Review

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Physiological Function of a Respiratory Complex, NAD(P)H Dehydrogenase in Chloroplasts: Dissection by Chloroplast Reverse Genetics

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Abstract

The chloroplast genome of higher plants contains eleven *ndh* genes encoding homologs of mitochondrial complex I subunits. The enigmatic occurrence of the respiratory complex in chloroplasts suggests the possibility that NAD(P)H dehydrogenase catalyzes cyclic electron flow around photosystem I and/or chlororespiratory electron flow, as it does in cyanobacteria. Establishment of a chloroplast transformation technique in tobacco facilitated the reverse genetic approach. Although some conclusions are still matters of controversy, it is sure that the gene product, NDH, functions to donate electrons to plastoquinone. Although NDH is dispensable under mild growth conditions, *ndhB* disruptant is more sensitive to high light stress, suggesting that NDH functions in minor compensation for the electron flow to cope with changes in environmental conditions. Here, we review the physiological function of NDH in chloroplasts, including important subjects that are still unclear.

Introduction

Plastid transformation was first established in unicellular green algae, *Chlamydomonas reinhardtii* (Boynton *et al.*, 1988), and subsequently in tobacco (Golds *et al.*, 1993; Svab and Maliga, 1993). Since DNA is incorporated into plastid DNA via homologous recombination, plastid transformation can be utilized in gene disruption and site-directed mutagenesis (for reviews, Rochaix, 1997; Tomizawa *et al.*, 2000). Although the transformation efficiency should be improved in higher plants, the technology has been almost completely established as far as in tobacco and is a powerful tool for *in vivo* analysis in plant molecular biology and physiology.

When the technology became available, prior targets of the chloroplast reverse genetics were eleven *ndh* genes encoding homologs of mitochondrial complex I subunits (Matsubayashi *et al.*, 1987). It was surprising to find genes encoding the respiratory component in the chloroplast genome. It has been speculated that the putative gene product, NDH, may be involved in cyclic electron flow around photosystem (PS) I and/or respiratory electron flow in chloroplasts (chlororespiration).

The contribution of cyclic electron flow around PSI in chloroplast energetics has been a topic of general interest in plant physiology (Fork and Herbert, 1993; Bendall and Manasse, 1995). Theoretically, it can modulate the production ratio of NADPH and ATP. Furthermore, the possible function of the electron flow in downregulation of PSII has been discussed (Heber and Walker, 1992). Cyclic electron flow around PSI can generate a proton gradient across the thylakoid membranes (ΔpH), which can induce nonradiative dissipation of absorbed light energy from PSII, especially under limiting conditions of the electron acceptor from PSI. Despite its physiological significance, the mechanism of the electron flow has been poorly understood.

Chlororespiration, respiratory electron flow in chloroplasts, is a classical but still unclear topic in plant physiology (Bennoun, 1982; 1994; Peltier *et al.*, 1987). The electron flow involves the reduction of plastoquinone (PQ) by NAD(P)H, with subsequent oxidation of plastoquinol (PQH_2) terminating with the reduction of molecular oxygen. It is probable that putative NAD(P)H dehydrogenase (NDH) encoded by chloroplastic *ndh* genes mediates the reduction of PQ in chlororespiration. It is

also possible that the chlororespiratory machinery catalyzing PQ reduction may also function in cyclic electron flow around PSI in light.

We can see the analogy of the proposed electron flow in cyanobacteria, in which respiratory and photosynthetic electron flows locate in the same membranes. Cyanobacterial NDH functions both in respiratory electron flow and cyclic electron flow around PSI (Mi *et al.*, 1992a; 1992b; 1994; 1995). A probable hypothesis is that chloroplasts retained the cyanobacterial respiratory complex as a regulatory mechanism of the photosynthetic electron flow.

Chloroplast reverse genetics must focus on two important questions. (1) Does NDH mediate cyclic electron flow around PSI and/or chlororespiration? (2) What is the physiological function of the NDH-mediated electron flow? Unfortunately, we are still not sure of the complete answer. Thus, this review describes current understanding of the available information, some of which is still a matter of discussion.

Electron transport mediated by chloroplastic NDH

When complete nucleotide sequences were determined in tobacco (Shinozaki *et al.*, 1986) and liverwort (Ohyama *et al.*, 1986), it was surprising that the genome contains eleven *ndh* genes encoding homologs of mitochondrial complex I (NADH dehydrogenase) subunits (Matsubayashi *et al.*, 1987). It has been and probably still is an enigma why plastid genomes encode subunits of the respiratory complex. Considering the prokaryotic origin of plastids, a gene product, NDH, may have originated from the respiratory complex in cyanobacteria, in which respiratory and photosynthetic electron transport pathways locate in the same membranes and interact with each other via a common electron pool. The hypothesis is supported by the fact that plastid *ndh* genes are more similar to cyanobacterial ones than to the mitochondrial counterparts in the same plant species.

Respiratory electron flow in *Chlamydomonas* chloroplasts has been a matter of interest for plant physiologists prior to the finding of chloroplast *ndh* genes (Bennoun , 1982; 1994; Peltier *et al.*, 1987). The electron flow referred to as chlororespiration was also suggested to be present in higher plants (Garab *et al.*, 1989; Feild *et al.*, 1998).

Findings of plastid *ndh* genes led to the serious consideration of the theory that NDH mediates the electron flow from NAD(P)H to PQ in chlororespiration. Another important breakthrough in understanding the physiological function of the respiratory electron flow in chloroplasts was the discovery of an *ndhB*-deficient mutant in *Synechocystis* PCC6803 (Ogawa, 1991). The mutant, *M55*, requires high CO_2 concentration for growth and is unable to transport CO_2 and HCO_3^- into the intracellular C_i pool. In *M55*, cyclic electron flow around PS I was severely impaired, indicating that NDH mediates this photosynthetic electron flow (Mi *et al.*, 1992a; 1992b; 1994; 1995). The phenotype of *M55* can be explained by the theory that cyclic electron transport mediated by NDH generates additional ΔpH to energize the machinery of inorganic CO_2 concentration. These results raised the possibility that NDH also mediates cyclic electron transport around PSI in light in chloroplasts of higher plants, as well as the chlororespiratory electron flow in the dark.

Fig. 1 summarizes the proposed electron flows in chloroplasts. Considering dual function of cyanobacterial NDH in respiratory electron flow and cyclic electron flow around PSI, chloroplastic NDH may also function in both electron flows. We realize that the determination of the photosynthetic electron transport pathway has not yet become common. Molecular identity of ferredoxin-PQ reductase (FQR) and terminal oxidase in chlororespiration (TO) has not been assigned and the physiological significance of the electron flows is still poorly understood.

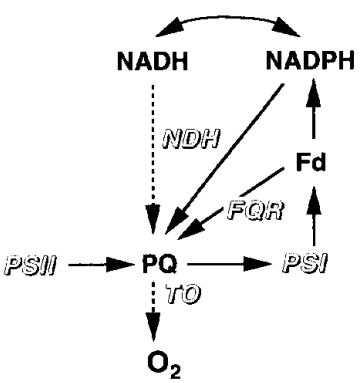

Fig. 1 Proposed electron flows in chloroplasts. Arrows with straight lines and dot lines depict photosynthetic electron flow and chlororespiratory electron flow, respectively. Cyclic electron flow around PSI is catalyzed both by ferredoxin-PQ reductase (FQR) and NAD(P)H dehydrogenase (NDH). Chlororespiration involves dark-reduction of PQ by NADH and subsequent oxidation of PQH_2 by unidentified terminal oxidase (TO). NADH and NADPH are interconvertible via transhydrogenase.

Disruption of *ndh* genes

As a technology became available, the interests of researchers concentrated on a phenotype of the *ndh* disruptants in higher plants. **Fig. 2** shows a scheme of the gene disruption by chloroplast transformation. We selected *ndhB* for the target (Shikanai *et al.*, 1998), since it was essential for electron transport in *Synechocystis* PCC6803 (Ogawa, 1991). *ndhB* was inactivated by the insertion of the aadA (aminoglycoside adenyl transferase) cassette conferring resistance to spectinomycin and streptomycin (**Fig. 2**). Transformation vector was delivered by bombardments into chloroplasts and then incorporated into the genome by homologous recombination. Since chloroplast genome is present in multiple copies, gene disruption requires repetitive regeneration processes on a medium containing antibiotics to eliminate the wild-type copy completely. Even though the target gene is essential for photosynthesis such as *rbcL*, it can be completely disrupted by culturing transformants on a medium containing sucrose (Kanevski and Maliga, 1994).

In addition to our group, three groups independently disrupted *ndh* genes. Medgyesy and Peltier group disrupted the same gene, *ndhB*, introducing a stop codon in the coding region (reported in 5th International Congress of Plant Molecular Biology, Singapore). Maliga and Nixon Group disrupted *ndhC*, *ndhK* and *ndhJ* (Burrows *et al.*, 1998). In addition to these three genes, Koop and Steinmüller group disrupted *ndhA*, *ndhI* and *ndhJ* (Kofer *et al.*, 1998). Although the strategy was straight forward, the resulting conclusions were quite controvertible

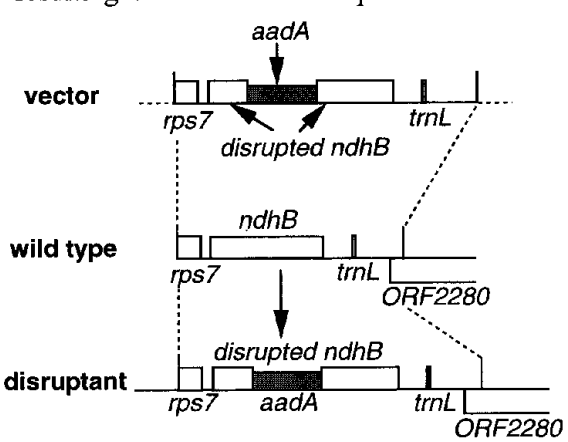

Fig. 2 Disruption of *ndhB*. *ndhB* was insertionally disrupted by aadA conferring spectinomycin and streptomycin resistance (vector). The sequence was delivered into chloroplasts and incorporated into the genome (wild type) by homologous recombination (disruptant).

(Maliga and Nixon, 1998; Koop *et al.*, 1998). Koop and Steinmüller group reported that *ndh* genes were essential for viability and partial gene disruption induced a strong phenotype, accumulation of starch in chloroplasts. In contrast, the remaining three groups came to the similar conclusion that the disruptants were rather normal under the mild culture conditions. The most critical point in this discrepancy is how the homoplasmic state was evaluated (Maliga and Nixon, 1998). A chloroplast contains approximately 100 copies of plastid DNA molecules and a cell contains 100 chloroplasts, indicating that 10,000 copies of plastid DNA molecules are present in a tobacco cell. The severe problem is the presence of copies of plastid sequences in mitochondria and nuclear genomes as promiscuous sequences, which are detected by the PCR analysis even in the complete disruptant. Thus, evaluation of homoplasmic state is rather difficult in higher plants (Hager *et al.*, 1999; Tomizawa *et al.*, 2000). We performed RT-PCR to show the homoplasmic state of the *ndhB*-disruptant, since promiscuous sequences are not spliced to mature transcripts even though they are accidentally transcribed (Shikanai *et al.*, 1998).

After the first screening, the genome of transformants often consisted of a mixture of the wild-type and transformed DNA molecules (heteroplasmic) and *ndhB* was partially disrupted. The genome composition was rather stable on the medium without the spectinomycin pressure, suggesting that the function of *ndhB* was dispensable under the culture conditions used. In contrast, continuous spectinomycin pressure was necessary to maintain the heteroplasmic state in the disruptants of *clpP* encoding a proteolytic subunit of ATP-dependent protease (Shikanai *et al.*, in preparation). When the partial *clpP* disruptants were cultured on the medium without antibiotics, the genome composition rapidly reverted to the wild type.

To examine the contribution of NDH in the possible electron flow from NAD(P)H to PQ, four groups focused on the same phenomenon, transient increase in chlorophyll fluorescence after actinic light illumination (Asada *et al.*, 1993). **Fig. 3** shows the principle of *in vivo* analysis. Chlorophyll fluorescence is emitted from PSII and reflects the reduction state of the primary quinone-type acceptor Q_A and consequentially the redox level of the PQ pool (Krause and Weis, 1991). In dark-adapted leaves, the PQ pool is almost completely oxidized and the minimum level of chlorophyll fluorescence (Fo) is emitted. Actinic light illumination induces fluorescence with complex kinetics terminating to the steady-state level of fluorescence

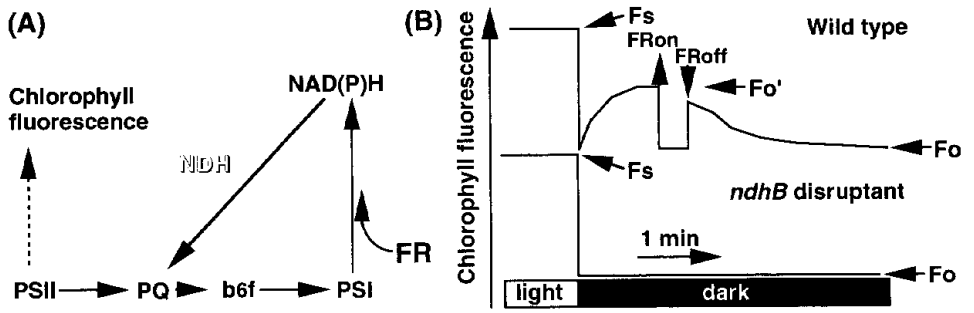

Fig. 3 *In vivo* analysis of the NDH-mediated electron transport. (A) Redox state of PQ can be monitored by chlorophyll fluorescence emitted from PSII. The PQ pool reduced by NAD(P)H which accumulates during actinic light illumination via the NDH-mediated pathway. (B) Transient increase in chlorophyll fluorescence in the dark (Fo'). The post-illumination Fo increase is due to the reduction of PQ, since it is quenched by far-red light (FR) activating PSI. In the *ndhB* disruptant, the transient Fo increase was arrested, indicating that NDH functions in the dark reduction of PQ. Fo and Fs stand for minimum chlorophyll fluorescence level in the dark and steady state level in the light, respectively.

(Fs). After the termination of actinic light, the PQ pool is once fully oxidized and then transiently reduced by electron donation possibly from NAD(P)H which is accumulated during the actinic illumination. The transient increase of chlorophyll fluorescence in the dark (Fo') is due to the reduction of PQ, since it is quenched by far-red light which activates PSI. In the *ndhB* disruptant, the transient increase in Fo was inhibited, indicating that NDH functions in the electron donation to PQ.

We also analyzed oxidation kinetics of P700 (reaction center chlorophyll of PSI) after the actinic light illumination under the background of far-red light (Shikanai *et al.*, 1998). Maliga and Nixon group showed dark reduction kinetics of P700 following far-red illumination (Burrows *et al.*, 1998). Both analyses demonstrate the dark reduction of PQ, which are essentially identical to the analysis of chlorophyll fluorescence. Thus, all results presented by the four groups were consistent with each other, demonstrating that NDH mediates the electron donation to PQ in the dark.

Although NDH can mediate the electron donation to PQ in the dark, the electron flow can also function in cyclic electron flow around PSI in the light. We could not find any significant difference in nonphotochemical quenching levels of chlorophyll fluorescence (NPQ) between the wild type and *ndhB* disruptant. The result indicates that contribution of the NDH-mediated pathway is rather small in the plants cultured under mild conditions.

What is an electron donor to NDH?

Rather subtle phenotypes of *ndh* disruptants made it difficult to speculate the physiological function of NDH by simple reverse genetic approaches. Thus, it is extremely important to determine the electron donor to NDH with the combination of biochemical approach. When NADPH is an electron donor, NDH can function in cyclic electron flow around PSI (**Fig. 1**), as can cyanobacteria (Mi *et al.*, 1992a; 1992b; 1994). When NADH is an electron donor, however, NDH functions in respiratory electron transport (**Fig. 1**), as does mitochondria (Chlororespiration). It may modulate cyclic electron transport around PSI by poising the reduction state of PQ (Casano *et al.*, 2000).

When the NDH complex was purified from *Synechocystis* PCC 6803, it accepted electrons from NADPH but not from NADH (Mi *et al.*, 1995; Matsuo *et al.*, 1998). The result is consistent with the fact cyanobacterial NDH functions in cyclic electron flow around PSI (Mi *et al.*, 1992a; 1992b; 1994). In higher plants, however, the purified NDH complex from barley (Casano *et al.*, 2000) and pea (Sazanov *et al.*, 1998) showed NADH-specific activity. In contrast with the results using purified complexes, spinach thylakoid membranes exhibited the reduction of PQ by NADPH but not by NADH, which was suppressed by inhibitors of the mitochondrial complex I (Endo *et al.*, 1997). Similar analysis using tobacco thylakoid membranes showed the bi-phasic inhibition of PQ reduction by antimycin A (Endo *et al.*, 1998). The activity less sensitive to antimycin A was impaired in the *ndhB* disruptant, suggesting that it was catalyzed by NDH (Endo *et al.*, 1998).

What is a reason for the discrepancy between experiments using purified complexes and thylakoid membranes? One possible explanation is that NDH complex is unstable *in vitro*. We showed that the

NADPH-dependent PQ reduction was found only when the intact chloroplast was ruptured in the buffer containing a high concentration of $MgCl_2$ (30 mM) in spinach, suggesting that the fraction involving NADPH-dependent activity is easily dissociated from the main complex (Endo *et al.*, 1997). To conclude the hypothesis, we must demonstrate the biochemical data on the fraction that is missing in the purified complex. Preliminary work was reported by Mi *et al.* (1998).

One reason why the problem of the electron donor is so serious is that chloroplast genomes do not encode homologs of subunits binding NADH in the mitochondrial complex I. The minimal structural framework of NADH dehydrogenase requires 14 subunits, which are common in mitochondrial and bacterial complexes (Friedrich *et al.*, 1995). However, genes encoding three subunits are missing in chloroplast genomes. Although these essential genes may be encoded in the nuclear genome, complete nucleotide sequence determination also failed to find three genes in *Synechocystis* PCC6803. Subunits encoded by the genes are functional in NADH binding, suggesting the possibility that cyanobacterial and chloroplastic NDH is equipped with other electron acceptor subunits. One possible idea is the involvement of ferredoxin-NADP reductase (FNR) in NDH activity (Guedeney *et al.*, 1996; Jose Quiles and Cuello, 1998).

Physiological function of NDH

Except for minor changes in the electron flow, the *ndhB* disruptant exhibited no phenotypes under the greenhouse culture conditions, indicating that NDH is dispensable under mild conditions (Shikanai *et al.*, 1998). However, it is highly probable that NDH functions in minor compensation of the electron flow, which is important to gain the maximum yield of photosynthesis in the field.

We found that the *ndhB* disruptant was more sensitive to a brief exposure to supra-saturating light (3,000 μmol quanta m^{-2} s^{-1} for 20 min) (Endo *et al.*, 1999). After the exposure, the reaction center of PSI (P700) was not fully oxidized by far-red light due to rapid charge recombination in PSI, which is explained by over-reduction of the stroma. We consider that NDH functions in a short time response to high light stress by preventing over-reduction of the stroma.

What is a function of NDH in preventing over-reduction of the stroma? The simplest theory is that under severe light-excessive conditions, NDH functions to generate additional ΔpH by cyclic electron flow around PSI, which is important to induce downregulation of PSII photochemistry. However, we failed to detect any difference in NPQ levels reflecting the regulation between the wild type and the *ndhB* disruptant (Shikanai *et al.*, 1998). Possibly the subtle difference in ΔpH generation, which cannot be detected by NPQ, may protect the stroma from over-reduction by irradiation of the supra-saturating light.

Although the contribution of the NDH-mediated pathway to inducing NPQ is subtle in a healthy plant, it may function significantly in a photo-damaged leaf. Photo-oxidative stress impairs a reaction center subunit of PSII, D1, which limits the electron transport from PSII. Under these conditions, the cyclic electron flow around PSI may function significantly to generate ΔpH inducing NPQ. It is also possible that the cyclic electron flow around PSI functions when the electron acceptor from PSI is limited such as under drought conditions. Maliga and Nixon group reported that NPQ induction was slightly affected in the *ndh* diruptants under water stress conditions (Burrows *et al.*, 1998).

Another possible function of the NDH-mediated pathway is a compensation of the NADPH/ATP production ratio. Although this rather classical idea on cyclic electron flow around PSI is unlikely under mild conditions, it may be indispensable to avoid stromal over-reduction under excessive light conditions.

Since photosynthesis is not affected in *ndh* disruptants, it appears that NADPH and ATP synthesis is well-balanced in C_3 plants without NDH. However, the idea is likely in C_4 plants. Expression of *ndh* genes is enhanced in bandle-sheath chloroplasts in C_4 plant *Sorghum bicolor*, suggesting that NDH is involved in cyclic electron flow around PSI to energize the machinery of CO_2 concentration (Kubicki *et al.*, 1996). NDH may function also in C_3 plants under the conditions in which extra ATP synthesis is required and C_4 plants may have utilized the mechanism of the minor compensation as a constitutive modulator of NADPH/ATP synthesis ratio in bandle-sheath cells.

Terminal oxidase functioning in chlororespiration

Oxygen uptake associated with chlororespiration appears to be catalyzed by a terminal oxidase which is inhibited by CN^- and CO in *Chlamydomonas* (Bennoun, 1982). Nonphotochemical reduction of PQ is enhanced by CO in sunflower, suggesting that terminal oxidase is also CO-sensitive in higher plants (Feild *et al.*, 1998). However, the terminal

oxidase has not been identified until now. One possible candidate is a chloroplast homolog of the mitochondrial alternative oxidase. It was identified as a mutant gene (*immuntans*) causing a variegated leaf phenotype in Arabidopsis (Wu *et al.*, 1999; Carol *et al.*, 1999). The oxidase oxidizes PQH_2 in the early steps of chloroplast development, in which PSI still does not function. Since phytoene desaturase requires the oxidized form of PQ for its activity, carotenoid biosynthesis is arrested during early chloroplast biogenesis in *immutans*, leading to photo-bleaching. The function of the oxidase in mature chloroplasts possibly in chlororespiration has not been assigned.

Recently, a new model of the terminal oxidation in chlororespiration was proposed (Casano *et al.*, 2000). In this model, electron transfer from PQH_2 to molecular oxygen results from sequential reactions, oxidation of PQH_2 by unidentified peroxidase, SOD and non-enzymatic reduction of O_2. The sensitivity of the putative terminal oxidase to CN^- can be explained by involvement of Cu/Zn-SOD in the reaction and CO inactivates the hemoprotein peroxidases. Their model does not require the terminal oxidase.

Future prospects

Despite introduction of the reverse genetic approach, physiological function of chloroplast *ndh* genes has been only poorly understood. Chloroplast reverse genetics should be coupled with high quality physiology and biochemistry to perform its maximum level.

The most important future work is to determine the electron donor to chloroplastic NDH, which must be very suggestive to speculate the physiological function. Identification of subunits functioning in the electron donor binding may determine it. A genetic approach using imaging of chlorophyll fluorescence may also be a breakthrough in identifying nuclear-encoded subunits of NDH (Niyogi, 1999; Shikanai *et al.*, 1999)

Differences in the stress sensitivity between the wild type and the *ndh* disruptants must be surveyed more extensively. Environmental conditions are variable and consequently very stressful to plants and are rather difficult to reproduce under experimental conditions. For instance, nobody doubts the physiological significance of thermal energy dissipation of absorbed light energy from PSII (Horton *et al.*, 1996), although Arabidopsis mutants lacking the machinery are rather tolerant of high light intensity (Niyogi *et al.*, 1998; Li *et al.*, 2000). For this purpose, the *ndh* disruptants should be produced in other plant species, since tobacco is rather tolerant to variable stresses.

Disruption of *ndh* genes using C_4 plants should be very informative, since the contribution of the NDH-mediated pathway is estimated to be very large (Asada *et al.*, 1993; Kubicki *et al.*, 1996). We desire the establishment of chloroplast transformation in C_4 plants.

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