologous to the subunits of bacterial and mitochondrial NADH dehydrogenase (complex I) were found in the plastid genome of most land plants. These genes encode subunits of the chloroplast NAD(P)H dehydrogenase (NDH) complex involved in photosystem I (PSI) cyclic electron transport and chlororespiration. Although the chloroplast NDH is believed to be closely and functionally related to the cyanobacterial NDH-1L complex, extensive proteomic, genetic and bioinformatic studies have discovered many novel subunits that are specific to higher plants. On the basis of extensive mutant characterization, the chloroplast NDH complex is divided into four parts, the A, B, membrane and lumen subcomplexes, of which subunits in the B and lumen subcomplexes are specific to higher plants. These results suggest that the structure of NDH has been drastically altered during the evolution of land plants. Furthermore, chloroplast NDH interacts with multiple copies of PSI to form the unique NDH–PSI supercomplex. Two minor light-harvesting-complex I (LHCI) proteins, Lhca5 and Lhca6, are required for the specific interaction between NDH and PSI. The evolution of chloroplast NDH in land plants may be required for development of the function of NDH to alleviate oxidative stress in chloroplasts. In this review, we summarize recent progress on the subunit composition and structure of the chloroplast NDH complex, as well as the information on some factors involved in its assembly. This article is part of a Special Issue entitled: Regulation of Electron Transport in Chloroplasts.

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1. Introduction

The existence of NAD(P)H dehydrogenase (NDH) in chloroplasts was first suggested by the complete sequencing of the two plastid genomes in tobacco (*Nicotiana tabacum*) and liverwort (*Marchantia polymorpha*) in 1986 [1,2]. Surprisingly, the plastid genomes contain 11 genes encoding homologs of the subunits of mitochondria NADH dehydrogenase (complex I), and they are actively expressed [3]. Because of the high similarity between subunits, the complex formed by these gene products in chloroplasts was designated as the NAD(P)H dehydrogenase complex, hereafter called the NDH complex [4,5]. Discovery of the *M55* mutant in *Synechocystis* sp. PCC 6803 opened the door to understanding the function of the NDH complex in chloroplasts [6]. The *M55* mutant is defective in the *ndhB* gene, which encodes a membrane subunit of the cyanobacterial NDH-1 complexes, and this mutant requires high levels of CO_2 for growth. This discovery was followed by physiological analyses suggesting that cyanobacterial NDH-1 is involved in photosystem I (PSI) cyclic electron transport [7–9]. These facts suggest that cyanobacterial NDH-1 is involved in multiple functions, including cellular CO_2 uptake and PSI cyclic electron transport [10,11]. The story became slightly more complicated. Subsequently, proteomic studies revealed that cyanobacterial NDH-1 contains two functionally distinct complexes, NDH-1L and NDH-1MS. While NDH-1L mediates respiration and PSI cyclic electron transport, NDH-1MS is required for CO_2 uptake. More comprehensive discussions of cyanobacterial NDH-1 can be found in the recent reviews [12–14].

It is generally accepted that the chloroplast NDH complex originated from cyanobacterial NDH-1 [15]. Although multiple *ndhD* and *ndhF* genes are present in the genome of *Synechocystis* sp. PCC 6803, the plastid genome contains only a single copy of each gene, which corresponds to *ndhD1*/*D2* and *ndhF1* of cyanobacteria, respectively. The products of *ndhD1*/*D2* and *ndhF1* are the components of cyanobacterial NDH-1L, suggesting that chloroplast NDH may also function in PSI cyclic electron transport and chlororespiration [12,13]. Targeted disruption of the plastid *ndh* genes in tobacco confirmed this hypothesis [16–18].

In chloroplasts, linear electron transport mediated by PSII and PSI produces both ATP and NADPH, whereas PSI cyclic electron transport preferentially contributes to ATP synthesis without the accumulation of NADPH. Characterization of Arabidopsis mutants identified at least two partially redundant pathways for PSI cyclic electron transport in chloroplasts [19–21]. The main pathway depends on PROTON GRADIENT REGULATION 5 (PGR5) and PGR5-Like 1 (PGRL1) [22,23], while the minor pathway is mediated by chloroplast NDH [20,24]. The main PGR5-dependent pathway is required for NPQ (non-photochemical quenching of chlorophyll fluorescence) induction and for balancing the ATP/

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NADPH production ratio [22], while the contribution of the NDH-dependent pathway is comparatively subtle [20,25]. However, chloroplast NDH is essential in the absence of PGR5 [20], and it functions to alleviate stromal over-reduction under stress conditions even in wild-type plants [26–31]. The exact molecular mechanism of how NDH prevents over-reduction of the stroma is still unclear [21].

The NDH-1 complex of *Escherichia coli* (*E. coli*) has a minimal composition that consists of 14 subunits (two subunits are fused into one protein). Its structure provides the simplest model for understanding the molecular mechanism of complex I as the proton pumping NAD(P)H-quinone oxidoreductase in various organisms. Recently, the crystal structure of NDH-1 was solved in the thermophilic bacterium *Thermus thermophilius* (*T. thermophilius*) and *E. coli* [32,33]. The *E. coli* NDH-1 complex is divided into two major parts, the membrane and peripheral segments. The membrane segments consist of seven hydrophobic subunits with 63 total transmembrane helices, and it is required for proton translocation [33]. The peripheral segment protrudes into the bacterial cytosol and contains other seven hydrophilic subunits carrying redox centers, flavin mononucleotide (FMN) and Fe-S clusters [32]. Electrons accepted by FMN from NADH are passed through seven conserved Fe-S clusters and finally reduce quinone at the quinine-binding site.

Blast searches have failed to detect any candidates for the homologs of the three bacterial subunits that function in NADH oxidation. This is a long-standing mystery of the chloroplast and cyanobacterial NDH, which must be equipped with a different "catalytic" domain. The nature of the "catalytic" domain is probably closely related to the molecular mechanism by which chloroplast NDH alleviates stromal over-reduction. In the last decade, extensive genetic, proteomic, bioinformatic and biochemical studies have been performed in search of the missing "catalytic" subunits of the NDH complex in angiosperms and cyanobacteria. A large number of proteins required for chloroplast NDH activity have been identified. Among them, dozens of proteins have been shown to be subunits of the chloroplast NDH complex. In this review, we focus on recent progress in understanding the subunit composition and structure of chloroplast NDH and the assembly of the NDH complex.

2. Structure of the chloroplast NDH complex

Single-particle analysis of electron microscopy (EM) projections identified many low-resolution structures of complex I, and statistical analyses clarified its L-shaped structure [34]. Based on this technique, the NDH-1 complexes of the cyanobacterium, *Thermosynechococcus elongatus*, were suggested to have relatively short hydrophilic arms compared with that of mitochondrial complex I in Arabidopsis [35]. This result is consistent with the fact that the cyanobacterial genomes do not code for NuoE-G homologs, and the cyanobacterial NDH-1 complexes may have a completely different "catalytic" domain than *E. coli* NDH-1, or alternatively the "catalytic" domain is easily dissociated from the main complex. On the basis of the analogy with *E. coli* and cyanobacterial NDH-1, it is likely that chloroplast NDH also has an L-shaped structure [36,37]. To date, 10 novel NDH subunits and 1 subunit candidate have been identified by various approaches in Arabidopsis and maize (*Zea mays*) and the number may yet increase (Table 1) [38–48]. Many of these novel subunits are not conserved in the genome of *Synechocystis* sp. PCC 6803. The three-dimensional structure of the chloroplast NDH complex should include the additional components for these subunits. Our structural model of the chloroplast NDH complex is still based on the approaches of genetics combined with biochemistry, where the stability of most subunit is evaluated in detail in the different mutant backgrounds (Fig. 1) [43,47,48].

In Arabidopsis, chloroplast NDH interacts with at least two copies of PSI to form a novel NDH–PSI supercomplex (see below). In blue native (BN)-PAGE, the NDH–PSI supercomplex migrates at a high-molecular mass position corresponding to band I [49]. In *chlororespiratory reduction 23* (*crr23*)/*ndhl* and *ndhm* mutants, which are defective in each subunit, band II with a slightly lower molecular mass than band I was detected [49]. Proteomic analyses of these high-molecular mass complexes showed that subunits NdhH–NdhO were absent in band II, while the other NDH subunits, including transmembrane subunits NdhA–NdhG, stably accumulate in the thylakoids, although NDH activity was completely lost in the absence of NdhH–NdhO [43]. NdhH–NdhO may form the peripheral arm corresponding to hydrophilic segment of cyanobacterial and *E. coli* NDH-1 complexes. NdhA–NdhG probably forms the membrane subcomplex, which may be involved in proton pumping and plastoquinone (PQ) binding. Since the chloroplast NDH complex is likely to have another arm protruding to the stroma, we designated the arm including NdhH–NdhO as subcomplex A [43]. Because subcomplex A includes NdhL, which contains three transmembrane domains [42], we hesitate to term it the hydrophilic arm. NdhL is also conserved in cyanobacterial NDH-1 [50,51], and it may provide the contact site for the lumen subcomplex of chloroplast NDH.

According to extensive proteomic and bioinformatic analysis, we have suggested locations for several novel subunits on the lumenal side of the NDH complex [43]. PPL2 (PsbP-Like 2), FKBP16-2 (FK506 Binding Protein 16-2), two PsbQ-Like (PQL) proteins, At1g14150 and At3g01440, and CYP20-2 are proposed to attach to the lumenal side of NDH [39,43,45,47,48]. The nomenclature for two *PQL* genes is currently confusing [43,47,48], and thus we use the AGI (Arabidopsis Genome Initiative) codes in this manuscript. PPL2, FKBP16-2 and At1g14150 are required for stabilizing each other, strongly suggesting that they form the same subcomplex on the lumenal side of NDH [43,47,48]. In the *ppl2*, *fkbp16*-2 and *at1g14150* mutants, subunits of the subcomplex A are almost completely absent [43,47,48], implying that the lumen subcomplex is required for the accumulation of subcomplex A. CYP20-2 is also likely to be a peripheral subunit of this lumen subcomplex, as it was absent in the *at1g14150* mutant (see below) [47]. The subunit accumulation pattern in the *at3g01440* mutant differs from that of the *ppl2*, *fkbp16*-2 and *at1g14150* mutants. In the *at3g01440* mutant, the levels of subcomplex A subunits and lumen subcomplex subunits, PPL2 and At1g14150, were decreased to about 25% of wild-type level, whereas subunits in another stroma-exposed subcomplex, subcomplex B, were more severely affected, suggesting that At3g01440 is more closely localized to subcomplex B rather than to the lumen subcomplex [48]. Although we previously classified At3g01440 in the lumen subcomplex [43], the most recent data suggest that this PQL protein is localized to the lumenal side of subcomplex B [47,48].

Besides At3g01440, other chloroplast-specific NDH subunits, including NDF1 (NDH-DEPENDENT CYCLIC ELECTRON FLOW 1)/NDH48, NDF2/NDH45, NDF4, NDF6 and NDH18, are also grouped into subcomplex B. In mutants of *ndf2*/*ndh45*, *ndh18* and *ndf6*, the subunit levels of subcomplex A and lumen subcomplex are reduced to ~10% of the wild type [43,48]. However, subunits of subcomplex B were almost completely missing in these mutants [43,48]. No information is available for the exact location of NDF4, but it does not have any signals for thylakoid lumen localization [46]. Since NDF4 was detected in band II, in which subcomplex A was missing [43], we classified it as a putative component of subcomplex B (see 3.3).

3. Subunit composition of the chloroplast NDH complex

3.1. Conserved membrane subunits NdhA–NdhG

Membrane subunits NdhA–NdhG are conserved in various types of NDH, including the *E. coli* NDH-1 and mitochondrial complex I. Mass spectrometry (MS) analysis of the Arabidopsis NDH–PSI supercomplex identified almost all of the membrane subunits except for NdhG [43], providing direct evidence for the presence of these subunits in the chloroplast NDH complex. Probably due to technical challenges, the same MS analysis also failed to detect the NdhG subunit in maize thylakoids [41]. However, NdhG was detected in the NDH-1 complexes

Table 1
Summary of the subunits of the chloroplast NDH complex.

	Name	AGI code	*Synechocystis* sp. PCC 6803	*E. coli*	*T. thermophilus*	References
Membrane subcomplex	NdhA	ATCG01100	NdhA	NuoH	Nqo8	
	NdhB	ATCG00890	NdhB	NuoN	Nqo14	
	NdhC	ATCG00440	NdhC	NuoA	Nqo7	
	NdhD	ATCG01050	NdhD1/D2	NuoM	Nqo13	
	NdhE	ATCG01070	NdhE	NuoK	Nqo11	
	NdhF	ATCG01010	NdhF1	NuoL	Nqo12	
	NdhG	ATCG01080	NdhG	NuoJ	Nqo10	
Subcomplex A	NdhH	ATCG01110	NdhH	NuoD	Nqo4	[42]
	NdhI	ATCG01090	NdhI	NuoI	Nqo9	[38]
	NdhJ	ATCG00420	NdhJ	NuoC	Nqo5	[38]
	NdhK	ATCG00430	NdhK	NuoB	Nqo6	[38]
	NdhL	AT1G70760	NdhL	–	–	
	NdhM	AT4G37925	NdhM	–	–	
	NdhN	AT5G58260	NdhN	–	–	
	NdhO	AT1G74880	NdhO	–	–	
Subcomplex B	NDF1 (NDH48)	AT1G15980	?	–	–	[44,46]
	NDF2 (NDH45)	AT1G64770	–	–	–	[44,46]
	NDF4	AT3G16250	–	–	–	[46]
	NDF6	AT1G18730	–	–	–	[40]
	NDH18	AT5G43750	–	–	–	[43]
	PQL*	AT3G01440	–	–	–	[47,48]
Lumen subcomplex	PPL2	AT2G39470	–	–	–	[39]
	CYP20-2	AT5G13120	–	–	–	[45]
	FKBP16-2	AT4G39710	–	–	–	[43]
	PQL*	AT1G14150	–	–	–	[47,48]
NDH candidate	CRR3	AT2G01590	–	–	–	[73]

Some subunits of the NDH-1 complexes from *Synechocystis* sp. PCC 6803, *E. coli* and *T. thermophilus* are also included to show the conserved subunits in various species. A question mark (?), a protein with weak similarity to NDF1/NDH48 was found in cyanobacteria; it is unclear whether this protein is a subunit of cyanobacterial NDH-1 complexes. *, nomenclature for two PQL genes is currently inconsistent in Suorsa et al. [47] and Yabuta et al. [48].

isolated from several cyanobacterial species [51–53], suggesting that NdhG may in fact be present in chloroplast NDH.

The three-dimensional structure of the *E. coli* NDH-1 membrane domain showed that subunits of NuoL, NuoM and NouN, corresponding to chloroplast NDH subunits NdhF, NdhD and NdhB, respectively (Table 1), are antiporter-like proteins, and each of them contains 14 conserved transmembrane helices [33]. In particular, two remarkable discontinuous helices are present in each subunit, which are thought to have a critical function in transporters and channels. Furthermore, the NuoL subunit includes a long amphipathic α-helix spanning almost the entire length of the membrane domain. Secondary structure predictions indicate that chloroplast NdhF also contains this kind of long α-helix, which may directly contact one of the discontinuous helices included in NdhB, NdhD and NdhF [33]. NuoA, NuoJ and NuoK correspond to chloroplast NdhC, NdhG and NdhE, respectively (Table 1). These subunits have a total of 11 transmembrane helices and are located at the extremity of the membrane domain [33]. NuoH, corresponding to chloroplast NdhA, contains eight transmembrane helices and is loosely associated with NuoA, NuoJ and NuoK. According to the working mechanism model of complex I proposed by Efremov et al. [33], electron movement from NADH to quinone is coupled to the conformational changes in the hydrophilic peripheral domain, and these changes are further transmitted to the long amphipathic α-helix in NuoL, leading to a piston-like motion of the amphipathic α-helix along the membrane domain. This kind of motion would tilt the three discontinuous helices in subunits NuoL, NuoM and NouN, finally resulting in translocation of three protons. The fourth proton may also be translocated at the interface of the two main domains. Consequently, NDH-1 accepts two

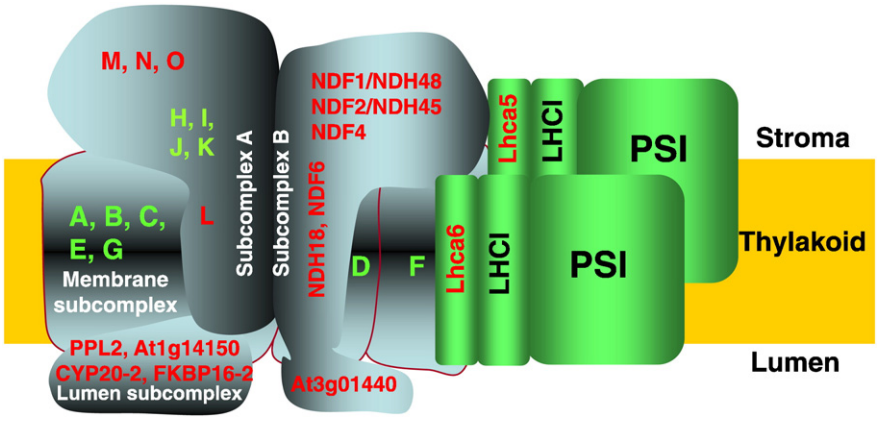

Fig. 1. A Schematic model of the NDH–PSI supercomplex in chloroplasts. The chloroplast NDH is divided into four subcomplexes: the membrane subcomplex, A and B subcomplexes, and the lumen subcomplex. Components of each subcomplex are listed in Table 1. On the basis of the analogy with *E. coli* NDH-1, it is likely that NdhD and NdhF are located at the distal (from the subcomplex A) end of the membrane domain [33]. The putative monomeric NDH with a molecular mass of ~700 kDa was detected in *lhca5 lhca6*, and NDH still interacted with PSI to form the smaller version of the supercomplex in the *lhca5* or *lhca6* single mutants, suggesting that NDH interacts with at least two copies of PSI via Lhca5 and Lhca6 (Peng and Shikanai, manuscript submitted). This model does not include information about subunit stoichiometry or the exact position of each NDH subunit and the PSI complexes. Eleven plastid-encoded subunits are depicted in green letters, while nucleus-encoded subunits are depicted in red letters. The electron donor-binding module is still unclear.

electrons from NADH and pumps four protons from the cytoplasm to the periplasm [33]. If this calculation is correct for the chloroplast NDH complex, eight total protons (another four protons being translocated via the Q-cycle of the cytochrome b_6f (Cyt b_6f) complex) would be translocated across thylakoid membranes coupled with the transfer of two electrons from the stromal electron donor to PQ via NDH, and then to the PSI complex via the Cyt b_6f complex. The NDH-dependent PSI cyclic electron transport may efficiently produce an extra proton gradient when it is physiologically needed.

Genetic approaches have identified several nucleus-encoded proteins required for biogenesis of the NDH membrane subunits. In Arabidopsis, the pentatricopeptide repeat (PPR) protein family includes approximately 450 members involved in multiple steps of RNA maturation [54,55]. A PPR protein CRR2 functions in the intergenic RNA cleavage between the *ndhB* and *rps7* gene, a process that is essential for the translation of *ndhB* [24]. Another PPR protein, CRR4, is a *trans*-factor required for RNA editing that creates the start codon of the *ndhD* transcript [56]. PROTON GRADIENT REGULATION 3 (PGR3) is also a PPR protein that stabilizes *petL* transcripts [57]. Recently, it was further shown that PGR3 is required for the translation of *petL* and *ndhA* (Cai et al., manuscript submitted). A sigma factor, SIG4, was suggested to be involved in the transcription of the *ndhF* gene [58]. Accumulation of the entire NDH complex is severely impaired in the *crr2, crr4, pgr3* and *sig4* mutants, suggesting that the membrane subunits affected in these mutants are required for the stability of the entire membrane domain.

PPR protein, CRR21, is required for RNA editing at site 2 of *ndhD* (ndhD-2), where C to U edition converts Ser-128 to Leu [59]. Another PPR protein, CRR22, is involved in RNA editing at ndhB-7 and ndhD-5, which converts Ser-249 to Phe of NdhB and Pro-296 to Leu of NdhD as well as at the site of rpoB-3 [60]. CRR28 is another PPR protein that is essential for editing at ndhB-2 and ndhD-3, which converts Pro-156 to Leu and Ser-293 to Leu, respectively [60]. All of these residues in NdhB and NdhD are predicted to be in the transmembrane helices [59,60]. Although the levels of NDH accumulation were identical in wild-type and *crr21*, *crr22* and *crr28* plants, NDH activity was lost in these three mutants [59,60], suggesting that the residues are required for NDH activity. A further PPR protein, ORGANELLE TRANSCRIPT PROCESSING 84 (OPT84) is required for RNA editing of ndhB-10 (Pro-494 to Leu) and ndhF-2 (Ser-97 to Leu), and these two amino acids are also located in the transmembrane helices [61]. In contrast to the *crr21*, *crr22* and *crr28* mutants, the stability of NDH was affected in the *opt84* mutant [61]. These discoveries suggest that certain residues and structures of transmembrane helices in NdhB, NdhD and NdhF are critical for maintaining the activity and/or stability of NDH.

3.2. Subunits of subcomplex A (NdhH-NdhO)

Four plastid-encoded subunits, NdhH–NdhK, were detected in several plant species including Arabidopsis, maize, tobacco, spinach (*Spinacia oleracea*) and pea (*Pisum sativum* cv. Little Marvel) by protein blot and/or MS analysis [16,62–64]. The crystal structure of the hydrophilic domain of NDH-1 isolated from *T. thermophilus* indicated that the cavity at the interface of subunits Nqo4, Nqo6, Nqo8 (which are homologous to chloroplast NdhH, NdhK and NdhA), and Nqo7, Nqo10, Nqo11 subunits (which correspond to the membrane subunits, NdhC, NdhG and NdhE), provides the quinone-binding site [33]. Homologs of chloroplast NdhH–NdhK bind three Fe-S clusters required for the electron transfer in the *T. thermophilus* complex [32]. These observations suggest that the route of electron transfer from NdhH–NdhK to the quinone-binding site is well conserved between chloroplast NDH and *T. thermophilus* NDH-1.

Extensive proteomic studies to identify the missing subunits involved in the electron donor binding in cyanobacteria have been unsuccessful, probably because these subunits are easily dissociated from the main complex in the presence of detergents [51–53,65,66]. Chromatography combined with MS was also used to investigate the components of chloroplast NDH [38]. NdhH was successfully tagged by 7-His at its N-terminus without affecting the function of NdhH using plastid transformation in tobacco, and an NDH subcomplex containing plastid-encoded subunits NdhA, NdhH–NdhK and three nucleus-encoded subunits NdhM, NdhN and NdhO was purified by Ni^{2+} affinity chromatography [38]. In this work, NdhA was co-purified with NdhH–NdhK, suggesting an interaction between NdhA and the hydrophilic domain. Novel subunits of NdhM, NdhN and NdhO were also identified in the complex, but they do not have any known domains suggesting their involvement in electron donor-binding. Absence of any one of these three subunits destabilizes the entire subcomplex A, suggesting that they may be required for stabilization or/and assembly of subunits NdhH–NdhK [38,43,49]. Recent single particle EM analyses of YFP-fusion NdhM, NdhN and NdhO subunits suggested that they are co-localized at the middle surface of the membrane domain in cyanobacterial NDH-1 [67]. However, this kind of experiment may be not suitable for chloroplast NDH because NdhL and NdhM fused with even a short tag, like 7-His or HA, at the C-terminus could not rescue NDH activity in their corresponding Arabidopsis mutants (Peng, unpublished observations).

NdhL was discovered in the characterization of the Arabidopsis mutant, *crr23/ndhl* [42]. NdhL is required for the accumulation of subcomplex A in Arabidopsis [43]. However, the cyanobacterial *M9* mutant strain defective in the expression of NdhL had no effect on the assembly of the NDH-1 complexes, but complex activity was severely suppressed [51], suggesting that NdhL is located close to the putative "catalytic" domain in cyanobacteria. This discrepancy may be explained by the fact that cyanobacterial NdhL, which contains two transmembrane domains, shows a weak similarity to Arabidopsis NdhL, which includes an additional transmembrane domain in the N-terminus [42]. It is possible that the N-terminal domain plays an important role in stabilizing subcomplex A, while the C-terminus interacts with the "catalytic" domain. Additionally, NdhL may mediate the binding of subcomplex A and the lumen subcomplex, which are separated by thylakoid membranes in chloroplasts [43].

3.3. Subunits of subcomplex B

The expression of genes encoding NDH subunits and NDH biogenesis factors are highly coregulated [39]. Based on this phenomenon, several NDH subunits and factors required for the biogenesis of NDH, called NDF (NDH-DEPENDENT CYCLIC ELECTRON FLOW), have been identified by *in silico* co-expression analysis [40,46,68]. Among these, NDF1 and NDF2 were also independently discovered by MS analysis and were designated as NDH48 and NDH45, respectively [44]. Both proteins co-migrate with the NDH complex in BN-PAGE [44,46]. While the putative stromal exposed part of NDF1/NDH48 is partially sensitive to mild treatment with trypsin, the NDF2/NDH45 protein is resistant to protease treatment, suggesting that NDF2/NDH45 is embedded in subcomplex B or in thylakoid membranes [44]. NDF1/NDH48 and NDF2/NDH45 are required for the accumulation of the NDH complex, and they are unstable in the mutants lacking the membrane subcomplex or subcomplex B, supporting the conclusion that they are NDH subunits [43,44,46]. NDF2/NDH45 is specific to higher plants, but a protein with a weak similarity to NDF1/NDH48 was found in cyanobacteria [44,46]. However, proteomic analyses of the cyanobacterial NDH-1 complexes did not detect this protein, suggesting that it may not be a subunit of cyanobacterial NDH-1.

NDF4 was also discovered by the same *in silico* strategy [46] and was detected in the NDH–PSI supercomplex [43]. Like other NDH subunits, its putative ortholog also accumulates in bundle sheath cells in maize [41]. NDF4 shows a significant sequence similarity to another predicted protein in Arabidopsis, At4g32590, whose function is still unclear [46]. Although the homolog (protein ID 174881) of NDF4 was also found in the green algae, *Chlamydomonas reinhardtii*, in which the NDH complex is absent, it is more closely related to At4g32590 than

NDF4. The protein of At4g32590 and 174881 may have similar functions that are independent of chloroplast NDH in both higher plants and green algae. NDF4 is predicated to contain an iron–sulfur (Fe-S) cluster-binding domain, and *in vitro* analysis of the NDF4 recombinant protein indicates that the Fe-S cluster of the NDF4 protein is redox-active, suggesting the involvement of NDF4 in electron transfer within the NDH complex [46]. NDF4 is the only novel subunit containing an Fe-S cluster that has been identified so far, and it may provide a clue for elucidating the routes taken by electrons within the chloroplast NDH complex.

Both NDF6 and NDH18 each have one transmembrane domain, suggesting that they are intrinsic membrane subunits [40,43]. In the *ndf6* and *ndh18* mutants, subunits of subcomplex A accumulated to approximately one-tenth of the wild-type levels, but NDF1 completely disappeared [43,48], suggesting tight interactions with other stroma-exposed subunits, including NDF1/NDH48 and NDF4, and also NDF2/NDH45. NDF6 and NDH18 were also unstable in the mutants lacking NdhB or NdhD, providing evidence that NDF6 and NDH18 are subunits of the NDH complex [40,43]. No homologs of NDF6 and NDH18 were found in cyanobacteria or *Chlamydomonas*, indicating that they are specific to higher plants.

The PsbQ-like (PQL) protein, At3g01440, is located on the lumenal side of the NDH complex [47,48]. The At3g01440 protein was completely missing in the *ndf6* mutant and *vice versa*, the accumulation of other subunits of subcomplex B was also drastically reduced in the absence of At3g01440, suggesting that it is a subunit of NDH complex and is tightly associated with subcomplex B [47,48]. A search for homologs revealed that PQL proteins are present in higher plants, but no homologs were found in cyanobacteria, algae, mosses or ferns [48], implying that they are also specific to higher plants.

3.4. Subunits of the lumen subcomplex

Several proteins have been proposed to be structural components of the lumenal protuberance of the NDH complex [39,43,45,47,48]. It is remarkable that most of them are homologous to PsbP and PsbQ, which are subunits of the oxygen-evolving complex associated with PSII [39,47,48,69]. In addition to PsbP, an intrinsic subunit of the oxygen-evolving complex, two PsbP-like (PPL) proteins, PPL1 and PPL2 that show significant sequence similarity to cyanoP (a cyanobacterial PsbP homolog), were found in higher plants. While PPL1 is involved in the efficient repair of photodamaged PSII, PPL2 is required for activity of the NDH complex [39].

Three PQLs are also present in the chloroplast. At least two of them, At1g14150 and At3g01440, together with PPL2, co-localize with NDH subunits on BN gels and were detected by mass analysis of the NDH–PSI supercomplex [43,47,48]. All of these proteins are essential for the accumulation of the intact NDH complex, and they are also unstable in the NDH-deficient mutants [39,47,48]. These results suggest that these proteins can be regard as NDH subunits. It should be noted that the third PsbQ-like protein, At2g01918, is also required for the accumulation of the NDH complex [48]. However, no signal of this protein was detected in the NDH–PSI supercomplex [43], and further studies are needed to examine whether it physically associates with NDH.

FKBP16-2 is a member of the FKBP-type peptidyl-prolyl *cis-trans* isomerase and it shows 40–50% sequence similarity with FKBP13, which interacts with the Rieske Fe-S subunit of the Cytb_6f complex [70]. The *FKBP16-2* gene was successfully knocked-down by RNA interference and the mutant is specifically defective in the accumulation of the NDH subcomplex A and also the lumen subcomplex [43]. FKBP16-2 is also absent in some mutant backgrounds lacking NDH. No proteins closely related to Arabidopsis FKBR16-2 were found in *Chlamydomonas* or cyanobacteria, demonstrating that FKBP16-2 is a novel NDH subunit and is essential for the complex stability/assembly.

In total, 16 immunophilins are present in the thylakoid lumen, but only CYP20-2 was shown to possess PPIase activity in Arabidopsis [71]. CYP20-2 was detected in the NDH–PSI supercomplex, which is consistent with the findings that CYP20-2 is co-localized with NDH–PSI on BN gels, and that the maize homolog of CYP20-2 co-migrates with NDH subunits [41,43,45]. The level of CYP20-2 was drastically reduced in the *crr2* and *at1g14150* mutants, supporting the conclusion that CYP20-2 is tightly associated with NDH [45,47]. Although it is possible that CYP20-2 (like FKBP16-2) is involved in protein folding or/and in complex assembly, the interaction between NDH and CYP20-2 is unlikely to be transient [43]. CYP20-2 may have dual functions as a structural component of NDH and active immunophilin. In contrast to other mutants defective in NDH subunits, however, the *cyp20-2* mutant shows almost normal NDH activity and only a slight reduction of At1g14150 levels in the mutant. Taken together with the finding that CYP20-2 was absent in the *at1g14150* mutant, these results indicate that CYP20-2 is closely localized to At1g14150 [47]. Interestingly, the wheat (*Triticum aestivum*) homolog of CYP20-2 was shown to be a negative regulator in the GA signaling pathway, and it regulates stem development, suggesting distinct roles for CYP20-2 in monocotyledon and dicotyledon [72].

3.5. An NDH subunit candidate

CRR3 was discovered in our *crr* mutant screen [73]. It has one transmembrane domain and is conserved among higher plants. NdhH is missing in the *crr3* mutant, and the NDH complex is also required for the accumulation of CRR3, suggesting that CRR3 is an NDH subunit specific to chloroplasts [73]. However, we failed to detect CRR3 in the NDH–PSI supercomplex by mass analysis [43]. It is possible that, like NdhG, the CRR3 protein could not be detected by linear ion-trap triple quadrupole (LTQ)-Orbitrap MS, possibly due to its hydrophobic nature and low molecular weight (13.7 kDa) [73]. Alternatively, the majority of CRR3 may be dissociated from the NDH–PSI complex in BN gels. CRR3 was absent in *crr6* and *crr7* [73], suggesting that CRR3 is a part of subcomplex A or the putative “catalytic” subcomplex.

4. Structure and physiological function of the NDH–PSI supercomplex

In 2005, two groups independently detected an NDH complex with high-molecular mass (more than 1000 kDa) in Arabidopsis and maize [63,74]. Because of the limitations in our knowledge of the NDH structure, this unique large complex has been overlooked or has been simply concluded to be an NDH dimer. However, recent genetic and biochemical studies have strongly suggested that NDH interacts with PSI to form a novel supercomplex in higher plants [43,49]. After mild solubilization of thylakoids and separation by BN-PAGE, a high-molecular-mass green band (band I) was visible at the top of the gel. This band was slightly down-shifted to a position corresponding to band II in the *ndhl*/*crr23* and *ndhm* mutants and was completely missing in the NDH mutants lacking membrane subunits, suggesting that these bands contain NDH subunits [49]. Subsequent immunoblot and MS analyses showed that the green band detected in wild-type plants represents the intact NDH–PSI supercomplex [43].

A question remains as to how NDH interacts with PSI. Perhaps their interaction requires specific proteins. In higher plants, four major LHCI proteins, Lhca1–Lhca4, bind to the PSI complex on the side of the PsaF/PsaJ subunits [75]. Additionally, the Arabidopsis genome encodes two minor LHCI subunits, Lhca5 and Lhca6, which were detected in our MS analysis of the NDH–PSI supercomplex [43,76]. Lhca5 associates with PSI monomer at the sub-stoichiometric level [77], while Lhca6 was not detected in PSI complexes isolated from various higher plants by MS analysis, although it shows high similarity with Lhca2 [78,79]. Furthermore, the *Lhca6* gene is co-expressed with the genes encoding NDH subunits and biogenesis factors [43]. Thus Lhca5 and Lhca6 may be required for the specific interaction between NDH and PSI. Indeed, characterization of

Arabidopsis *lhca5* and *lhca6* mutants showed that the intact NDH–PSI supercomplex disappeared in the absence of Lhca5 or Lhca6. Instead, a smaller NDH–PSI supercomplex with a slightly higher mobility in BN gels than that of the largest PSII supercomplex (which has a predicted molecular mass of ~1300 kDa) was detected in *lhca5* and *lhca6* [43]. These results suggest that NDH still interacts with PSI in the absence of Lhca5 or Lhca6, and that NDH may associate with multiple copies of PSI. This idea is supported by our recent finding that a putative NDH monomer with a molecular mass of ~700 kDa was detected in the *lhca5 lhca6* double mutant (Peng and Shikanai, manuscript submitted). On the basis of the molecular mass of the PSI monomer (530 kDa) and the largest PSII supercomplex from higher plants (~1300 kDa), we propose that the smaller NDH–PSI supercomplexes detected in *lhca5* and *lhca6* contain only one copy of PSI and NDH, respectively, and that NDH interacts with two copies of PSI to form the intact NDH–PSI supercomplex via Lhca5 and Lhca6 (Fig. 1). However, more direct studies, such as EM analysis, are required to confirm this model.

The binding sites of Lhca5 and Lhca6 in the NDH–PSI supercomplex can be inferred on the basis of the mutant phenotypes. Subcomplex A was unstable in the *crr23*/*ndhl* mutant, but Lhca5 and Lhca6 still associated with the remaining parts of NDH [43], suggesting that subcomplex A is not required for the interaction between PSI and NDH. In the *crr2-2* mutant defective in the expression of membrane subunit NdhB, a putative sub-supercomplex was detected that includes subcomplex B subunits and Lhca6 as well as PsaA. This suggests that some subunits included in subcomplex B provide the binding sites for Lhca6 [43]. Subcomplex B or/and the membrane subcomplex may mediate the binding of Lhca5 and NDH because Lhca5 still associates with the residual parts of NDH in the absence of subcomplex A [43]. Some lumenal NDH subunits were also proposed to mediate the interaction with PSI by contacting the lumenal loops of Lhca5 and Lhca6 [47]. Lhca5 has been shown to bind to Lhca2/Lhca3 subunits of the PSI complex [80]. Although it is unclear how Lhca6 interacts with the PSI complex, Lhca6 was found to associate with the PSI monomer in the absence of NDH, suggesting the affinity of Lhca6 for PSI [43].

NDH levels were reduced in mature leaves of the *lhca6* mutant (~50%), but the defect was milder in immature leaves. In the mature leaves of *lhca6*, the *in vitro* Fd-dependent PQ reduction activity was also not completely impaired in the presence of Antimycin A, which inhibits the PGR5/PGRL1-dependent PSI cyclic electron transport, suggesting that the samller NDH-PSI supercomplex detected in *lhca6* still retains activity. However, the *lhca6 pgr5* double mutant showed severe phenotypes in growth rate and photosynthesis, as did the *crr3 pgr5* and *crr4-2 pgr5* double mutants, which were defective in both pathways of PSI cyclic electron transport [20,43]. A possible explanation for this discrepancy is that supercomplex formation is required for the efficient operation of NDH *in vivo*, which cannot be evaluated by Fd-dependent PQ reduction activity in ruptured chloroplasts. Due to the lack of a reliable technique for quantitatively measuring NDH activity *in vivo*, we cannot eliminate this possibility. Another hypothesis based on experimental results is that supercomplex formation is required for stabilizing NDH, and that, under a certain threshold level of NDH, the phenotype becomes evident in the *pgr5* mutant background. In this case, NDH monomers may have similar NDH activity as those in the supercomplex, but stability and/or assembly of NDH is affected. We recently discovered that supercomplex formation is required to protect the NDH complex from high-light stress (Peng and Shikanai, manuscript submitted).

Supercomplex formation between NDH-1 and PSI was also reported in cyanobacteria, as some NDH subunits, including NdhH–NdhK, NdhN and NdhO, were co-purified with PSI from *Synechocystis* sp. PCC 6803 cells by Ni^{2+} affinity chromatography [81]. However, no homologs of Lhca5 and Lhca6 were found in cyanobacteria [43], suggesting that cyanobacteria and higher plants use different strategies for supercomplex formation. It is unclear whether the cyanobacterial NDH–PSI supercomplex has a similar physiological function as the NDH–PSI supercomplex present in chloroplasts.

5. Assembly of the NDH complex

Like other thylakoid protein complexes, such as PSI and PSII, the NDH complex consists of multiple subunits encoded by both plastid and nuclear genomes. Extensive genetic and biochemical studies of the PSII and PSI assembly imply that the functional assembly of NDH subunits also likely requires multiple steps. However, the application of biochemistry, which is routinely used in the study of PSII and PSI assembly, is challenging for NDH because of its low abundance and fragility. On the basis of the quantitative immunoblotting studies, the level of chloroplast NDH complex was estimated to be only ~1.5% of the level of PSII on a molar basis [16]. Genetics is a powerful tool in such a situation, and several nuclear-encoded assembly factors have been discovered by screening for Arabidopsis mutants specifically defective in NDH activity. By combining these data with high-sensitivity mass spectrometry analysis, it has become feasible to study the assembly of the NDH complex.

NDH subunits were separated into complexes with distinct molecular masses on BN gels in different laboratories. The 500-kDa complex includes PPL2 and NdhH [39], while the 300-kDa complex contains NdhE, and the 250-kDa complex contains NdhH, NdhI, and NdhK [63]. These results reflect the fragile nature of chloroplast NDH, and most likely represent NDH subcomplexes dissociated from the intact NDH–PSI supercomplex during experimental procedures. These results also imply that the stability or/and the assembly of the individual subcomplex is partly independent of the other parts of NDH, at least during the purification procedure. The most striking example is subcomplex A, and even in its absence, other parts of the NDH–PSI supercomplex are still stable *in vivo*, suggesting that subcomplex A is not required for the assembly and stabilization of the other parts of the NDH–PSI supercomplex. As for the assembly of subcomplex A, we recently discovered several subcomplex A assembly intermediates (about 800, 500, and 400 kDa) in the stroma [82].

The *crr6* and *crr7* mutants were discovered based on their lack of NDH activity [83,84]. CRR6 and CRR7 are stromal proteins, and both proteins are required for the accumulation of subcomplex A, but they do not contain any known motifs that indicate their function [82]. The expression of plastid-encoded genes encoding subcomplex A subunits was not affected in the mutants, suggesting that CRR6 and CRR7 are required for the assembly of subcomplex A. Although CRR6 did not co-migrate with any assembly intermediates of subcomplex A in clear native (CN)-PAGE, it was co-purified with NdhH by affinity chromatography [82]. Mass analysis of the total proteins co-purified with CRR6 clarified several novel assembly factors, as well as subunits of subcomplex A (Peng and Shikanai, unpublished results). However, CRR7 was not found in the CRR6 co-purified samples, and it is likely that CRR7 functions in a different but related step of subcomplex A biogenesis from CRR6 [82]. CRR6 and CRR7 are also conserved in cyanobacteria, although it is still unclear whether they play the same role as they do in higher plants.

CRR1 is homologous to dihydrodipicolinate reductase (DHPR), which functions in lysine biosynthesis [85]. The Arabidopsis genome encodes three DHPR paralogs, including CRR1. The other two proteins are likely to be intrinsic DHPR, but the dihydrodipicolinate-binding motif is not conserved in CRR1 [85]. Like CRR6 and CRR7, CRR1 is also a stromal protein whose stability is independent of the NDH complex, suggesting that CRR1 is not a subunit of NDH. Taken together with the results that NDH activity was specifically impaired in *crr1*, these facts suggest that CRR1 is involved in NDH biogenesis rather than in lysine biogenesis in Arabidopsis. As CRR1 contains a well conserved NAD(P)H-binding motif, it may be an NAD(P)H dehydrogenase, and the reduction of an unknown substrate may be required for the assembly of the NDH complex. CRR1 is considered to have evolved from the

cyanobacterial sll1058 [85]. SLL1058 may be essential for lysine biosynthesis because *Synechocystis* sp. PCC 6803 does not encode any DHPR-like gene other than sll1058. It is possible that SLL1058 has dual functions in both lysine biosynthesis and the accumulation of NDH-1 in cyanobacteria. However, CRR1 may have evolved to have a specific role in the assembly of the NDH complex.

NDF5 was also identified on the basis of *in silico* co-expression analysis [68]. This protein shows a weak homology to the NDH subunit NDF2/NDH45 (only 27% identity in amino acid sequence). NDF5 is a thylakoid membrane protein, although it is predicted to have no transmembrane domain. NDF5 was not detected in the NDH–PSI supercomplex by mass analysis [43], suggesting that NDF5 is not a subunit of the NDH complex. Instead, it may be involved in the stability or biogenesis of both A and B subcomplexes, as accumulation of the NDH subunits NdhH, NDF1/NDH48 and NDF2/NDH45 were severely affected in the *ndf5* mutant [68]. However, NDF5 over-accumulated in the *ndho* mutant [68], and NDF5 was found in the sub-supercomplex corresponding to band II isolated from the *ndhl* mutant [43]. These results suggest that NDF5 binds to subcomplex B in the absence of subcomplex A. NDF5 may be a factor required for the assembly and/or the stability of subcomplex B. It is also possible that NDF5 associates with subcomplex B to recruit the assembly intermediate of subcomplex A to the proper position in the super-complex. NDF5 is not conserved in cyanobacteria, although a single gene, which is similar to both *NDF5* and *NDF2*, was identified in the moss, *Physcomitrella patens*, suggesting that NDF2 and NDF5 are paralogs that originated from a single ancestor related to PpNDF2/NDH5 [68]. It is unclear whether this *P. patens* homolog has NDF2-like function (subunit) or NDF5-like function (assembly factor) or both.

6. Evolution of the NDH complexes from cyanobacteria to higher plants

As mentioned above, various lines of evidence suggest that not only the structure of the NDH complex, but also its biogenesis, has been drastically altered during the evolution from cyanobacteria to higher plants. What is the reason for the drastic evolution of chloroplast NDH? Although chloroplast NDH is part of the machinery of PSI cyclic electron transport and chlororespiration, its contribution to proton gradient formation during steady-state photosynthesis is subtle [20,25], and it is mainly involved in alleviating oxidative stress in chloroplasts [26–31]. However, recent results imply that the chloroplast NDH monomer is sensitive to high-light stress (Peng and Shikanai, manuscript submitted). To develop a novel function for stress resistance and to make the machinery itself resistant to the stress, chloroplast NDH may have changed drastically during the evolution of land plants. In order to further understand the basis for these evolutionary changes, it is necessary to study the exact molecular mechanism of how chloroplast NDH alleviates oxidative stress.

7. Concluding remarks

Despite the substantial progress that has been made in elucidating the structure of the NDH complex and its subunit composition over the last decade, the identity of the electron donor of the chloroplast and cyanobacterial NDH complex(es) is still unclear. We remain certain that identification of the putative “catalytic” domain, which will include the electron donor-binding subunits, is the most straightforward strategy to answer this fundamental question. However, a vast number of publications suggest that this putative catalytic domain is easily dissociated from the main parts of the NDH complex during the experimental procedures. Even so, it is also possible that a trace amount of the “catalytic” subcomplex is still associated with the NDH–PSI supercomplex, in which a great number of unknown proteins were detected in our mass analysis [43]. The key components may be already in our hands.

An emerging interest in the field is the dynamics of NDH assembly processes in higher plants. Given its extremely low abundance and the fragile nature of this complex, the chloroplast NDH complex has not been a target for biogenesis research. However, recent developments in mass analysis as well as in molecular genetics have made it feasible to identify specific assembly factors. As described in this review, the chloroplast NDH complex underwent drastic evolution in land plants. It is likely that its evolution also required some specific assembly factors. This research will clarify what is necessary for chloroplasts to have created the novel machinery of the NDH–PSI supercomplex for the resistance of stress during the evolution of land plants.

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