A Eukaryotic Factor Required for Accumulation of the Chloroplast NAD(P)H Dehydrogenase Complex in Arabidopsis

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The NAD(P)H dehydrogenase (NDH) complex in chloroplasts mediates photosystem I cyclic and chlororespiratory electron transport. Eleven chloroplast genes and three nuclear genes have been identified as encoding Ndh subunits, but the entire subunit composition is still unknown. An Arabidopsis (Arabidopsis thaliana) chlororespiratory reduction (crr3) mutant was isolated based on its lack of transient increase in chlorophyll fluorescence after actinic light illumination; this was due to a specific defect in accumulation of the NDH complex. The CRR3 gene (At2g01590) encodes a novel protein containing a putative plastid-targeting signal and a transmembrane domain. Consistent with the gene structure, CRR3 localized to the membrane fraction of chloroplasts. In addition to the essential function of CRR3 in stabilizing the NDH complex, the NDH complex is also required for the accumulation of CRR3. These results suggest that CRR3 interacts with the NDH complex in the thylakoid membrane. In contrast to other subunits in the chloroplast NDH complex, CRR3 is not conserved in cyanobacteria from which the chloroplast NDH complex is believed to have originated. We propose that CRR3 is a subunit of the NDH complex, which is specific to the chloroplast.

Chloroplasts are derived from the integration of prokaryotic cyanobacteria into eukaryotic cells via endosymbiosis. Consequently, chloroplasts consist of proteomes that originated from both prokaryotes and eukaryotes. The basic machinery for photosynthesis and housekeeping functions exhibits high similarity with the prokaryotic versions, whereas the regulatory machinery is often specific to eukaryotes. For example, whereas the system of chloroplast gene expression is similar to that of prokaryotes (Sugiura et al., 1998), it is regulated by members of the pentatricopeptide repeat family, which is specific to eukaryotes (Lurin et al., 2004).

Eleven subunits of the chloroplast NAD(P)H dehydrogenase (NDH) complex, which is involved in PSI cyclic electron transport (Munekage and Shikanai, 2005) and chlororespiration (Peltier and Cournac, 2002) are encoded in the chloroplast genome. Although the chloroplast NDH complex is functionally and structurally homologous to the mitochondrial NDH dehydrogenase complex (complex I), chloroplast NDH is more similar to the cyanobacterial NDH complex than to the mitochondrial complex in the same species. In addition to 11 chloroplast-encoded subunits (NdhA–NdhK), three nuclear-encoded subunits (NdhM–NdhO) were recently identified in chloroplasts using a biochemical approach (Rumeau et al., 2005). Because their orthologs were discovered in cyanobacteria (Prommeenate et al., 2004; Battachikova et al., 2005), three nucleus-encoded ndh genes are also likely to have originated in cyanobacteria and to have transferred from the chloroplast genome to the nuclear genome. Thus, it is generally accepted that the chloroplast NDH complex originated in cyanobacteria.

In the cyanobacterium Synechocystis PCC 6803, the NdhD and NdhF subunits are encoded by six and three genes, respectively. As a result of modification of the subunit composition, the cyanobacterial NDH complex is involved in multiple functions in respiration, PSI cyclic electron transport, and CO₂ uptake (Ohkawa et al., 2000; Shibata et al., 2001; Zhang et al., 2004, 2005). In higher plants, however, both subunits are encoded by single-copy genes in the chloroplast genome (Matsubayashi et al., 1987). Chloroplasts NdhD and NdhF are most similar to cyanobacterial NdhD1, NdhD2, and NdhF1, respectively. These subunits are included in the NDH1-L complex that is involved in respiration and probably also in PSI cyclic electron transport. This is consistent with the fact that the NDH complex is involved in PSI cyclic electron transport and chlororespiration in higher plants (Burrows et al., 1998; Shikanai et al., 1998).

Although the chloroplast NDH complex originated in cyanobacteria, it is not involved in CO₂ uptake. Consistent with their difference in function, the genes
specifically involved in this process (ndhD3, ndhD4, ndhF3, cupA, and cupB) are absent from the Arabidopsis (Arabidopsis thaliana) genome. Although the chloroplast NDH complex mediates PSI cyclic electron transport, its contribution is much lower in higher plants than in cyanobacteria. Instead of the NDH-mediated pathway, the PROTON GRADIENT REGULATION 5 (PGR5)-dependent pathway significantly contributes to PSI cyclic electron transport in higher plants (Munekage et al., 2002, 2004).

Although 14 subunits have been identified in the chloroplast NDH complex, the subunits involved in electron donor binding are still unclear (for review, see Shikanai and Endo, 2000). This is closely related to the long debate on the electron donor to the NDH complex. The NDH complex purified from cyanobacteria accepts electrons from NADPH, but not from NADH (Mi et al., 1995; Matsuo et al., 1998). In contrast, the purified chloroplast NDH complex favors NADH over NADPH as an electron donor (Sazanov et al., 1998; Casano et al., 2000; Rumeau et al., 2005). Despite extensive efforts to identify additional subunits, the entire subunit composition remains unclear. The difficulty is partially due to the fragile nature of the complex. Our genetic approach focusing on the chlorophyll fluorescence change depending on NDH activity has led to the identification of chlororespiratory reduction (crr) mutants that are specifically defective in NDH activity (Hashimoto et al., 2003). Here, we report the characterization of crr3 in which a novel chloroplast protein is impaired. Although CRR3 is not found in cyanobacteria, the nature of CRR3 is similar to the subunits of the NDH complex.

RESULTS

crr3 Is Specifically Defective in the Accumulation of the Chloroplast NDH Complex

The chloroplast NDH complex mediates electron transport from the stromal reducing pool to plastoquinone (PQ; Fig. 1A). After actinic light (AL) illumination, the NDH complex still donates electrons to PQ in the dark to the extent that the reducing equivalents are available, resulting in a transient increase in chlorophyll fluorescence in the wild type (Fig. 1B). The fluorescence level is roughly proportional to the reduction of the PQ pool, which depends on the activity of the chloroplast NDH complex. Arabidopsis crr mutants were isolated based on a lack of this chlorophyll fluorescence change using two-dimensional fluorescence imaging (Hashimoto et al., 2003). crr3 is a recessive mutant defective at a single locus (data not shown) and did not show any increase in chlorophyll fluorescence after AL illumination (Fig. 1B).

The contribution of the chloroplast NDH complex in photosynthetic electron transport is minor and the Arabidopsis mutants specifically defective in NDH activity do not show any distinct phenotype under mild growth conditions in a growth chamber (Hashimoto et al., 2003; Kotera et al., 2005; Munshi et al., 2005, 2006). To assess whether the crr3 defect is specific to the NDH complex, the light intensity dependence of two chlorophyll fluorescence parameters were compared between crr3 and the wild type (Fig. 2). Whereas the electron transport rate (ETR) reflects the relative rate of electron transport through PSI, nonphotochemical quenching (NPQ) is mainly related to dissipation of excessive absorbed light energy as heat from PSI (Niyogi et al., 2005). These parameters reflect rather subtle defects in photosynthetic electron transport and have often been used for primary characterization of mutants defective in photosynthesis. In crr3, both parameters showed the same light-intensity dependence as the wild type (Fig. 2). We conclude that the crr3 defect is specific to the NDH complex.

![Figure 1. Monitoring of NDH activity using chlorophyll fluorescence analysis. A, Schematic model of NDH function. The NDH complex functions in electron transport from an unidentified electron donor, possibly NADP(H) or ferredoxin (Fd) to PQ. PQ reduction was monitored by chlorophyll fluorescence emitted from PSII. PQ reduction in the dark depends on NDH activity and can be monitored as a transient increase in chlorophyll fluorescence after AL illumination. PC, Plastocyanin; FNR, Fd-NADP⁺ oxidoreductase. B, Analysis of the transient increase in chlorophyll fluorescence after turning off AL. The bottom curve indicates a typical trace of chlorophyll fluorescence in the wild type (WT). Leaves were exposed to AL (50 μmol photons m⁻² s⁻¹) for 5 min. AL was turned off and the subsequent change in chlorophyll fluorescence level was monitored. Insets are magnified traces from the boxed area. The fluorescence levels were normalized by Fm levels. ML, Measuring light; SP, saturating pulse of white light; crr3 + CRR3, crr3 complemented by introduction of wild-type genomic CRR3.](https://www.plantphysiol.org)
The crr3 phenotype specifically defective in activity of the NDH complex may be due to impaired accumulation of the complex. To assess this possibility, the protein level of NdhH, a subunit of the NDH complex, was evaluated in crr3 (Fig. 3). In crr3, the NdhH level was drastically reduced to below the detection limit (at least 12.5% of the wild type). In contrast, the level of cytochrome f, a subunit of the cytochrome b_{6}f complex, was not affected in crr3. These data are consistent with the results of chlorophyll fluorescence analysis, which suggests a specific loss of NDH activity in crr3. We conclude that the accumulation of the NDH complex is specifically impaired in crr3.

CRR3 Encodes a Novel Membrane Protein That Is Specific to Higher Plants

The gene responsible for the crr3 phenotype was identified based on the genetic map. The crr3 mutant (Columbia gil background) was crossed with the polymorphic wild type (Landsberg erecta). Fine mapping using approximately 150 F_{2} plants pinpointed the 190-kb region between markers RGA and F14H20 at the top of chromosome 2. Because the crr3 phenotype is specific to chloroplast NDH activity, the nucleotide sequences of candidate genes that encode proteins with predicted target signal to plastids (Predotar [http://urgi.infobiogen.fr/predotar/predotar.html] and TargetP [http://www.cbs.dtu.dk/services/TargetP]) were determined. Finally, one nucleotide substitution from C to T was discovered in At2g01590.

Direct sequencing of the reverse transcription-PCR products showed that At2g01590 consists of three exons and two introns (Fig. 4A). A single-nucleotide substitution in crr3 generates a stop codon in the first exon, strongly suggesting that crr3 completely lacks the function of At2g01590. To verify that the mutation is responsible for the defect in the NDH complex, the wild-type genomic sequence containing At2g01590 was introduced into crr3. This transformation fully complemented the transient increase in chlorophyll fluorescence after AL illumination (Fig. 1B) and also the accumulation of NdhH protein (Fig. 3). We thus concluded that the crr3 phenotype is caused by the mutation in At2g01590 (CRR3).

The CRR3 gene encodes a protein consisting of 174 amino acids. The first 54 amino acids were predicted to be a target signal to plastids by TargetP (Fig. 4B). CRR3 contains one transmembrane domain at the C-terminal region, suggesting that CRR3 anchors the thylakoid membranes. Although CRR3 is conserved in rice (Oryza sativa), their homologs have not been identified in cyanobacteria from which the chloroplast NDH complex is believed to have originated. Furthermore, CRR3 was not detected in Chlamydomonas reinhardtii in which the chloroplast NDH complex is absent (http://genome.jgi-psf.org/chlre2/chlre2.home.html). These results suggest that CRR3 is a novel factor required for the accumulation of the NDH complex that is specific to higher plants.
CRR3 Localizes to Chloroplast Thylakoids

For the biochemical characterization of CRR3, an antibody was raised against it. Trials to fuse the T7 epitope tag to the C-terminal end and also prior to the transmembrane domain resulted in failure to complement the CRR3 function in the mutant, suggesting the essential function of the C-terminal region of CRR3. The mature CRR3 without the C-terminal end containing the transmembrane domain was therefore expressed as a fusion protein with NusA and His tags in *Escherichia coli*. The recombinant CRR3 was purified, released from the tags, and then used as the antigen.

The obtained antibody recognized a protein whose mobility in gel was consistent with the predicted molecular mass of CRR3 (13.7 kD; Fig. 5). The signal was absent in *crr3*, supporting our conclusion that *crr3* is a null allele. Furthermore, the signal was detected in *crr3* transformed with the wild-type genomic CRR3 in which NDH activity (Fig. 1B) and the accumulation of NDH complex (Fig. 3) were restored. We conclude that the antibody specifically recognizes CRR3. Consistent with the fact that CRR3 contains a transmembrane domain, CRR3 was detected in the membrane fraction of the chloroplast, but not in the stroma. We conclude that CRR3 localizes to thylakoid membranes, although we cannot eliminate the possibility that CRR3 is a plastid envelope protein.

The NDH Complex Is Essential for CRR3 Accumulation

CRR3 is essential for the accumulation of the chloroplast NDH complex (Fig. 3), but is not conserved in cyanobacteria from which the chloroplast NDH complex is believed to have originated (Fig. 4). This is in contrast to the nucleus-encoded subunit genes, *ndhM*, *ndhN*, and *ndhO* (Rumeau et al., 2005), which are common in cyanobacteria. CRR3 may be a regulatory factor that is essential for the expression or assembly of the NDH complex. If this were true, the accumulation of CRR3 would be independent of the NDH complex. To study this possibility, the CRR3 level was analyzed in the mutant backgrounds defective in the accumulation of the NDH complex (Fig. 6A). CRR2 is involved in the intergenic RNA cleavage between *rps7* and *ndhB* in the chloroplast and is possibly essential for the translation of *ndhB* encoding an Ndh subunit (Hashimoto et al., 2003). CRR7 is a candidate for a novel Ndh subunit and is essential for stabilizing the NDH complex (Munshi et al., 2005). In contrast to CRR7, CRR6 is stable against a mutant background lacking the NDH complex, but is essential to the accumulation of the NDH complex (Munshi et al., 2006). Under these three mutant backgrounds, the CRR3 level was drastically reduced below the detection limit (Fig. 6A). These results indicate that the NDH complex is essential for stabilizing CRR3.

In higher plants, PSI cyclic electron transport consists of two partially redundant pathways (Munekage et al., 2004). PGR5 is an essential factor in the non-NDH PSI cyclic electron transport (Munekage et al., 2002). Although the NDH complex somehow complements the function of PGR5-dependent PSI cyclic electron transport.

Figure 4. Positional cloning of *crr3*. A, Structure of CRR3. Exons (boxes) and introns (horizontal lines) were determined by direct sequencing of the reverse transcription-PCR products. Position of the *crr3* mutation (asterisks) is indicated. B, Alignment of CRR3 homolog sequences. Predicted cleavage site of the target signal (TargetP) is indicated by a vertical arrow. Arabidopsis CRR6 (At) and its rice homolog (Os) are aligned. The rice sequence was predicted from the genome information. The position of the *crr3* mutation is indicated by an asterisk. A horizontal bar indicates the transmembrane domain.

Figure 5. Protein-blot analysis of CRR3. A, Immunodetection of CRR3 protein using a polyclonal antibody against recombinant CRR3. Chloroplast preparations were further fractionated to obtain a membrane fraction and a stromal fraction. Large subunits of ribulose 1,5-bisphosphate carboxylase/oxygenase (RbcL) and the Cytf were detected as the control for fractionation. Lanes were loaded with protein samples corresponding to 2.5 μg (CRR3), 0.5 μg (Cytf), and 0.01 μg (RbcL) chlorophyll. *crr3* + CRR3, *crr3* transformed by genomic CRR3.
transport in the *pgr5* mutant (Munekage et al., 2004), the CRR3 level was not altered in *pgr5* (Fig. 6A).

To confirm that the NDH complex is directly required to stabilize CRR3 as a partner that coaccumulates in the thylakoid membranes, we analyzed the CRR3 level in two alleles of *crr4*. Whereas *crr4*-3 is a null allele defective in the accumulation of the NDH complex, *crr4*-4 is a weak allele in which the level of the NDH complex is mildly affected (Kotera et al., 2005). Protein-blot analysis indicated that the CRR3 levels were reduced to approximately 25% in *crr4*-4 and less than 12.5% in *crr4*-3 of that in the wild type (Fig. 6B). These were proportional to the NdhH levels in *crr4*-4 (50%-25%) and *crr4*-3 (25%-12.5%). Taking all the results together, we propose that CRR3 is a subunit of the NDH complex, which is specific to the chloroplast.

**DISCUSSION**

crr3 was isolated based on its lack of transient increase in chlorophyll fluorescence after AL illumination (Hashimoto et al., 2003). The fluorescence change strictly depends on NDH activity under non-stress conditions in the air (Hashimoto et al., 2003), facilitating the isolation of mutants specifically defective in the NDH complex (*crr* mutants). Because the contribution of the NDH complex to PSI cyclic electron transport is rather minor in higher plants, a specific mutation in the NDH complex has little effect on overall electron transport, which can be monitored according to the chlorophyll fluorescence parameters of ETR and NPQ (Fig. 2). Combined with conventional chlorophyll fluorescence analyses, our genetic strategy clarified the genes specifically involved in NDH activity.

Previously, we characterized four genes, *CRR2*, *CRR4*, *CRR6*, and *CRR7*, which are specifically required for the accumulation of the NDH complex (Hashimoto et al., 2003; Kotera et al., 2005; Munshi et al., 2005, 2006). Both *CRR2* and *CRR4* encode the members of the pentatricopeptide repeat family and are specifically involved in RNA maturation of the chloroplast *ndh* genes (Hashimoto et al., 2003; Kotera et al., 2005). *CRR2* and *CRR4* were probably acquired by higher plants to control the expression of chloroplast *ndh* genes. In contrast, *CRR6* and *CRR7* do not contain any motifs suggesting their function and are conserved in cyanobacteria (Munshi et al., 2005, 2006). As well as *CRR2* and *CRR4*, *CRR6* and *CRR7* are specifically required for the accumulation of the NDH complex, implying that they were simultaneously transferred with the NDH complex from cyanobacteria to eukaryotic cells. We first assessed the simplest possibility—that the identified genes encode novel subunits of the NDH complex. In photosynthetic protein complexes, a mutation primarily affecting a single subunit results in a pleiotropic defect in the accumulation of other subunits (for review, see Wollman et al., 1999; Choquet and Vallon, 2000). The same story is true for the NdH subunits (Burrows et al., 1998; Kofer et al., 1998; Hashimoto et al., 2003; Kotera et al., 2005; Rumeau et al., 2005). Whereas the NDH complex is essential for stabilizing *CRR7*, *CRR6* is stable even against a mutant background lacking the NDH complex (Munshi et al., 2005, 2006). Based on all this combined information, we proposed that *CRR7* was a candidate for a novel subunit of the NDH complex. In contrast, *CRR6* may be a nonsubunit factor required for stabilizing the NDH complex.

*CRR3* was required for the accumulation of the NDH complex (Fig. 3) and, conversely, *CRR3* was destabilized in *crr2*, *crr6*, and *crr7* (Fig. 6A). This character of *CRR3* was identical to that of *CRR7*. Furthermore, the *CRR3* levels were proportional to the NdhH level in two alleles of *crr4* in which the accumulation of the NDH complex was affected to a different extent (Fig. 6B), although the stoichiometry of *CRR3* to the NDH complex is unclear. The *crr4* defect in NdhD accumulation may affect the accumulation of *CRR3* more severely than that of NdhH because both NdhD and *CRR3* are membrane proteins. However, NdhH is likely to be a subunit of the connecting subcomplex. As the simplest working model, we propose that *CRR3* is a subunit of the chloroplast NDH complex. *CRR7* is a soluble protein and may be included in an unidentified subcomplex involved in binding to an electron donor. It is also possible that *CRR7* localizes between the subcomplex binding to the electron donor and the membrane subcomplex.
Chlorophyll Fluorescence Analysis

Chlorophyll fluorescence was measured using a MINI-pulse-amplitude modulation portable chlorophyll fluorometer (Walz). Minimal fluorescence at open PSI centers in the dark-adapted state \( (F_0) \) was excited by a weak measuring light (650 nm) at a light intensity of 0.05 to 0.1 \( \mu \text{mol} \text{ photons m}^{-2} \text{ s}^{-1} \) for 30 s. A saturating pulse of white light (800 nm, 3,000 \( \mu \text{mol} \text{ photons m}^{-2} \text{ s}^{-1} \)) was applied to determine the maximal fluorescence of closed PSII centers. The transient increase in chlorophyll fluorescence after turning off AL was monitored as described previously (Shikanai et al., 1998).

**MATERIALS AND METHODS**

**Plant Materials and Growth Conditions**

*Arabidopsis (Arabidopsis thaliana)* seedlings were grown in soil under growth chamber conditions (50 \( \mu \text{mol} \text{ photons m}^{-2} \text{ s}^{-1} \)) for 3 to 4 weeks. *crr3* was mutagenized by ethyl methanesulfonate (Hashimoto et al., 2003). As another example, despite the conserved function of the oxygen-evolving center in PSII, there is a diversity of its subunit composition between cyanobacteria and higher plants (De Las Rivas et al., 2004). Even though the chloroplast NDH complex originated in cyanobacteria, the subunit composition may be slightly divergent between cyanobacteria and higher plants.

Since the discovery of 11 *ndh* genes in the chloroplast genome, the chloroplast NDH complex has been discussed by analogy with the cyanobacterial complex (Shikanai and Endo, 2000). However, the cyanobacterial complex is involved in multiple functions by modifying the subunit composition, which is unlikely in the chloroplast complex. In chloroplasts, the NDH complex is considered to alleviate various oxidative stresses (Endo et al., 1999; Horváth et al., 2000; Munné-Bosch et al., 2005; Wang et al., 2006), whose exact mechanisms are not yet clear. In contrast to cyanobacteria, PGR5-dependent PSI cyclic electron transport significantly contributes to chloroplast energetics (Munekage et al., 2002, 2004). It may be necessary to take into account the diversity of the NDH complex between cyanobacteria and chloroplasts with respect to structure and even function.

**Isolation of Chloroplasts and Protein Analysis**

Chloroplasts were isolated from the leaves of 4- to 5-week-old plants as described previously (Munshi et al., 2006). The protein samples were separated by 12.5% SDS-PAGE and used for immunodetection (Hashimoto et al., 2003).

**Antibody Preparation**

The internal *crr3* sequence that does not encode the transit peptide or membrane-spanning domain was amplified using cDNA synthesized from RNA extracted from Arabidopsis leaves by PCR with the synthetic oligonucleotide primers 5'-GGATCCCAAATGGGAAGTCAAAACC-3' and 5'-CTCGAGTCACATCTAAXCCTGTTICC-3'. These primers provided BsalHI and BsmBI sites (underlined) for cloning. The amplified sequence was ligated into the pET-43.1a vector (Novagen), which provided the NusA and hexahistidine tags at the N terminus of CRR3. *Escherichia coli* BL21 (DE3) cells transformed with the plasmid were incubated at 37°C in 4 L of Luria-Bertani medium. Expression of the recombinant protein was induced by addition of 1 mM isopropylthiogalactoside at an OD600 of 0.4 to 0.6 for 6 h. Cells were harvested and resuspended in 4 volumes of buffer A (50 mM Tris-HCl, pH 7.5, 0.3 mM NaCl, 7 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride). The following steps were performed at 4°C. The cells were disrupted by sonication and centrifuged at 15,000g for 30 min to remove cell debris. The supernatant was loaded onto a 1-mL HisTrap column (GE Healthcare Bio-Sciences) that had been equilibrated with buffer B (0.01 mM Na2HPO4, 0.01 mM NaH2PO4, and 0.5 mM NaCl) containing 10 mM imidazole. The column was washed with buffer B containing 40 mM imidazole. The recombinant CRR3 protein was eluted with buffer B containing 500 mM imidazole. The purified protein was analyzed by SDS-PAGE. To remove the NusA and His tags, the recombinant protein was treated with thrombin protease and further purified via a nickel-chelating column as a through fraction, then used as an antigen.

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**LITERATURE CITED**


A Eukaryotic Factor Stabilizing NAD(P)H Dehydrogenase


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