In Planta Mutagenesis of Src Homology 3 Domain-like Fold of NdhS, a Ferredoxin-binding Subunit of the Chloroplast NADH Dehydrogenase-like Complex in Arabidopsis

A CONSERVED ARG-193 PLAYS A CRITICAL ROLE IN FERREDOXIN BINDING

Hiroshi Yamamoto and Toshiharu Shikanai

From the 1Department of Botany, Graduate School of Science, Kyoto University, Kyoto 606-8502 and 2CREST, Japan Science and Technology Agency, Chiyoda-ku, Tokyo 102-0076, Japan

Background: NdhS is a ferredoxin-binding subunit of chloroplast NADH dehydrogenase-like (NDH) complex.

Results: Mutagenesis of the Src homology 3-like domain of NdhS revealed that its positively charged pocket is required for high affinity binding of ferredoxin.

Conclusion: The positive charge of Arg-193 plays a critical role in electrostatic interaction with ferredoxin.

Significance: Our results provide insights into the evolution of the electron input module of chloroplast NDH.

Chloroplast NADH dehydrogenase-like (NDH) complex mediates cyclic electron transport around photosystem I and chlororespiration in angiosperms. The Src homology 3 domain (SH3)-like fold protein NdhS/CRR31 is an NDH subunit that is necessary for high affinity binding of ferredoxin, indicating that chloroplast NDH functions as a ferredoxin:plastoquinone oxidoreductase. However, the mechanism of the interaction between NdhS and ferredoxin is unclear. In this study, we analyzed their interaction in planta by using site-directed mutagenesis of NdhS. In general, binding of ferredoxin to its target proteins depends on electrostatic interaction. In silico analysis predicted the presence of a positively charged pocket in the SH3-like domain of NdhS, where nine charged residues are highly conserved among plants. Systematic alteration of these sites with neutral glutamine revealed that only arginine 193 was required for high NDH activity in vivo. Further replacement of arginine 193 with negatively charged aspartate or glutamate or hydrophobic alanine significantly decreased the efficiency of ferredoxin-dependent plastoquinone reduction by NDH in ruptured chloroplasts. Similar results were obtained in in vivo analyses of NDH activity and electron transport. From these results, we propose that the positive charge of arginine 193 in the SH3-like domain of NdhS is critical for electrostatic interaction with ferredoxin in vivo.

Light reactions of photosynthesis in the thylakoid membrane of chloroplasts convert light energy into NADPH and ATP. The process consists of two types of electron transport: linear electron transport and cyclic electron transport around photosystem I (CET). In linear electron transport, photosystem II (PSII) oxidizes water to dioxygen in its manganese cluster, and the electrons derived are transported to photosystem I (PSI) via plastoquinone (PQ), the cytochrome b6f complex, and plastocyanin. PSI reduces ferredoxin (Fd), an iron-sulfur electron carrier protein, and Fd donates electrons to ferredoxin:NADP+ oxidoreductase (FNR) to reduce NADP+ to NADPH for CO2 fixation by the Calvin-Benson cycle. Proton translocation from the stroma to the thylakoid lumen, coupled with the Q-cycle in the cytochrome b6f complex and water oxidation in PSI, produces the proton motive force to produce ATP by ATP synthase. In contrast, CET is driven solely by PSI, and electrons are recycled fromFd to PQ (1). Consequently, CET generates ΔpH without reducing NADP+. Two physiological roles of CET have been proposed (2–4): 1) regulation of the ATP to NADPH production ratio in photosynthesis and 2) acidification of the thylakoid lumen to induce the thermal dissipation of absorbed excess photon energy in PSI, which can be monitored as a Dq component of non-photochemical quenching (NPQ) of chlorophyll fluorescence. Acidification of the thylakoid lumen down-regulates the cytochrome b6f complex to prevent overreduction of PSI, especially under fluctuating light conditions (5). In angiosperms, backflow of electrons from Fd to PQ operates via two redundant pathways, namely a PGR5 (proton gradient regulation 5)/PGRL1 (PGR5-like photosynthetic phenotype 1) complex-dependent anticyanin A(AA)-sensitive pathway (6–9) and an NDH dehydrogenase-like complex (NDH)-dependent anticyanin A-insensitive pathway (10–12).

The complete sequencing of plastid genomes has revealed 11 genes, ndhA to ndhK, that encode proteins homologous to subunits of mitochondrial NADH dehydrogenase (complex I) (13). Chloroplast NDH is more similar to cyanobacterial NDH-1, which is believed to be the origin of chloroplast NDH, than to...
mitochondrial NADH dehydrogenase present in the same species (14). Extensive genetic and proteomic analyses have revealed that the chloroplast NDH complex is composed of five subcomplexes, i.e. the A, B, membrane, lumen, and electron donor-binding subcomplexes (15, 16). Chloroplast NDH is further associated with PSI to form a >1-MDa supercomplex via minor light-harvesting complex I (LHCl) proteins (15, 16). Recently, the fine tertiary structure of complex I of the bacterium *Thermus thermophilus* was resolved (17–19). The bacterial complex I forms an L-shaped structure consisting of a peripheral arm and a membrane-embedded domain. The top of the peripheral arm is equipped with three subunits, Nqo1 to -3, involved in the oxidation of NADH (17). Although the L-shaped skeleton is conserved in chloroplast and cyanobacterial NDH, the complex does not include subunits corresponding to Nqo1 to -3. For this reason, the nature of the electron donor and electron donor-binding subunits has long been unclear in chloroplast and cyanobacterial NDH.

A novel NDH subunit, NdhS/CRR31 (cholororespiratory reduction 31), has been identified by proteomic and genetic analyses of the NDH-PSI supercomplex in *Arabidopsis thaliana* (12). NdhS is a thylakoid peripheral protein, and its C-terminal region forms the Src homology 3 domain (SH3)-like fold that is known to be involved in protein-protein interaction (20). NdhS interacts with subcomplex A of NDH complex via the J-proteins NdhT/CRRJ and NdhU/CRRRL (12), forming an electron donor-binding subcomplex in chloroplast NDH (14). NDH with high affinity forFd has been reconstituted in ruptured chloroplasts isolated from *Arabidopsis* null allele *ndhs-1 (crr31-1)* by the addition of recombinant NdhS (12). From these results, we propose that the chloroplast NDH complex functions as a PGR5/PGRRL1-independent, Fd-dependent PQ reductase and that it should be renamed as NADH dehydrogenase-like complex (12).

Chloroplast Fds are small, two-iron two-sulfur proteins that serve as electron donors in various metabolic pathways other than photosynthesis, such as nitrogen and sulfur assimilation (21–23). The surface net charge of Fd is highly negative, and this property plays a crucial role in electrostatic interaction with its target proteins, such as FNR, nitrite reductase, and sulfite reductase (24). To address the mechanisms underlying the interaction between NdhS and Fd, we conducted site-directed mutagenesis of nine highly conserved charged amino acids in the SH3-like domains from land plant NdhS homologs and analyzed the function of NdhS variants in planta.

**EXPERIMENTAL PROCEDURES**

**Plant Materials and Growth Conditions—*A. thaliana* (Columbia) was grown in soil in a growth chamber (50 μmol of photons m⁻² s⁻¹, 16-h photoperiod, 23 °C) for 3–4 weeks. Biochemical and physiological analyses were performed on plants before the initiation of bolting. *ndhs-1/crr31-1* was provided by GABI-Kat, and *ndhs-1 pgr5* was produced as reported previously (12).

In *Planta* Site-directed Mutagenesis of NdhS—The wild-type genomic sequence containing NdhS cloned into pDONR/Zeo (Invitrogen) was used as a template for site-directed mutagenesis (12). NdhS has no intron sequences. Site-directed mutagenesis of triplet codons for the positively or negatively charged amino acids in the SH3-like domain (K178Q, R193Q, R193K, R193H, R193D, R193E, R193A, E204Q, D209Q, R210Q, K232Q, R222Q, and K224Q) was done with a QuikChange site-directed mutagenesis kit (Stratagene). All of the mutated sequences were confirmed by sequencing and then transferred to the binary vector pGWBN1 by LR Clonase reaction (Invitrogen). *Agrobacterium tumefaciens* C58 was transformed by electroporation with the resultant plasmids, and the bacteria were used to transform *Arabidopsis ndhs-1* and *ndhs-1 pgr5* mutants by using the floral dip method (25). Transformed plants were selected on solidified 1/2 Murashige-Skoog medium containing 7.5 μg ml⁻¹ BASTA, and insertion of the BASTA resistance gene (bar) into the genome was verified by PCR. The T₂ generation of transgenic lines was used for physiological and biochemical analyses.

In Vivo Chlorophyll Fluorescence Analyses—Chlorophyll fluorescence was measured with a pulse-amplitude modulation portable chlorophyll fluorometer, MINI-PAM (Walz), as described before (12). Three or four plants of each genotype were analyzed, and average values and standard deviations were calculated. Minimum fluorescence at open PSII centers in the dark-adapted state (*F₀*) was excited with a weak measuring light (wavelength 650 nm) at a photon flux density (PFD) of 0.05–0.1 μmol of photons m⁻² s⁻¹. A saturating pulse of white light (800 ms, 8000 μmol of photons m⁻² s⁻¹) was applied to determine the maximum fluorescence at closed PSII centers in the dark-adapted state (*Fₘ*) and during actinic light (AL) illumination (*Fₘ’*). The steady-state fluorescence level (*Fₛ*) was recorded during AL illumination (5–1000 μmol of photons m⁻² s⁻¹). The maximum quantum yield of PSII was calculated as *Fₛ/Fₘ*. Electron transport rate (ETR) was calculated as *Φₛ/Fₘ* × PFD (μmol of photons m⁻² s⁻¹). *qL*, the fraction of open PSII center (27), was calculated as (*Φₛ/Fₘ’*)/(*Φₛ/Fₘ’’*) × (NPQ + 1) (28). The transient increase in chlorophyll fluorescence after the AL was turned off was monitored as described in the legend for Fig. 2 (10). Intensity of the measuring light was 1 μmol of photons m⁻² s⁻¹.

In Vitro Assay of Fd-dependent PQ Reduction—In vitro assay of Fd-dependent PQ reduction was performed as described previously (12). Intact chloroplasts (20 μg of chlorophyll ml⁻¹) were osmotically ruptured in 50 mM HEPES-NaOH (pH 8.0) for each assay. NADPH (0.25 mM) and the indicated concentrations of spinach Fd (Sigma) were added, and the increase in apparent minimum chlorophyll fluorescence (*Fₘ’*) was recorded with a MINI-PAM. Fluorescence levels were normalized against *Fₘ* levels. To measure NDH-dependent PQ reduction, 5 μM AA (Sigma) was added to the assay to inhibit PGR5-PGRRL1-dependent PQ reduction activity, as reported previously (29).

**SDS-PAGE and Immunoblot Analyses**—Intact chloroplasts were purified from the leaves of 3–4-week-old plants, as described previously (6). The purified chloroplasts were suspended in 20 mM HEPES-KOH (pH 7.6) containing 5 mM MgCl₂ and 2.5 mM EDTA. The insoluble fraction containing thylakoids and envelopes was separated from the stromal frac-
tion by centrifugation for 5 min at 15,000 × g. The chlorophyll concentration was determined as described previously (30). Proteins in the insoluble fraction separated by 12.5% (w/v) SDS-PAGE were electrotransferred onto polyvinylidene fluoride membranes. The antibodies were added, and the protein-antibody complexes were labeled by using an ECL Plus Western blotting detection kit (GE Healthcare). Chemiluminescence was detected with a lumino-image analyzer LAS3000 (FUJIFILM) and analyzed with MultiGauge Version 3.0 software (FUJIFILM). Antibody against FNR was purchased from Agrisera.

**Homology Modeling and Calculations of Surface Net Charge and Hydrophobicity**—Homology models of the SH3-like domain in NdhS variants were constructed by using Swiss-Model, with a *Synechocystis* sp. PCC 6803 NdhS homolog (Protein Data Bank (PDB): 3C4S) as a template. The surface net charges of the homology models were calculated with PDB2PQR Server version 1.7 by using the Poisson-Boltzmann equation (31). PROPKA was used to assign the protonation states of the positively charged residues, only Arg-193 was necessary for Fd binding (32).

**Accession Numbers**—Sequence data from this article can be found in the *Arabidopsis* Genome Initiative or GenBank™/European Molecular Biology Laboratory (EMBL) databases under the following accession numbers: *A. thaliana* NdhS (AT4G23890), *Vitis vinifera* NdhS (XP_002269878), *Populus trichocarpa* NdhS_1 (XP_002297973), *P. trichocarpa* NdhS_2 (XP_002304561), *Oryza sativa* NdhS (NP_001059119, Os07g0196200), *Sorghum bicolor* NdhS (XP_002445975), *Zea mays* NdhS (NP_001143622), *Physcomitrella patens* subsp. *patens* NdhS (XP_001770269), *Triticum urartu* BP-1 (NP_681425, TLR0636, *Thermosynechococcus elongatus* BP-1), *N. 7120* (NP_4846981, ASRO654, *Nostoc* sp. PCC 7120), *S. 6803* (NP_442353, SSL0352, *Synechocystis* sp. PCC 6803), and *UCYN-A* (NC_013771.1, cyanobacterium UCYN-A).

**RESULTS**

Identification of Conserved Charged Amino Acids and Positively Charged Pocket of the SH3-like Domain of NdhS—Fig. 1A shows multiple alignments of the C-terminal region of NdhS homologs from land plants and cyanobacteria. Although the ocean cyanobacterium UCYN-A does not encode genes for PSII subunits or Calvin-Benson cycle enzymes, ndhS is conserved as well as other genes for NDH subunits of A and membrane subcomplexes (36). In the cyanobacterium *Synechocystis* sp. PCC 6803 (S. 6803), the function of NdhS as a subunit of the NDH complex is conserved (37). By homology modeling on the basis of the tertiary structure of S. 6803 NdhS (PDB: 3C4S), we revealed that *Arabidopsis* NdhS had a conserved SH3-like domain composed of five β-sheets (Fig. 1, A and B) (10). The SH3-like domain was sandwiched between land plant-specific domains (corresponding to Glu-57–Leu-168 and Arg-222–Pro-250 of AtNdhS). The N-terminal extension is not essential for the function of NdhS (12). Several positively or negatively charged amino acids were highly conserved among the SH3-like domains; in particular, Arg-193, Lys-198, and Glu-204 were completely conserved among cyanobacteria and land plants (note that this numbering refers to AtNdhS) (Fig. 1A). Asp-209 was also conserved, except in the case of UCYN-A. Lys-178, Arg-210, Arg-222, Lys-224, and Lys-234 were specifically conserved in land plants. Prediction of the protein surface on the basis of models produced by the PDB2PQR server (31) and Q-SiteFinder (33) indicated the presence of a positively charged pocket in the SH3-like domain in both *Arabidopsis* and S. 6803 NdhS (Fig. 1, C and E). The pocket of AtNdhS was likely to be more positively charged than that of S. 6803 NdhS. Two conserved positively charged amino acids, Arg-193 and Lys-198, were localized to the edge of this pocket in AtNdhS (Fig. 1, B and D). The volume of the positively charged pocket in AtNdhS was estimated to be 86 Å³ by the Q-SiteFinder. Prediction by using e-surf and the Pocket-Finder Pocket Detection server also suggested that the pocket around Arg-193 was positively charged and hydrophilic in S. 6803 NdhS (data not shown).

Electrostatic interaction plays a crucial role in the binding of Fd to target proteins (24). Because our previous study indicated that the N-terminal extension of AtNdhS is not essential for its function in vivo (12), for the mutagenesis, we focused on nine highly conserved charged amino acids at the C-terminal region, summarized in Fig. 1A.

**Site-directed Mutagenesis of Nine Conserved Charged Amino Acids in the SH3-like Domain in *Planta*—An Arabidopsis ndhs-1/crr31-1 mutant is a knock-out allele of the ndhs gene (12). To assess the contribution of the nine charged residues conserved in the SH3-like domains, Lys-178, Arg-193, Lys-198, Glu-204, Asp-209, Arg-210, Arg-222, Lys-224, and Lys-232 were replaced by neutral Gln, and the mutated versions of the ndhs gene were introduced into the *Arabidopsis ndhs-1* mutant.

Chloroplast NDH mediates electron flow from Fd to PQ (Fig. 2A). After AL is turned off, NDH still reduces PQ in the dark. This non-photochemical reduction of PQ by NDH in vivo can be monitored as a transient increase in chlorophyll fluorescence after AL is turned off (F₀/F₁ rise) (10, 38). As reported previously (12), ndhs-1 did not show an F₀/F₁ rise, indicating that NdhS is required for efficient operation of NDH in vivo. Expression of the wild-type and truncated form (NdhSΔ57–168) of NdhS in *ndhs-1* led to recovery of the rise in F₀/F₁, indicating that the N-terminal extension is not essential for NdhS function (Fig. 2B) (12). In a series of Gln scannings, only R193Q significantly decreased the F₀/F₁ rise (Fig. 2B). Immunoblot analyses indicated that this replacement did not affect NdhS stability (Fig. 2C). Except for R193Q, none of substitutions of conserved amino acids with Gln affected the F₀/F₁ rise or NdhS stability (data not shown). Our antibody does not detect NdhSΔ57–168 (12). Point mutations or a lack of NdhS did not affect the accumulation of NdhL, a subunit of subcomplex A of the NDH complex (Fig. 2C), as reported previously (12). Of the nine conserved positively charged residues, only Arg-193 was necessary for NdhS function.

**Mutations of Arg-193 Alter the Surface Net Charge of the Pocket of SH3-like Domain**—Arg-193 was included in the positively charged pocket that could form an Fd-binding site via
electrostatic interaction (Fig. 1). To predict the role of Arg-193 in formation of the positively charged pocket, the surface net charges of homology models of the SH3-like domain were calculated in NdhS variants (Fig. 3). Substitution of Arg-193 with positively charged Lys did not affect the surface net charge in NdhSR193K. The positive charges of the pockets of NdhSR193H, NdhSR193Q, and NdhSR193A were predicted to be decreased. Furthermore, upon substitution with the negatively charged residues Asp and Glu, the surface net charges of NdhSR193D and NdhSR193E were predicted to be neutral and slightly negative, respectively. Arg-193 was predicted to form a hydrophilic patch on the edge of the pocket (data not shown). In NdhSR193A, the hydrophilic patch was predicted to be hydrophobic.

**Positive Charge at the Position of the 193rd Amino Acid is Essential for High Affinity Binding of Fd to the NDH Complex**

To test for a link between the positively charged surface of the SH3-like domain of NdhS and NDH activity, we analyzed Fd-dependent PQ reduction activity in ruptured chloroplasts isolated from ndhs-1 mutant plants accumulating NdhS variants by monitoring the increase in chlorophyll fluorescence due to PQ reduction (Fig. 4A). Because the PGR5-PGRL1-dependent pathway contributes mainly to PQ reduction activity, a subtle change in PQ reduction activity was observed in the original ndhs-1 plants and ndhs-1 plants accumulating NdhS variants in the absence of AA, except in the case of pgr5 (Fig. 4, B and C).

**FIGURE 1. Structure of the SH3-like domain of Arabidopsis NdhS.** A, multiple alignment of amino acid sequences of the SH3-like domain in NdhS homologs from plants and cyanobacteria. Identity of amino acids at values of 100, 80, and 60% among homologs is colored black, dark gray, and gray, respectively. Multiple alignment was done by using ClustalW2 (European Bioinformatics Institute (EBI)). Positively (Lys and Arg) or negatively (Asp and Glu) charged amino acids, which were highly conserved among organisms and mutagenized in this study, are indicated by blue (positively charged) or red (negatively charged) characters, and predicted secondary structures are indicated at the bottom. At, A. thaliana; Vv, V. vinifera; Pt, P. trichocarpa; Os, O. sativa; Sb, S. bicolor; Zm, Z. mays; Pp, P. patens subsp. patens; T, T. BP-1, T. elongatus BP-1; N, 7120, Nostoc sp. PCC 7120; S, 6803, Synechocystis sp. PCC 6803; UCYN-A, Cyanobacterium UCYN-A. B, homology model of the SH3-like domain in AtNdhS. The tertiary structure of the C-terminal region (Leu-168–Glu-223) of AtNdhS was predicted by using SwissModel on the basis of the structure of the S. 6803 homolog (SLL0352, PDB: 3C4S) as a template. C, surface net charge of the SH3-like domain in AtNdhS was calculated by using PDB2PQR Server version 1.7 and the Poisson-Boltzmann equation. Positively and negatively charged surfaces are indicated by blue and red, respectively. The positively charged pocket is indicated by a dashed box. D, overlay of B and C. E, surface net charge of S. 6803 NdhS. All images were produced by using UCSF Chimera 1.5.3rc.
NdhS is not essential for NDH activity but is required for high affinity binding of Fd to NDH (12). To evaluate the function of NdhS, we monitored the Fd concentration dependence of PQ reduction activity (Fig. 4, D–L) in the presence of AA. PQ reduction activity was almost saturated at 3 μM Fd in the wild type (Fig. 4 D). In ndhs-1, however, even in the presence of 20 μM Fd, PQ reduction activity was slightly lower than that in the wild type with 1 μM Fd (Fig. 4 E). Introduction of NdhS<sub>R139K</sub> almost fully complemented PQ reducing activity, as in plants that expressed wild-type NdhS (Fig. 4, F and G). However, introduction of NdhS<sub>R139A</sub> and NdhS<sub>R139Q</sub> only partly complemented PQ reducing activity (Fig. 4, H and I). Introduction of NdhS<sub>R139A</sub>, NdhS<sub>R139D</sub> or NdhS<sub>R139Q</sub> did not complement PQ reducing activity at all (Fig. 4, J–L), although the plants accumulated wild-type levels of NdhS proteins (Fig. 2C). The level of FNR, whose reverse reaction is required in the assay system, was not affected in any genotypes (Fig. 2C). Similar results were obtained by analyzing a different set of measurement using independent transgenic lines (supplemental Fig. S1).

Physiological Significance of Arg-139 in Photosynthesis—In Arabidopsis, chloroplast NDH is dispensable under growth chamber conditions but is essential in the pgr5 mutant background (4). To evaluate the physiological significance of the positive surface charge of the SH3-like domain, the ndhs-1 pgr5 double mutant was transformed by using NdhS variants in which the light intensity dependence of ETR, NPQ, and 1-qL was analyzed. The ndhs-1 defect scarcely affected NPQ induction, even under the pgr5 mutant background (data not shown). However, ETR and 1-qL were drastically affected in the ndhs-1 pgr5 double mutant (Fig. 5, A–D), as reported previously (12). For representative results, we selected two points of light intensity (95 and 692 μmol of photons m<sup>−2</sup> s<sup>−1</sup>).
DISCUSSION

A key to elucidating the function of NdhS in the chloroplast NDH complex was the finding that the predicted tertiary structure of the C-terminal domain of NdhS forms an SH3-like domain that is similar to that of PsaE, which forms the Fd-binding site of PSI with PsaC and PsaD (12, 39). In cyanobacteria, PsaE was reported to be involved in CET in an unspecified manner (40). On the basis of our in silico prediction, again, we discovered a putative positively charged pocket in the SH3-like domain of NdhS (Fig. 1). Among nine charged residues that are conserved among NdhS proteins, only Arg-193 was essential for efficient operation of NDH in vivo (Figs. 2 and 5) and in ruptured chloroplasts (Fig. 4). In silico prediction of the tertiary structure and surface net charge of a protein is a powerful tool for clarifying the function of the protein, especially when the information is combined with that from in vitro mutagenesis.

Arg-139 is a critical site for the high affinity binding of NDH to Fd. As observed in the interaction between FNR and Fd, electrostatic interaction is likely to play an important role (22). A similar case was observed in Arg-39 of PsaE, the SH3-like domain of which also forms the site for the docking of PSI to Fd (41). As observed in NDH activity both in vivo (Figs. 2 and 5) and in ruptured chloroplasts (Fig. 4), the \( K_d \) value between PsaE and Fd depends strictly on the charge of the Arg-39 site. It is 0.22 \( \mu M \) in the case of PsaE_{R39} in the wild type, whereas it is 1.5 \( \mu M \) for PsaE_{R39E}, 56 \( \mu M \) for PsaE_{R39Q}, and more than 100 \( \mu M \) for PsaE_{R39D} and PsaE_{R39K} (41). Most likely, NdhS is involved in high affinity binding with Fd via the same molecular mechanism as with PsaE.

Even when the acidic amino acids Asp or Glu were substituted for Arg-139, the lines showed higher NDH activity than the ndhs-1 null allele (Figs. 2 and 5). This contrasts with the fact that similar substitutions in Arg-39 of PsaE increase the \( K_d \) value even higher than that in the pseA null allele (41). In NdhS, other amino acid residues also contribute to the high affinity binding of NDH to Fd, although eight of the charged residues conserved in NdhS proteins do not have this role.

PsaE serves as a high affinity Fd-docking protein of PSI, but it is not essential for photoreduction of Fd by PSI (42, 43). Besides PsaE, PsaC and PsaD also contribute to Fd binding (39). Similarly, NdhS is required for high affinity binding of NDH to Fd. We do not eliminate the possibility that electron transport from Fd is partly affected in the ndhs mutant, but NdhS is not essential for NDH activity (12). Complex I is believed to have originated from [NiFe]-hydrogenase, which accepts electrons from Fd rather than NADH (44). In this enzyme, the subunit EchF is involved in oxidation of Fd in Methanosarcina barkeri. EchF is structurally similar to NdhI in chloroplast NDH, implying that NdhI directly accepts an electron from Fd. If this is the case, NdhS may be closely localized to NdhI to form the Fd-binding site. How does Fd still interact with NdhI in the ndhs mutant (12)? In genuine NADH dehydrogenase in Escherichia coli, a subunit corresponding to NdhI accepts electrons from an NADH-binding module consisting of NuoE, -F, and -G (44). It is unlikely that the L-shaped skeleton of the NADH dehydrogenase-related...
Ferredoxin-binding Site of Chloroplast NDH

A. Diagram showing the chlorophyll fluorescence pathway involving NDH, Fd, NADPH, FNR, PGR5-PGRL1, and PSII.

B. Graphs showing chlorophyll fluorescence over time for different conditions.

C. Additional graphs for comparison and analysis.

D. Graphs for WT and ndhs-1 conditions.

E. Graphs for ndhs-1 and Ndhs R193K #6 conditions.

F. Graphs for ndhs-1 + Ndhs R193Q #2 conditions.

G. Graphs for ndhs-1 + Ndhs R193E #6 conditions.

H. Graphs for ndhs-1 + Ndhs R193D #9 conditions.

I. Graphs for ndhs-1 + Ndhs R193A #6 conditions.

J. Graphs for ndhs-1 + Ndhs R193E #6 conditions.

K. Graphs for ndhs-1 + Ndhs R193D #9 conditions.
complex possesses a site for high affinity binding to Fd. In *Campylobacter jejuni*, complex I lacks subunits corresponding to NuoE and NuoF. In the absence of these proteins, CJ1574 protein, which is not conserved in complex I in other organisms, mediates electron flow from reduced flavodoxin to the complex (45, 46). As in *C. jejuni*, the remaining NDH activity in the ndhs-1 mutant may depend on additional accessory NDH subunits that form the Fd-binding site, as well as on NdhS (12). In Arabidopsis, NdhS forms the electron donor-binding subcomplex with the J protein NdhT/CRRJ and the J-like protein NdhU/CRRL. In contrast to NdhS, NdhT and NdhU are essential for NDH activity (12). It is also possible that unknown factors are still lacking in the model (14).

**FIGURE 4.** Fd-dependent PQ reduction activity in ruptured chloroplasts. A, schematic model of Fd-dependent PQ reduction in ruptured chloroplasts. AA inhibits the PGR5-PGRL1 complex-dependent PQ reduction. The increase in chlorophyll fluorescence level was monitored after consecutive addition of 0.25 mM NADPH and 5 μM Fd under weak illumination (1 μmol of photons m⁻² s⁻¹) in osmotically ruptured chloroplasts (20 μg of chlorophyll ml⁻¹). The increase in fluorescence reflects NDH-dependent PQ reduction in the presence of AA. B and C, total Fd-dependent PQ reduction activities in ruptured chloroplasts isolated from various genotypes as indicated. A.u., arbitrary units. D–L, NDH-dependent PQ reduction activity was measured in the presence of 5 μM AA.

**FIGURE 5.** Chlorophyll fluorescence analysis of pgr5 mutants expressing NdhS variants. A–D, light intensity dependence of chlorophyll fluorescence parameters, ETR at 95 (A) and 692 μmol of photons m⁻² s⁻¹ (B), and the reduced state of the PQ pool (1-qL) at 95 (C) and 692 μmol of photons m⁻² s⁻¹ (D) were analyzed. A representative line from each construction was selected, and three independent T2 seedlings were analyzed. Columns with the same letters are not significantly different (Tukey-Kramer test, p < 0.05). Error bars indicate mean ± S.D. E, immunodetection of NdhS, NdhL, FNR, and Cytf in chloroplast membranes. Membrane proteins corresponding to 1 μg of chlorophyll were loaded. NdhL, FNR, and Cytf were detected as controls for the NDH complex and thylakoid membrane protein, respectively. Cytf, cytochrome f.
REFERENCES

1. Shikanai, T. (2007) Cyclic electron transport around photosystem I; genetic approaches. Annu. Rev. Plant Biol. 58, 199–217
2. Heber, U., and Walker, D. (1992) Concerning a dual function of coupled cyclic electron transport in leaves. Plant Physiol. 100, 1621–1626
3. Bendall, D. S., and Manasse, R. S. (1995) Cyclic phosphorylation and electron transport. Biochim. Biophys. Acta. 1229, 23–38

4. Munekage, Y., Hashimoto, M., Miyake, C., Tomizawa, K., Endo, T., Tsukagoshi, M., and Shikanai, T. (2004) Cyclic electron flow around photosystem I is essential for photosynthesis. Nature 429, 579–582

5. Suorsa, M., Järvi, S., Grieco, M., Nurmi, M., Pietrzykowska, M., Rantala, M., Kangasjärvi, S., Paakkarinen, V., Tikkanen, M., Janssen, S., and Aro, E.-M. (2012) PROTON GRADIENT REGULATIONS is essential for proper acclimation of Arabidopsis photosystem I to naturally and artificially fluctuating light conditions. Plant Cell 24, 2934–2948

6. Munekage, Y., Hojo, M., Meurer, J., Endo, T., Tasaka, M., and Shikanai, T. (2002) PGR5 is involved in cyclic electron flow around photosystem I and is essential for photoprotection in Arabidopsis. Cell 110, 361–371

7. DalCorso, G., Pesaresi, P., Masiero, S., Aseaeva, E., Schünnemann, D., Finazzi, G., Joliot, P., Barbato, R., and Leister, D. (2008) A complex containing PGR1 and PGR5 is involved in the switch between linear and cyclic electron flow in Arabidopsis. Cell 132, 273–285

8. Hertle, A. P., Blunder, T., Wunder, T., Pesaresi, P., Pribil, M., Armbuster, U., Leister, D. (2013) PGR1 is the elusive ferredoxin-plastoquinone reductase in photosynthetic cyclic electron flow. Mol. Cell. 49, 511–523

9. Sugimoto, K., Okegawa, Y., Tohri, A., Long, T. A., Covert, S. F., Hisabori, M., Hidaka, T., Meng, B. Y., Ohto, C., Tanaka, M., Kato, A., Maruyama, T., and Sugiura, M. (1987) Six chloroplast genes (ndhA-F) homologous to human mitochondrial genes encoding components of the respiratory chain NDH dehydrogenase are actively expressed: Determination of the splice sites in ndhA-F pre-mRNAs. Mol. Gen. Genet. 210, 385–393

10. Shikanai, T., Endo, T., Hashimoto, M., Yamasaki, Y., Asada, K., and Yokota, A. (1998) Directed disruption of the tobacco ndhB gene impairs cyclic electron flow around photosystem I. Proc. Natl. Acad. Sci. U.S.A. 95, 9705–9709

11. Hashimoto, M., Endo, T., Peltier, G., Tsukagoshi, M., and Shikanai, T. (2003) A nucleus-encoded factor, CRR2, is essential for the expression of chloroplast NADH dehydrogenase-like complex in Arabidopsis. Plant Cell 15, 210, 1621–1626

12. Yamamoto, H., Peng, L., Fukao, Y., and Shikanai, T. (2011) An Src homology 3 domain-like fold protein forms a ferredoxin binding site for the chloroplast NDH dehydrogenase-like complex in Arabidopsis. Plant Cell 23, 1480–1493

13. Matsubayashi, T., Wakasugi, T., Shinozaki, K., Yamaguchi-Shinozaki, K., Zaita, N., Hidaka, T., Meng, B. Y., Ohno, C., Tanaka, M., Kato, A., Maruyama, T., and Sugiyama, M. (1987) Six chloroplast genes (ndhA-F) homologous to human mitochondrial genes encoding components of the respiratory chain NDH dehydrogenase are actively expressed: Determination of the splice sites in ndhA-F and ndhB pre-mRNAs. Plant Cell Physiol. 25, 1560–1566

14. Peng, L., Fukao, Y., Fujimori, M., Takami, T., and Shikanai, T. (2009) Efficient operation of NAD(P)H dehydrogenase requires supercomplex formation with photosystem I via minor LHCl in Arabidopsis. Plant Cell 21, 3623–3640

15. Peng, L., and Shikanai, T. (2011) Supercomplex formation with photosystem I is required for the stabilization of the chloroplast NDH dehydrogenase-like complex in Arabidopsis. Plant Physiol. 155, 1629–1639

16. Sanazan, L. A., and Hinchliffe, P. (2006) Structure of the hydrophilic domain of respiratory complex I from Thermus thermophilus. Science 311, 1430–1436

17. Efremov, R. G., Baradaran, R., and Sanazan L. A. (2010) The architecture of respiratory complex I. Nature 465, 441–445

18. Völkl, I., Gori, G., and Sazanov, L. A. (2011) Crystal structure of the entire respiratory complex I. Nature 479, 443–448

19. Kishan, K. V. R., and Agrawal, V. (2005) SH3-like fold proteins are structurally conserved and functionally divergent. Curr. Protein Pept. Sci. 6, 143–150

20. Knaff, D. B., and Hirasawa, M. (1991) Ferredoxin-dependent chloroplast enzymes. Biochim. Biophys. Acta 1056, 93–125

21. Akashi, T., Matsumura, T., Ideguchi, T., Ikawai, K., Kawakatsu, T., Taniuchi, H., and Hase, T. (1999) Comparison of the electrostatic binding sites on the surface of ferredoxin for two ferredoxin-dependent enzymes, ferredoxin-NADP + reductase and sulfite reductase. J. Biol. Chem. 274, 29399–29405

22. Hanke, G., and Mulo, P. (2013) Plant type ferredoxin and ferredoxin-dependent metabolism. Plant Cell Environ. 36, 1071–1084

23. Hase, T., Schürmann, P., and Knaff, D. B. (2006) The interaction of ferredoxin with ferredoxin-dependent enzymes. in Photosystem I (Goebel J., ed) pp. 477–498, Springer, Dordrecht, The Netherlands

24. Aro, E.-M. (2012) PROTON GRADIENT REGULATION5 is essential for photoprotection in Arabidopsis thaliana. J. Biol. Chem. 287, 87–92

25. Kramer, D. M., Johnson, G., Kiriats, O., and Edwards, G. E. (2004) New fluorescence parameters for the determination of Qy vs. redox state and extinction energy fluxes. Photosynth. Res. 79, 209–218

26. Miyake, C., Amako, K., Shiraishi, N., and Sugimoto, T. (2009) Acclimation of tobacco leaves to high light intensity drives the plastoquinone oxidation system—relationship among the fractions of open PSII centers, non-photochemical quenching of Chl fluorescence and the maximum quantum yield of PSII in the dark. Plant Cell Physiol. 50, 730–743

27. Okegawa, Y., Kagawa, Y., Kobayashi, Y., and Shikanai, T. (2008) Characterization of factors affecting the activity of photosystem I cyclic electron transport in chloroplasts. Plant Cell Physiol. 49, 825–834

28. Porra, R. J., Thompson, W. A., and Kriedemann, P. E. (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophylls standards by atomic absorption spectroscopy. Biochim. Biophys. Acta 975, 384–394

29. Dolinsky, T. J., Nielsen, J. E., McCammon, J. A., and Baker, N. A. (2004) PDB2PQR: an automated pipeline for the setup, execution, and analysis of Poisson-Boltzmann electrostatic calculations. Nucleic Acids Res. 32, W665–W667

30. Li, H., Robertson, A. D., and Jensen, J. H. (2005) Very fast empirical prediction and rationalization of protein pKa values. Proteins 61, 704–721

31. Laurie, A. T. R., and Jackson, R. M. (2005) Q-SiteFinder: an energy-based method for the prediction of protein-ligand binding sites. Bioinformatics 21, 1908–1916

32. Kyte, J., and Doolittle, R. F. (1982) A simple method for displaying the hydrophatic character of a protein. J. Mol. Biol. 157, 105–132

33. Pettersen, E. F., Goddard, T. D., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) UCSF Chimera – a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1505–1512

34. Tripp, H. I., Bench, S. R., Turk, K. A., Foster, R. A., Desany, B. A., Niazi, F., Affourtit, J. P., and Zehr, J. P. (2010) Metabolic streaming in an open-ocean nitrogen-fixing cyanobacterium. Nature 464, 90–94

35. Batchikova, N., Wei, L., Du, L., Bersanini, L., Aro, E.-M., and Ma, W. (2015) Identification of a novel Sl0352 protein (NdhS), essential for efficient operation of cyclic electron transport around photosystem I, in NADPH-plastoquinone oxidoreductase (NDH-1) complexes of Synechocystis sp. PCC 6803. J. Biol. Chem. 286, 36992–37001

36. Burrows, P. A., Sanazan, L. A., Svab, Z., Maliga, P., and Nixon, P. J. (1998) Identification of a functional respiratory complex in chloroplasts through analysis of tobacco mutants containing disrupted plastid ndh genes.
39. Sétif, P., Fischer, N., Lagoutte, B., Bottin, H., and Rochaix, J. D. (2002) The ferredoxin docking site of photosystem I. Biochim. Biophys. Acta 1555, 204–209

40. Yu, L., Zhao, J., Muhlenhoff, U., Bryant, D. A., and Golbeck, J. H. (1993) PsaE is required for in vivo cyclic electron flow around photosystem I in the cyanobacterium Synechococcus sp. PCC 7002. Plant Physiol. 103, 171–180

41. Barth, P., Guillouard, I., Sétif, P., and Lagoutte, B. (2000) Essential role of a single arginine of photosystem I in stabilizing the electron transfer complex with ferredoxin. J. Biol. Chem. 275, 7030–7036

42. Barth, P., Lagoutte, B., and Sétif, P. (1998) Ferredoxin reduction by photosystem I from Synechocystis sp. PCC 6803: toward an understanding of the respective roles of subunits PsaD and PsaE in ferredoxin binding.

Biochemistry 37, 16233–16241

43. Xu, Q., Jung, Y-S., Chitnis, V. P., Guikema, J. A., Golbeck, J. H., and Chitnis, P. R. (1994) Mutational analysis of photosystem I polypeptides in Synechocystis sp. PCC 6803. Subunit requirements for reduction of NADP+ mediated by ferredoxin and flavodoxin. J. Biol. Chem. 269, 21512–21518

44. Hedderich, R., and Forzi, L. (2005) Energy-converting [NiFe] hydrogenases: more than just H2 activation. J. Mol. Microbiol. Biotechnol. 10, 92–104

45. Finel, M. (1998) Does NADH play a central role in energy metabolism in Helicobacter pylori? Trends Biochem. Sci. 23, 412–413

46. Weerakoon, D. R., and Olson, J. W. (2008) The Campylobacter jejuni NADH:ubiquinone oxidoreductase (complex I) utilizes flavodoxin rather than NADH. J. Bacteriol. 190, 915–925