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The NAD(P)H Dehydrogenase Complex in Photosynthetic Organisms: Subunit Composition and Physiological Function

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ABSTRACT

The chloroplast NAD(P)H dehydrogenase (NDH) complex is believed to originate from the cyanobacterial NDH-1 complex because of their similarity in structure and function. In both cyanobacteria and chloroplasts, NDH is involved in respiratory electron transport (chlororespiration in chloroplasts) and photosystem I cyclic electron transport. By modifying its subunit composition, cyanobacterial NDH also contributes to CO_2 concentration. In *Arabidopsis thaliana*, photosystem I cyclic electron transport consists of two partly redundant pathways. Although the knockout of chloroplast NDH gives only a subtle phenotype, it causes a severe defect in photosynthesis under the mutant background of *pgr5* (*proton gradient regulation 5*), in which another pathway of photosystem I cyclic electron transport is impaired. Although the characteristics of the mutant phenotype suggest that PGR5-dependent photosystem I cyclic electron transport contributes markedly to ATP synthesis during photosynthesis, chloroplast NDH may function as a safety valve to prevent the over-reduction of stroma. In cyanobacterial and chloroplast NDH, we have not discovered the electron donor-binding subunits that are conserved in bacterial NDH-1. This fact implies that the NDH complex is equipped with a different electron-donor binding module in cyanobacteria and chloroplasts, and thus the identity of the electron donor to the complex is still unclear.

Keywords: Arabidopsis, chloroplast, chlororespiration, cyclic electron transport, NDH, photosystem I, photosynthesis
Abbreviations: AOX, alternative oxidase; **cyt**, cytochrome; **Fd**, ferredoxin; **NDH**, NAD(P)H dehydrogenase; **NPQ**, non-photochemical quenching of chlorophyll fluorescence; **PSI and PSII**, photosystem I and II; **PQ**, plastoquinone; **PTOX**, plastid terminal oxidase

CONTENTS

INTRODUCTION

Respiratory electron transport through the inner membranes of mitochondria is mediated by four major protein complexes: NADH dehydrogenase (complex I), succinate dehydrogenase (complex II), the cytochrome (cyt) bc_1 complex (complex III), and cyt *c* oxidase (complex IV) (**Fig. 1**). Complex I catalyzes NADH-dependent reduction of ubiquinone and this action is coupled with proton pumping from the matrix to the inter-membrane space. In addition to electron transport through complexes III and IV (cyt pathway), which contributes to proton translocation, ubiquinone is also oxidized by alternative oxidase (AOX), which directly transfers electrons to O_2. AOX does not translocate any protons across the inner mitochondrial membranes, and it is considered to be involved in energy dissipation in the mitochondria (Umbach *et al*. 2005; Yoshida *et al*. 2006). Interestingly, proteins related to the respiratory machinery (complex I and AOX) were discovered in plastids (Matsubayashi *et al*. 1987; Carol *et al*. 1999; Wu *et al*. 1999). In higher plants, the plastid genome contains 11 genes (*ndhA-K*) encoding proteins homologous to the complex I subunits (Matsubayashi *et al*. 1987), although some exceptions have been discovered, for example, in black pine (*Pinus thunbergii*) (Wakasugi *et al*. 1994). In chloroplasts these Ndh subunits form a protein complex, NAD(P)H dehydrogenase (NDH), the main focus of this review. The chloroplast NDH complex is more similar to the bacterial NDH (NDH-1) than to the mitochondrial complex I in the same species. In particular, cyanobacterial NDH-1 is closely related to chloroplast NDH in structure and function and is believed to be the origin of chloroplast NDH (Friedrich and Weiss 1997).

In this decade our knowledge of chloroplast NDH has improved greatly. Advances in our genetics knowledge of cyanobacteria and the higher plants tobacco (*Nicotiana tabacum*) and Arabidopsis (*Arabidopsis thaliana*) have contributed markedly to this progress. It may already not be an enigma that the plastid genome encodes the genes for the respiratory complex, as it previously had been (Shikanai and Endo 2000). However, it is also true that we still cannot an-

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Invited Review

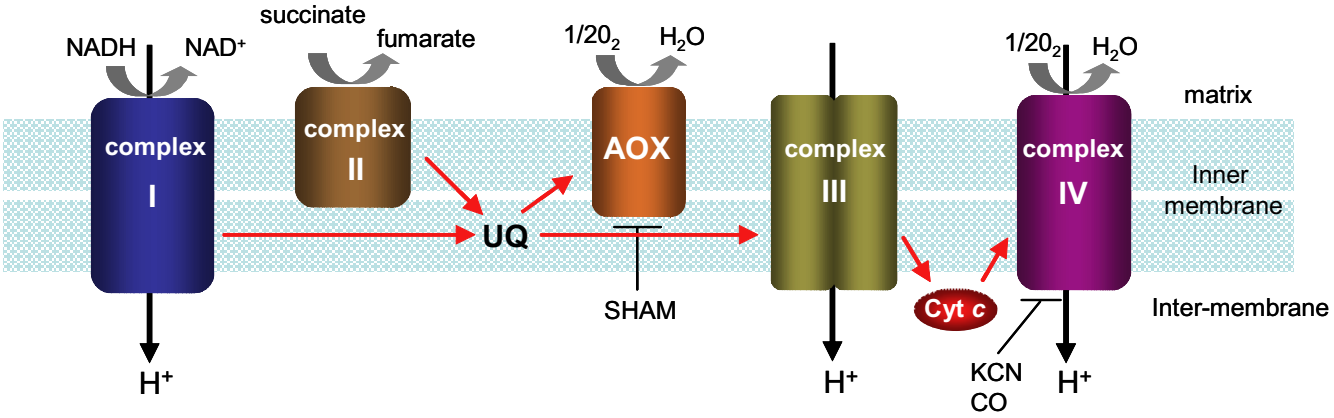

Fig. 1 Schematic representation of plant respiratory electron transport in inner mitochondrial membranes. Red and black arrows indicate the movements of electrons and protons, respectively. UQ, ubiquinone.

swer the fundamental question: what is the electron donor to the complex in chloroplasts? In this article, I summarize the current status of knowledge on the chloroplast and cyanobacterial NDH complexes and also what remains to be clarified.

CHLORORESPIRATION

Chlororespiration consists of respiratory electron transport in thylakoid membranes and interacts with photosynthetic electron transport via the plastoquinone (PQ) pool (Peltier and Cornac 2002). For chloroplast molecular biologists, the physiological term chlororespiration was convenient to explain the enigmatic occurrence of 11 *ndh* genes (*ndhA-K*) encoding proteins homologous to subunits of mitochondrial NADH dehydrogenase in the plastid genome. Ironically, however, chlororespiration was discovered and well characterized in the green alga, Chlamydomonas (*Chlamydomonas reinhardtii*), in which the chloroplast NDH complex is probably absent (Bennoun 1982). In pioneer works, chlororespiration was detected as an effect of the respiratory inhibitors KCN, CO and salicylhydroxamic acid (SHAM) on photosynthetic electron transport in Chlamydomonas (Bennoun 1982) and also in higher plants (Garab *et al*. 1989). On the basis of the recent accumulation of molecular information, we can draw a model of chlororespiratory electron transport in the thylakoid membranes (**Fig. 2**). The chloroplast NDH complex non-photochemically reduces PQ. In Chlamydomonas, type-2 NAD(P)H dehydrogenase (NDH-2) may mediate this electron transport (Peltier and Cornac 2002). NDH-2 consists of a single subunit that catalyzes NAD(P)H-dependent PQ reduction, which is probably not accompanied by proton translocation across the membranes (Mus *et al*. 2005). NDH-2 is distinguished from the complex I-type NAD(P)H dehydrogenase that is designated as NDH-1.

PTOX (plastid terminal oxidase) was discovered in an Arabidopsis variegated mutant, *immutans* (Carol *et al*. 1999; Wu *et al*. 1999). PTOX is homologous to mitochondrial AOX and is likely to mediate electron transport from PQ to O_2 independently of proton translocation across the thylakoid membranes. PQ is non-photochemically reduced by NDH and subsequently oxidized by PTOX in the dark. This route taken by electrons implies the presence of complete respiratory electron transport in chloroplasts (**Fig. 2**). However, the variegated phenotype of *immutans* suggests the distinct function of PTOX in chloroplast development. Because phytoene accumulates in the white sectors of *immutans* leaves, PTOX is proposed to be essential for phytoene desaturation, an essential step in carotenoid biosynthesis (Josse *et al*. 2000). Phytoene desaturase requires oxidized PQ as an electron acceptor.

Even in mature chloroplasts in which PQ is oxidized by photosystem I (PSI), PTOX may function as an alternative electron sink. PTOX can theoretically make excessive electrons leak from the intersystem without generating reactive oxygen species. Because of the variegated phenotype in *immutans*, however, it is not simple to evaluate the physiological function of PTOX in the chloroplasts of its mature leaves. However, the available information does not support the idea that PTOX contributes greatly to this process of photoprotection (Joët *et al*. 2002; Rosso *et al*. 2006).

PTOX is homologous to mitochondrial AOX which is insensitive to KCN. The pioneer work indicated that chlororespiratory oxidation of PQ is sensitive to KCN and CO, as the cyt pathway in mitochondria (Garab *et al*. 1989). However, the homolog of complex IV has not yet been discovered in plastids so far. We cannot eliminate the possibility that PTOX has functional redundancy with an unknown terminal oxidase and that the phenotype can be detected only in the double mutant defective in all the PQ oxidation reactions in the dark.

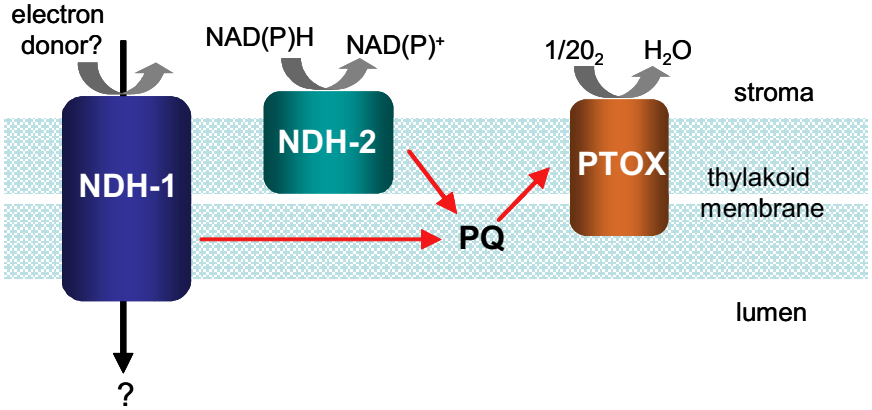

Fig. 2 Schematic representation of chlororespiratory electron transport in thylakoid membranes. Electron donor to NDH is not known. It is unclear whether NDH has proton pumping activity in chloroplasts.

CHLOROPLAST NDH AS MACHINERY OF PHOTOSYSTEM I CYCLIC ELECTRON TRANSPORT

In the light, PQ reduced by the chloroplast NDH complex is oxidized by the cyt b_6f complex via PSI activity, not by PTOX (Shikanai *et al*. 1998). Given that the electron donor to the NDH complex is NAD(P)H or ferredoxin (Fd), the mode of electron transport is cyclic (**Fig. 3**). PSI cyclic electron transport was discovered in the 1950s, but the machinery involved has long been unclear (Bendall and Manasse 1995; Shikanai 2007). As discussed later, the idea that the chloroplast NDH complex mediates PSI cyclic electron transport originated from researches on cyanobacteria (Ogawa 1992; Mi *et al*. 1992, 1994, 1995). A plastid transformation technique established in tobacco (Svab and Maliga 1993) enabled us to test this possibility by directly disrupting the *ndh* genes encoded by the plastid genome (Burrows *et al*. 1998; Kofer *et al*. 1998; Shikanai *et al*. 1998). The knockout lines exhibit a defect in non-photochemical reduction of PQ in the dark and also a reduction in the size of electron input into PQ under far-red light illumination. These results indicate that the chloroplast NDH complex mediates PSI cyclic electron transport in the light.

Since the route taken by electrons is cyclic, PSI cyclic electron transport generates proton gradient across the thylakoid membranes (ΔpH) without an accumulation of reducing power. It has long been discussed whether PSI cyclic electron transport is required to balance the ATP and NADPH production ratio (Allen 2002) or for photoprotection via regulation of the lumen pH (Heber and Walker 1992). Excessive light energy is safely dissipated from photosystem II (PSII) as heat (thermal dissipation), the regulation of which is triggered by monitoring the acidification of the thylakoid lumen and detected as a qE component of NPQ (non-photochemical quenching) in chlorophyll fluorescence (Müller *et al*. 2001). The physiological significance of PSI cyclic electron transport was discussed in detail elsewhere (Munekage *et al*. 2002, 2004; Munekage and Shikanai 2005; Shikanai 2007). In the *ndh* knockout lines, however, no phenotypes were detected, except for the minor alteration in electron transport at least under the greenhouse conditions (Burrows *et al*. 1998; Shikanai *et al*. 1998). Although the chloroplast NDH complex can mediate PSI cyclic electron transport, its contribution is too small to affect photosynthesis via ATP generation or to induce the regulatory process of photosynthesis. The chloroplast NDH complex mediates PSI cyclic electron transport, a result that was expected from research on cyanobacterial NDH, as described below. A more important discovery from the phenotype of tobacco *ndh* knockout lines may have been that the chloroplast NDH complex is dispensable under greenhouse conditions.

In higher plants, PSI cyclic electron transport consists of two partly redundant pathways, the NDH-dependent pathway and the antimycin A-sensitive pathway (Joët *et al*. 2001; Munekage *et al*. 2004). In the latter, a small thylakoid protein PGR5 is essential, although the exact function of PGR5 is unclear (Munekage *et al*. 2002). In higher plants, the main route of PSI cyclic electron transport depends on PGR5 and contributes markedly to both photosynthesis and photoprotection. Under growth chamber conditions, the Arabidopsis mutant *pgr5*, which is defective in this electron transport, can grow as well as the wild type. In contrast, double mutants lacking both pathways of PSI cyclic electron transport exhibit a severe defect in growth and are photo-damaged, even at very low light intensity of less than 50 μmol photons m^{-2} sec^{-1} (Munekage *et al*. 2004). This result clearly indicates that the NDH complex is required for survival under the *pgr5* mutant background.

In *pgr5*, an imbalance between linear and PSI cyclic electron transport causes over-reduction of the stroma; this can be monitored as a reduction in the level of $P700^+$ (oxidized PSI reaction center chlorophylls) (Munekage *et al*. 2002). This over-reduction can be detected in wild-type plants, which probably operate PGR5-dependent PSI cyclic electron transport in the field (Endo *et al*. 2005). This result suggests that the chloroplast NDH complex alleviates oxidative stress, even in the wild type, under certain stressed conditions. Consistently with this idea, tobacco mutants defective in the accumulation of NDH are sensitive to various environmental stresses (Endo *et al*. 1999; Horváth *et al*. 2000; Li *et al*. 2004; Munné-Bosch *et al*. 2005; Wang *et al*. 2006). However, it is also true that the phenotype is subtle even under the stress conditions and there is a debate whether chloroplast NDH is really involved in the stress responses (Barth and Krause 2002). Plants have redundant mechanisms to protect their photosynthetic machinery from oxidative stress, and this redundancy may be essential for adaptation to fluctuating environmental conditions.

In light of the mutant phenotype, the chloroplast NDH complex is likely to be a safety valve to prevent the over-reduction of stroma. We are still not sure of the exact mechanism by which the chloroplast NDH complex alleviates stress. It is unlikely that the mechanism is via NPQ, since the double mutant of *npq4 pgr5* is similar to the single mutant of *pgr5*, in contrast to the severe phenotype observed in *crr2 pgr5* (Munekage *et al*. 2004). Whereas *crr2* is defec-

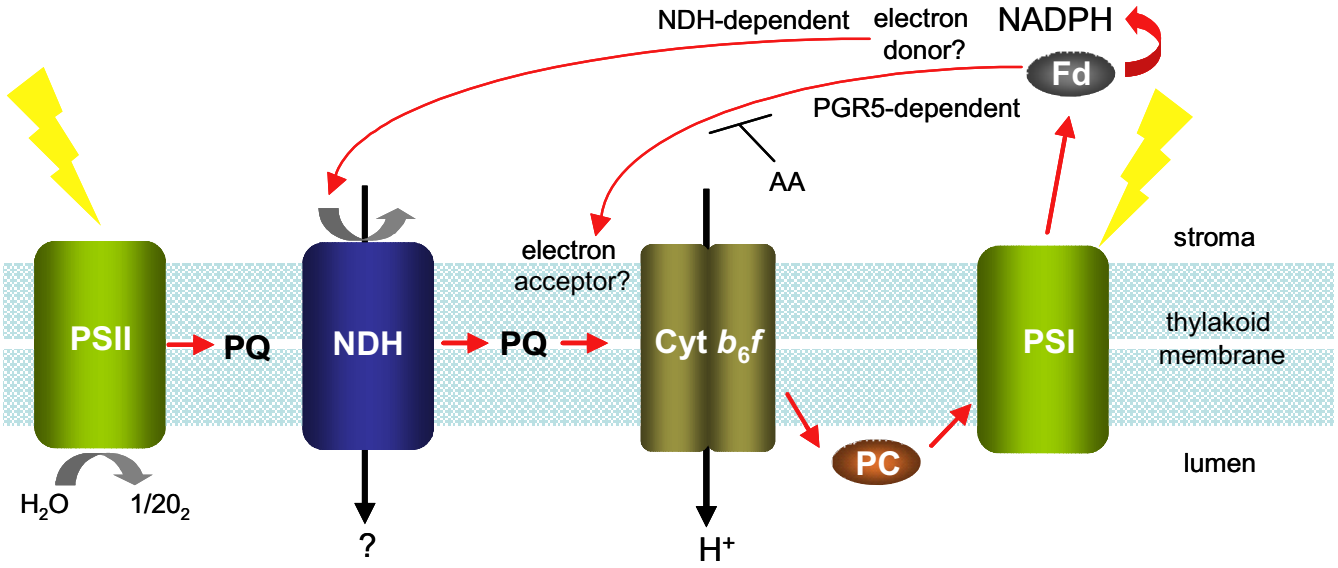

Fig. 3 Schematic representation of PSI cyclic electron transport in higher plants. Electron donor to NDH is not known. Direct electron acceptor in the PGR5-dependent pathway is unclear (Okegawa *et al*. 2005). Antimycin A (AA) inhibits the PGR5-dependent PSI cyclic electron transport. Exact location of PGR5 is not determined. PC, plastocyanin.

tive in the chloroplast NDH complex (Hashimoto *et al*. 2003), *npq4* is defective in PsbS, a pH sensor that is essential for qE induction (Li *et al*. 2000). From the available information, the simplest working model is that the NDH complex mediates PSI cyclic electron transport, which is essential to alleviate stromal over-reduction by modifying the ratio of ATP/NADPH production. Even though the rate of electron transport mediated by NDH is small compared with PGR5-dependent PSI cyclic electron transport or linear electron transport, this fine-tuning may be essential to prevent severe imbalance. This topic needs future research.

CO_2 CONCENTRATION AND PSI CYCLIC ELECTRON TRANSPORT MEDIATED BY CYANOBACTERIAL NDH

The M55 mutant of *Synechocystis* PCC6803 was isolated based on its phenotype requiring high CO_2 levels for growth (Ogawa 1991). It was shown later that CO_2 uptake was more strongly affected than HCO_3^--uptake in M55 (Ohkawa *et al*. 2000). M55 is defective in *ndhB* encoding a subunit of the NDH complex (Ogawa 1991). This discovery was followed by extensive physiological examinations, which demonstrated the impairment of respiration and PSI cyclic electron transport in M55 (Mi *et al*. 1992, 1994, 1995). These results suggest that cyanobacterial NDH mediates both respiratory and PSI cyclic electron transports. Because PSI cyclic electron transport produces ATP, in pioneer works the NDH complex was hypothesized to energize CO_2 concentrating machinery in *Synechocystis* PCC6803.

In *Synechocystis* PCC6803, most of the genes encoding the subunits of the NDH complex are present as single copies, except for *ndhD* and *ndhF*, which form small gene families consisting of six and three genes, respectively. Members of the *ndhD* family are divided into three pairs on the basis of their similarity (Ohkawa *et al*. 2000). The Δ*ndhD1*/*ndhD2* double mutant cannot grow under photoheterotrophic conditions, indicating that two genes are essential for respiratory electron transport. In contrast, the Δ*ndh3*/*ndh4* mutant requires high CO_2 levels for growth. NdhD3 is involved in the inducible high-affinity CO_2 uptake system, as are NdhF3, CupA and Sll1735 (Shibata *et al*. 2001; Maeda *et al*. 2002). In contrast, a system depending on NdhD4, ndhF4, and CupB has lower affinity for CO_2 and operates constitutively. These results, which are based on genetics, indicate that the NDH complex is involved in the distinct functions of respiratory electron transport, inducible high-affinity CO_2 uptake, and constitutive low-affinity CO_2 uptake by modifying its subunit composition. Because NdhB is shared by all the complexes, the phenotype of M55 is ascribed to a mixture of defects in several functions mediated by distinct complexes. It became necessary to reconsider the simple relationship between PSI cyclic electron transport and CO_2 uptake. Interestingly, the similar story of CO_2 concentration and the NDH complex revived in C_4 plants. The NDH complex is specifically expressed in bundle sheath cells and may energize the C_4 system (Kubicki *et al*. 1996; Takabayashi *et al*. 2006).

Cyanobacterial NDH is involved in multiple functions by modifying its subunit composition. This model, established by genetics, was shown to be consistent with direct analysis of the subunit composition by biochemistry (Zhang *et al*. 2004). The NDH-1L (large) complex consists of at least 15 subunits that were clarified so far and includes NdhD1 and NdhD2 subunits. The NDH-1M (medium size) complex has a subunit composition similar to that of the NDH-1L complex, but it lacks NdhD1 and NdhD2. The NDH-1M complex associates with two small complexes, NDH-$1S_1$ and NDH-$1S_2$ (Zhang *et al*. 2005). The NDH-$1S_1$ complex consists of NdhD3, NdhF3, CupA, and Sll1735. Accumulation of NDH-1M and NDH-1S complexes is induced in response to low CO_2 levels (Zhang *et al*. 2004).

One question remaining in the study of cyanobacterial NDH is which complex mediates PSI cyclic electron transport. The involvement of NDH in PSI cyclic electron transport was extensively studied in M55, in which accumulation of both NDH-1L and NDH-1M is impaired (Mi *et al*. 1992, 1994, 1995). Although the half-time of $P700^+$ reduction in the dark after far-red light illumination, by which electron input to the PQ pool can be estimated, was significantly slower in M55 than the wild type, it was not affected in ΔndhD1/D2 (Zhang *et al*. 2004). This result suggests that the NDH-1MS complex, rather than the NDH-1L complex, mediates PSI cyclic electron transport in *Synechocystis* PCC680. This is inconsistent with the fact that P700 is oxidized more under low-intensity far-red light in ΔndhD1/D2, which is similar to the phenotype of M55, suggesting that NDH-1L mediates both PSI cyclic electron transport and respiratory electron transport (Ohkawa *et al*. 2000). Because the measuring methods were different between the two experiments, the results cannot be directly compared. NDH-dependent PSI cyclic electron transport may consist of multiple pathways in cyanobacteria.

APPLICATION OF FORWARD GENETICS TO THE CHLOROPLAST NDH COMPLEX

Since plastid transformation is not routinely available in Arabidopsis, the use of forward genetics was essential to identify the Arabidopsis mutants specifically defective in NDH activity. Characterization of the double mutants defective in both pathways of PSI cyclic electron transport required Arabidopsis mutants (Munekage *et al*. 2004). We focused on the subtle change in chlorophyll fluorescence after actinic light illumination in the *ndhB* knockout tobacco (Shikanai *et al*. 1998), and we modified the technique of chlorophyll fluorescence imaging (Shikanai *et al*. 1999; Hashimoto *et al*. 2003). Under a CCD camera, NDH activity is visualized as an image of chlorophyll fluorescence, facilitating the isolation of *chlororespiratory* (*crr*) mutants defective in NDH activity. At present, we have identified 20 loci that are required for NDH activity in Arabidopsis. Our collection still does not contain mutants for *ndhM* and *ndhO*, which were discovered by a proteomics approach (Remeau *et al*. 2005), suggesting that more genes are related to the *crr* phenotype. From the mutants we have cloned 12 genes so far: five encode proteins required for the expression of plastid *ndh* genes and another five for Ndh subunits or candidates. One is probably required for assembly of the complex and one has an unknown function.

The mutant pool can be utilized for two future prospects of the research on chloroplast NDH. Mutants defective in the nuclear-encoded subunits are detected on the basis of their *crr* mutant phenotypes, and they may provide clues to identification of the subunits involved in electron donor binding, a piece of information that is still missing from our model (see below). Eleven subunits of the NDH complex are encoded in the plastid genome, and mutants defective in the expression of these *ndh* genes have also been isolated (Hashimoto *et al*. 2003; Yamazaki *et al*. 2004; Kotera *et al*. 2005). Although the function of these genes is not directly related to NDH, it may be involved in the regulation of plastid *ndh* gene expression to cope with environmental changes. Although many researches are involved in the regulation of plastid gene expression, the link to the regulation of photosynthesis has been poorly understood and the 11 plastid *ndh* genes may be the best targets for this purpose. I would like to discuss the current status of two topics, subunit composition of the NDH complex and the regulation of the *ndh* gene expression, further in detail in the following sections.

STRUCTURE OF THE NDH COMPLEX

The plastid genome of higher plants contains 11 genes encoding subunits of the NDH complex (NdhA-K). In *Escherichia coli*, the NDH-1 complex consists of a minimum set

Table 1 Subunit composition of the NDH complexes.

Arabidopsis plastid	**Synechocystis PCC6803**	***E. coli***	**Bovine mitochondria**	**Cofactors, etc**
NdhA	NdhA	NuoH	ND1	quinone binding
NdhB	NdhB	NuoN	ND2	
NdhC	NdhC	NuoA	ND3	
NdhD	NdhD1-6	NuoM	ND4	
NdhE	NdhE	NuoK	ND4L	
NdhF	NdhF1-3	NuoL	ND5	
NdhG	NdhG	NuoJ	ND6	
NdhH	NdhH	NuoD	49K	
NdhI	NdhI	NuoI	TYKK	2x[4Fe-4S]
NdhJ	NdhJ	NuoC	30K	[4Fe-4S]
NdhK	NdhK	NuoB	PSST	
CRR23	NdhL			
NdhM (At4g37925)*	NdhM (slr1623)*			
NdhN (At5g58260)*	NdhN (sll1262)*			
NdhO (At1g74880)*	NdhO (ssl1690)*			
		NuoE	24K	[2Fe-2S]
		NuoF	51K	NADH binding, FMN; [4Fe-4S]
		NuoG	75K	[2Fe-2S]; 3x[4Fe-4S]
	CupA			
	CupB			
	Sll1735			

Information for *E. coli* and bovine complex I is based on Yagi and Matsuo-Yagi (2003). The subunits encoded by the plastid genome are indicated by green. *The confusion of gene names suggested by Ogawa and Mi (2007) has been corrected between two organisms in Rumeau *et al*. (2006).

of 14 subunits (Friedrich and Weiss 1997; Yagi and Matsuo-Yagi 2003). Comparison of the subunit composition reveals that it is clear that the plastid genome does not encode the orthologs of NuoE, NuoF and NuoG (**Table 1**). Complete determination of the Arabidopsis nuclear genome did not clarify the genes encoding missing subunits even in the nuclear genome. Consistently with this fact, genes encoding homologs of NuoE, NuoF, and NuoG were not discovered in the cyanobacterial genomes. These three subunits are involved in binding to electron donors (NADH in *E. coli*), and thus the chloroplast NDH complex and also the cyanobacterial NDH complex still lack the electron donor-binding module in our model (**Fig. 4**). Thus, we cannot conclude that NDH is really NAD(P)H dehydrogenase in the chloroplast (see below).

The three-dimensional structure of NADH dehydrogenase has not been determined in any organisms so far. However, electron microscopic analysis has indicated that NADH dehydrogenase isolated from mitochondria of *Neurospora crassa* (Guenebaut *et al*. 1997) and Arabidopsis (Dudkina *et al*. 2005) has an L-shaped structure. Recently, the same technique was applied to the NDH complex of cyanobacterium *Thermosynechococcus elongatus* (Arteni *et al*. 2006). It is also L-shaped with a relatively short hydrophilic arm. There are variations in the length of the membrane-embedded arm, probably corresponding to the NDH-1L and NDH-1M complexes. In a small population, the hydrophilic arm has a longer extension, as in the *E. coli* NDH-1 complex, suggesting that the cyanobacterial NDH complex also has an electron donor-binding module corresponding to NuoE, NuoF, and NuoG. Interestingly, some complexes contain an additional hydrophilic arm on the membrane-embedded arm, forming a U-shape rather than an L-shape (Arteni *et al*. 2006). Topology analysis of the chloroplast NDH complex revealed that the hydrophobic arm extrudes to the stromal side; this is consistent with its predicted physiological function (Casano *et al*. 2004).

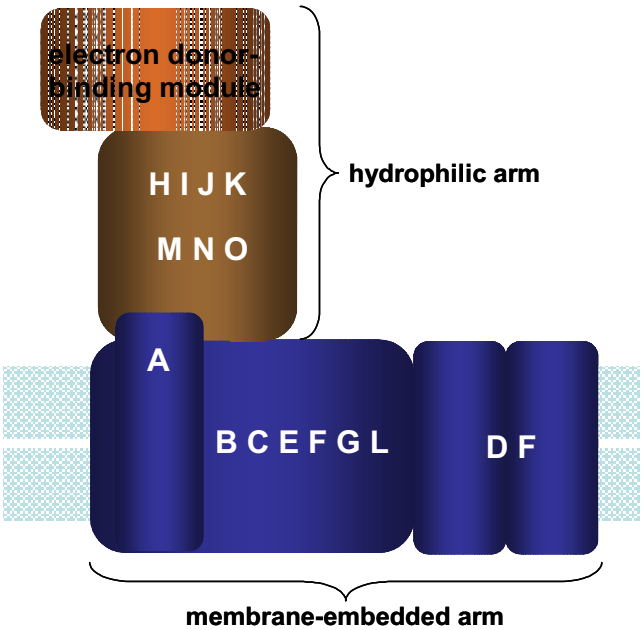

Fig. 4 Schematic model of the subunit structure of the chloroplast NDH complex. NdhA is purified with the hydrophilic arm, although it is a component of the membrane-embedded arm (Rumeau *et al*. 2005). By analogy with the cyanobacterial NDH complex, NdhD and NdhF are considered to associate peripherally to the other subunits in the thylakoid membranes (Zhang *et al*. 2004, 2005). The electron donor-binging module is still unclear.

By analogy with the cyanobacterial NDH complex, the membrane-embedded arm is considered to consist of at least 7 subunits, NdhA-G. An additional subunit of NdhL was discovered because its mutant phenotype required high CO_2 levels for growth (Ogawa 1992), and it was recently rediscovered by a proteomics approach (Battchikova *et al*. 2004). We also identified its Arabidopsis ortholog from the *crr23* mutants defective in NDH activity (Shimizu and Shikanai unpublished). In Arabidopsis, CRR3, which was also isolated from our *crr* mutant pool, is essential for stabilizing the NDH complex (Muraoka *et al*. 2006). Other subunits of NDH are also required to stabilize CRR3, suggesting that CRR3 is a subunit of the chloroplast NDH complex. Although the conclusion should await biochemical confirmation, CRR3 may interact with other Ndh subunits in the thylakoid membranes, since CRR3 contains one *trans*-membrane domain (Muraoka *et al*. 2006). In contrast to other NDH subunits in chloroplasts, the gene encoding the CRR3 homolog has not beed discovered in cyanobacterial genomes, indicating that CRR3 is specific to the chloroplast NDH complex.

Four plastid-encoded subunits, NdhH-K, are considered to form a hydrophilic arm. To identify the missing subunits involved in electron donor binding, NdhH was His-tagged by using plastid transformation and the NDH subcomplex containing NdhH was purified through Ni^{2+} affinity chromatography (Rumeau *et al*. 2005). NdhH was co-purified

with the other hydrophilic subunits (NdhI, NdhJ and NdhK), as well as with one membrane-embedded subunit NdhA. This subcomplex also contains three new subunits, NdhM, NdhN, and NdhO, which are encoded in the nuclear genome in higher plants. NdhH does not accumulate in Arabidopsis mutants defective in *ndhM*, *ndhN*, and *ndhO*, and NdhN and NdhO were also unstable in the tobacco *ndhB* knockout line (Rumeau *et al*. 2005). From these results, it is clear that three proteins are subunits of the chloroplast NDH complex. These proteins do not contain any known motifs suggesting their electron donor binding. Homologs of NdhM, NdhN, and NdhO were also discovered in cyanobacteria and are components of both NDH-1L and NDH-1M complexes (Prommeenate *et al*. 2004; Zhang *et al*. 2004). If we take all of this information together, we can see that it is likely that the hydrophilic arm, which still lacks the electron donor-binding module in the current model, consists of 7 subunits of NdhH-K encoded by the plastid genome and NdhM-O by the nuclear genome (**Fig. 4**).

CRR7 was discovered in the Arabidopsis mutant, *crr7*, and it is a candidate for the new subunit of the chloroplast NDH complex (Munshi *et al*. 2005). CRR7 is a hydrophilic protein and is likely to be a component of the hydrophilic arm. Although *CRR7* is conserved in cyanobacterial genomes, it was not discovered in the cyanobacterial NDH complex by a proteomics approach (Zhang *et al*. 2004). CRR7 may be too small (10 kDa) to be detected in the two-dimensional gel. It is also possible that CRR7 is a subunit of the electron donor-binding module, which can be easily dissociated from the NDH-1L or NDH-1M complexes.

CRR6 is also essential for stabilizing the chloroplast NDH complex and was discovered in the Arabidopsis mutant (Munshi *et al*. 2006). CRR6 is conserved in the cyanobacterial genomes but not in Chlamydomonas in which chloroplast NDH is probably absent. Although CRR6 is a soluble protein, it localizes to the thylakoid membranes, suggesting that CRR6 is anchored to an unknown membrane protein. In contrast to CRR3 and CRR7, CRR6 stably accumulates in *crr2*. Because *crr2* is defective in the expression of *ndhB*, almost all the Ndh subunits are likely to be unstable in this mutant, as in the cyanobacterial M55 (Hashimoto *et al*. 2003). Although this result does not suggest that CRR6 is a subunit of the NDH complex, we cannot eliminate this possibility. The NDH-1S complexes are stable in the absence of the NDH-1M complex, and CRR6 may peripherally associate with the NDH complex in chloroplasts.

The NDH complex consists of at least 15 subunits in chloroplasts and cyanobacteria. Knockout of one subunit gene generally destabilizes the entire complex. However, the impact of knockout on the complex stability is often specific to each subunit, a fact that is informative in presuming the subunit structure of the complex. Although the cyanobacterial NDH-1M complex is stable in the absence of the NdhD subunit, NdhD is essential for stabilizing the NDH complex in chloroplasts (Kotera *et al*. 2005). This fact suggests that the structure of the NDH complex is somewhat different between chloroplasts and cyanobacteria, despite the high similarity. Although the Ndh-1M complex is an intermediate for both NDH-1MS and NDH-1L complexes in cyanobacteria (Zhang *et al*. 2005), it is unlikely that chloroplast NDH has this plasticity of subunit structure.

Distinct stability is also observed in members of the NdhD and NdhF family. Even in the absence of NdhB, NdhD3 accumulates in the thylakoid membranes in M55, indicating that the NDH-$1S_1$ complex is stable in the absence of the NDH-M complex (Zhang *et al*. 2004). In contrast, NdhF1, which is a component of NDH-1L complex, is unstable in M55.

ELECTRON DONOR TO THE NDH COMPLEX

The chloroplast NDH complex is structurally similar to the bacterial NDH-1 and is likely to be NAD(P)H dehydrogenase. However, this idea has not been supported by any solid experimental evidence. Lack of information on the electron donor-binding subunits makes this suspicion more serious. The most straightforward strategy for clarifying the electron donor is characterization of the purified enzyme. However, because of its fragile nature *in vitro*, purification of the enzyme has not been technically simple. The complex purified from pea chloroplasts has a molecular mass of approximately 550 kDa and consists of at least 16 subunits (Sazanov *et al*. 1998). This purified enzyme prefers NADH as an electron donor. This result is consistent with the fact that the hydrophilic arm containing NdhA has NADH-oxidizing activity (Rumeau *et al*. 2005). However, this subcomplex is unlikely to contain the electron donor-binding module, and it is unclear whether it reflects activity of the NDH complex *in vivo*. In contrast to the results in chloroplasts, NDH purified from cyanobacteria prefers NADPH rather than NADH (Matsuo *et al*. 1998). This story is more complex, since PQ reduction depending on chloroplast NDH requires Fd as well as NADPH in ruptured chloroplasts (Endo *et al*. 1997; Munekage *et al*. 2004). Binding of FNR (Fd-$NADP^+$ oxidoreductase) to the chloroplast NDH complex has also been suggested (Guedeney *et al*. 1996). In conclusion, it is essential to identify the electron donor-binding subunits and purify the complex that includes them.

REGULATION OF ELECTRON TRANSPORT VIA CHLOROPLAST GENE EXPRESSION

The NDH complex alleviates the oxidative stress in chloroplasts. Given that this is true, the accumulation of the chloroplast NDH complex would be regulated to respond to the environmental stresses. In barley (*Hordeum vulgare*), high light intensity at low temperatures photoinactivates PSI and induces a marked increase in the level of the chloroplast NDH complex (Teicher *et al*. 2000). This response is accompanied by alteration of chloroplast gene expression and is possibly regulated via H_2O_2-mediated signaling by oxidative stresses (Casano *et al*. 2001). In addition to elevated gene expression, the chloroplast NDH is also activated via phosphorylation of the NdhF subunit (Lascano *et al*. 2003). It is essential to assess whether these stress responses of the chloroplast NDH complex are specific to barley or general to higher plants. At least the level of NDH complex is not altered under the *pgr5* background in *Arabidopsis*, in which PSI is sensitive to high light intensity (unpublished data). If gene expression is generally regulated by oxidative stress, then it would be interesting to use Arabidopsis, in which the regulatory machinery of chloroplast gene expression is becoming clearer, to study the regulation of chloroplast gene expression in response to an environmental cue.

Plastid gene expression is transcriptionally regulated during chloroplast development by conversion of the transcriptional machinery from the nucleus-encoded phage-type RNA polymerase (Hedtke *et al*. 1997) to the plastid-encoded bacterial-type RNA polymerase (Mullet 1988). The promoter specificity of the plastid-encoded polymerase is determined by nucleus-encoded sigma factors (Kanamaru and Tanaka 2004; Shiina *et al*. 2005). Knockout of SIG4, one of six sigma factors encoded by the Arabidopsis genome, specifically impairs transcription of the chloroplast *ndhF* gene (Favory *et al*. 2005). If the *ndhF* transcription were rate-limiting for accumulation of the NDH complex, SIG4 would be involved in regulation of the NDH level. It is also possible that environmental stimuli may activate several regulatory processes of *ndh* gene expression and that SIG4 is part of this mechanism.

In addition to *ndhF*, 10 chloroplast genes and at least three nuclear genes encode the subunits of the NDH complex (Matsubayashi *et al*. 1987; Rumeau *et al*. 2005). If regulatory accumulation of the chloroplast NDH complex were true, expression of all the genes would have to be coordinately regulated to respond to fluctuating environments. Arabidopsis mutants specifically defective in the expression of chloroplast *ndh* genes were also identified as having

the *crr* phenotype. RNA editing is a process of altering the genetic information from that encoded by the genome at the RNA level (Shikanai 2006). In *ndhD* encoding a subunit of the NDH complex, the translational initiation codon is encoded by ACG in the genome and is edited to AUG by RNA editing in Arabidopsis. An Arabidopsis mutant, *crr4*, is specifically defective in this RNA editing (Kotera *et al*. 2005). CRR4 is a member of the PPR (pentatricopeptide repeat) family, which contains approximately 450 members in Arabidopsis (Lurin *et al*. 2004). PPR proteins are believed to be involved in the steps of maturation of chloroplast or mitochondrial RNA. CRR4 is an RNA-binding protein that specifies the site of RNA editing (Okuda *et al*. 2006). Interestingly, the RNA editing at this site is developmentally regulated, and the extent of the editing is roughly proportional to the expression level of *ndhD* in tobacco (Hirose and Sugiura 1997). Although the story looks plausible, there is no experimental evidence to support the physiological significance of the regulation. In monocots, the site is encoded by ATG in the genome, implying that regulation via RNA editing is dispensable. Once the molecular mechanism of the *ndh* gene expression becomes clear, this possibility of regulation via RNA editing should be re-evaluated.

An additional two PPR protein genes have been identified in the screening of *crr* mutants (Hashimoto *et al*. 2003). PGR3 is involved in RNA stabilization and the translation of at least two distinct target RNAs (Yamazaki *et al*. 2004). Although CRR3 is also involved in accumulation of the cyt b_6f complex, the *pgr3-3* allele was isolated on the basis of its rather specific defect in the NDH complex. Despite the drastic difference in accumulation level, expression of the cyt b_6f complex and the NDH complex is regulated by a single factor, PGR3. Another PPR protein, CRR2, recognizes a unique target of the intergenic region between *rps7* and *ndhB* and is essential for RNA cleavage at this site (Hashimoto *et al*. 2003). Since this RNA cleavage is likely to be essential for *ndhB* translation, CRR2 may be involved in the regulation of *ndhB* translation. In the chloroplast, an intergenic RNA cleavage often balances the translational efficiency of the intervening two genes. *ndhD* is co-transcribed with an upstream gene, *psaC*, encoding a subunit of PSI. The accumulation level of PSI is much higher than that of the NDH complex (approximately 100 times), thus requiring regulation of translation. In most precursor RNA, RNA cleavage between *psaC* and *ndhD* takes place to disrupt the *ndhD* coding region, resulting in repression of *ndhD* translation (del Campo *et al*. 2002). An intact *ndhD* mRNA is less frequently generated via the RNA cleavage that occurs in the intergenic region, and the resulting monocistronic *ndhD* mRNA is also regulated via RNA editing for translation (Hirose and Sugiura 1997).

We did not identify any nuclear transcriptional factors in the *crr* mutant pool. Since at least four genes, *ndhL-O*, encode the Ndh subunits in the nuclear genome, it is plausible that some transcriptional factors are specifically involved in the *ndh* gene expression to respond to environmental signals. Because our strategy of map-based cloning focused on genes encoding plastid-targeting proteins, we may have eliminated transcriptional factors in the nuclei. The machinery of plastid gene expression may be also under the control of transcriptional factors. The 11 chloroplast *ndh* genes may be good targets for clarifying the network that coordinately regulates the expression of nucleus- and plastid-encoded genes.

CONCLUDING REMARKS

Despite progress in our knowledge of the chloroplast NDH complex, several fundamental questions remain. Priority should be given to the identification of electron donor-binding subunits and the electron donor itself. We have discussed the physiological function of the enzyme, but we are still not sure of the reaction mediated by the enzyme. On the basis of recent advances in molecular genetics and proteomics, it seems unlikely that it will take long to get an answer. This review will hopefully be rewritten soon under a new concept. PSI cyclic electron transport is one of the last enigmas remaining in the field of research on photosynthesis, but this statement is becoming less true for some topics.

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