CHLORORESPIRATORY REDUCTION 6 Is a Novel Factor Required for Accumulation of the Chloroplast NAD(P)H Dehydrogenase Complex in Arabidopsis[1]

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The chloroplast NAD(P)H dehydrogenase (NDH) complex is involved in photosystem I cyclic electron transport and chlororespiration in higher plants. An Arabidopsis (*Arabidopsis thaliana*) *chlororespiratory reduction 6* (*crr6*) mutant lacking NDH activity was identified by means of chlorophyll fluorescence imaging. Accumulation of the NDH complex was impaired in *crr6*. Physiological characterization of photosynthetic electron transport indicated the specific defect of the NDH complex in *crr6*. In contrast to the CRR7 protein that was recently identified as a potential novel subunit of the NDH complex by means of the same screening, the CRR6 protein was stable under the *crr2* mutant background in which the NDH complex does not accumulate. The *CRR6* gene (At2g47910) encodes a novel protein without any known motif. Although CRR6 does not have any transmembrane domains, it is localized in the thylakoid membrane fraction of the chloroplast. *CRR6* is conserved in phototrophs, including cyanobacteria, from which the chloroplast NDH complex has evolutionally originated, but not in *Chlamydomonas reinhardtii*, in which the NDH complex is absent. We believe that CRR6 is a novel specific factor for the assembly or stabilization of the NDH complex.

The light reactions of photosynthesis involve electron transport through the thylakoid membrane in the chloroplast. Two photosystems, PSI and PSII, convert light energy into the chemical energy that drives electron transport. Coupled with this electron transport, the cytochrome (Cyt) b_6f complex is involved in generating the proton gradient across the thylakoid membrane (ΔpH), which is utilized by H^+-ATPase for ATP synthesis. In addition to this linear electron transport, PSI cyclic electron transport is solely driven by PSI and generates ΔpH without any net accumulation of NADPH (for review, see Munekage and Shikanai, 2005). In higher plants, PSI cyclic electron transport consists of two partially redundant pathways (Munekage et al., 2004). The main pathway requires a thylakoid protein, PROTON GRADIENT REGULATION 5 (PGR5), and is essential for photoprotection (Munekage et al., 2002). The chloroplast NAD(P)H dehydrogenase (NDH) complex functions in the minor pathway and is essential under certain stress conditions (Endo et al., 1999; Horváth et al., 2000; Munné-Bosch et al., 2005; Wang et al., 2006). Notably, the NDH complex is crucial under the mutant background of *pgr5*, strongly supporting the idea that the NDH complex protects the chloroplast from stromal overreduction (Munekage et al., 2004).

The machinery of photosynthetic electron transport is embedded in the thylakoid membrane and consists of multiple subunits encoded by both the nuclear and the chloroplast genomes in plants. Their functional assembly requires multiple steps that include gene expression in both the nuclear and the chloroplast genomes, which are developmentally and environmentally regulated (for review, see Barkan and Goldschmidt-Clermont, 2000), targeting and insertion of proteins into the thylakoid membrane (for review, see Bauer et al., 2001), and protein folding with cofactors to form an active complex (for review, see Pilon et al., 2006). Because the plastids have evolutionally originated from cyanobacteria, both prokaryotic and eukaryotic machinery are utilized in the biogenesis of the photosynthetic apparatus in the chloroplast. For example, the system of plastid gene expression is essentially similar to that of prokaryotes (for review, see Sugiura et al., 1998). In contrast, gene expression is posttranscriptionally regulated by numerous nuclear-encoded factors, including members of the pentatricopeptide repeat family that are specific to eukaryotes (Lurin et al., 2004). For another example, assembly of PSI requires a plastid-encoded gene, *ycf3*, which originated from cyanobacteria (Boudreau et al., 1997). On the other hand, a member of the ACCUMULATION OF PHOTOSYSTEM ONE1 (APO1) family that is specific to eukaryotes is involved in the cofactor assembly in PSI (Amann et al., 2004). To survey the genes involved in the biogenesis of the photosynthetic apparatus, the genetic approaches focusing on the activity

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of the specific complex have significantly contributed (Miles, 1980; Meurer et al., 1996; Shikanai et al., 1999; Hashimoto et al., 2003).

Activity of the chloroplast NDH complex is monitored as a transient increase in chlorophyll fluorescence after turning off actinic light (AL; Burrows et al., 1998; Kofer et al., 1998; Shikanai et al., 1998). This fluorescence change was monitored under a CCD camera to screen the Arabidopsis (*Arabidopsis thaliana*) *chlororespiratory reduction* (*crr*) mutant specifically defective in NDH activity (Hashimoto et al., 2003). Because 11 subunits of the NDH complex are encoded by the chloroplast genome (Matsubayashi et al., 1987), several *crr* mutants are defective in the expression of chloroplast *ndh* genes. CRR2 is essential for intergenic RNA cleavage between *rps7* and *ndhB* (Hashimoto et al., 2003), whereas CRR4 is involved in the RNA editing that creates the translational initiation codon of *ndhD* (Kotera et al., 2005). Both genes encode members of the pentatricopeptide repeat family, which is specific to eukaryotes (Lurin et al., 2004).

In addition to the 11 chloroplast *ndh* genes, the nuclear genome also encodes several subunits of the NDH complex (Rumeau et al., 2005). Although 14 subunits have been identified for the chloroplast NDH complex so far, the subunit functioning in electron donor binding is still unclear (for review, see Shikanai and Endo, 2000). Most probably, the chloroplast NDH complex consists of more than 14 subunits. However, due to the fragile nature of the NDH complex during purification, it has been difficult to derive the entire subunit composition of the NDH complex in both the chloroplast and the cyanobacteria. The genetic approach focusing on the activity of the NDH complex complemented this biochemical approach. The Arabidopsis CRR7 protein was identified from the *crr7* mutant and is a candidate for a subunit of the most fragile subcomplex in the chloroplast NDH complex (Munshi et al., 2005). Here we report characterization of the Arabidopsis *crr6* mutant, which is distinctly different from *crr7*.

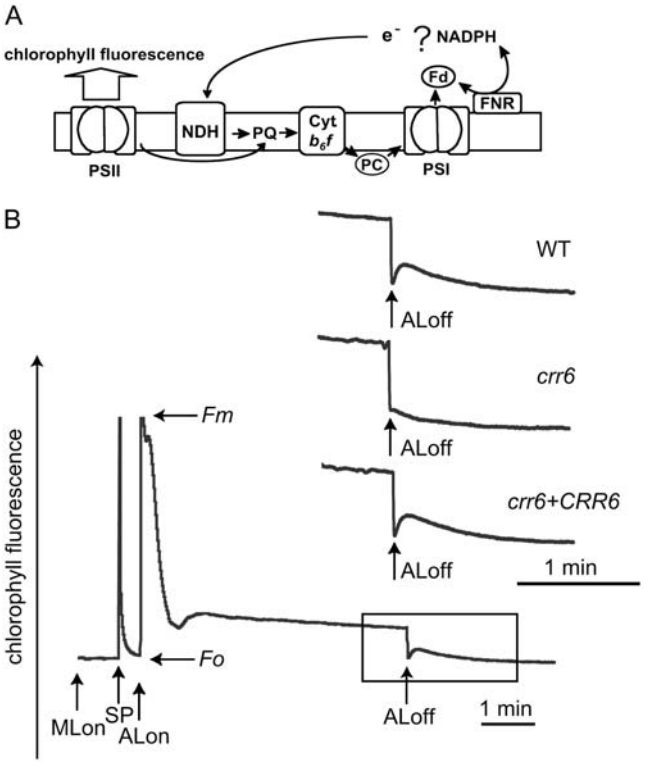

Figure 1. Monitoring of NDH activity using chlorophyll fluorescence analysis. A, Schematic model of NDH function. The NDH complex functions in electron transport from an unidentified electron donor, possibly NAD(P)H or ferredoxin (Fd) to PQ. PQ reduction was monitored by chlorophyll fluorescence emitted 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, ferredoxin-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 F_m levels. ML, Measuring light; SP, saturating pulse of white light; *crr6 + CRR6*, *crr6* complemented by introduction of the wild-type genomic *CRR6*.

RESULTS

crr6 Is Defective in Accumulation of the Chloroplast NDH Complex

The NDH complex is involved in PSI cyclic electron flow in both the chloroplast and the cyanobacteria. Arabidopsis *crr* mutants, specifically defective in NDH activity, were identified based on monitoring of chlorophyll fluorescence levels under a CCD camera (Hashimoto et al., 2003). In the wild type, the chlorophyll fluorescence level transiently increased after turning off AL (Fig. 1, A and B). This change in chlorophyll fluorescence level is ascribed to the reduction of plastoquinone (PQ) via NDH activity (Burrows et al., 1998; Kofer et al., 1998; Shikanai et al., 1998) and was impaired in the *crr6* mutant (Fig. 1B). Although PQ is also reduced via a non-NDH pathway after heat stress (Sazanov et al., 1998), the fluorescence change exclusively depends on NDH activity under the conditions used in this study (Hashimoto et al., 2003).

To characterize photosynthetic electron transport in *crr6*, the light intensity dependence of two chlorophyll fluorescence parameters was compared between the wild type and *crr6* (Fig. 2). These parameters are influenced by even subtle defects in photosynthetic electron transport. The electron transport rate (ETR) reflects the relative rate of electron transport through PSII and is not affected in *crr6* (Fig. 2A). Nonphotochemical quenching (NPQ) is mainly related to the size of energy dissipation as heat from PSII (thermal dissipation), which is a protective mechanism of PSII from oxidative damage. Thermal dissipation is triggered by acidification of the thylakoid lumen under excessive light conditions (Niyogi et al., 2005).

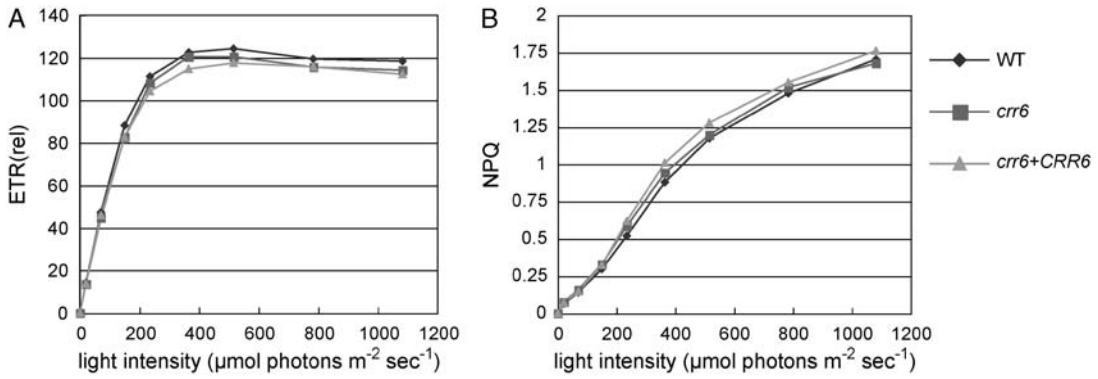

Figure 2. In vivo analysis of electron transport activity. A, Light-intensity dependence of ETR. ETR was depicted relative to Φ_{PSII} × light intensity (μmol photons m^{-2} s^{-1}). SDs are <10% of values (n = 5). B, Light-intensity dependence of NPQ of chlorophyll fluorescence. SDs are <20% of values (n = 5). *crr6 + CRR6*, *crr6* transformed with the genomic wild-type *CRR6*.

Although PGR5-dependent PSI cyclic electron flow is essential for the induction of NPQ via ΔpH generation (Munekage et al., 2002), the NDH complex scarcely contributes to NPQ induction at least in the air (Shikanai et al., 1998; Munekage et al., 2004). In *crr6*, the light-intensity dependence of NPQ was not altered (Fig. 2B), unlike in other *crr* mutants (Hashimoto et al., 2003; Kotera et al., 2005; Munshi et al., 2005). We conclude that *crr6* is specifically defective in the activity of the chloroplast NDH complex.

To assess the possibility that the level of the NDH complex is reduced in *crr6*, accumulation of NdhH, a subunit of the NDH complex, was analyzed in a protein blot (Fig. 3). The NdhH subunit is unstable without other Ndh subunits (Munekage et al., 2004; Munshi et al., 2005; Rumeau et al., 2005) and the protein blot can be used to monitor accumulation of the NDH complex. In *crr6*, the NdhH level was drastically reduced to an undetectable level (at least <12.5% of the wild type). In contrast, the level of Cyt *f*, a subunit of the Cyt b_6f complex, was not affected in *crr6*. These results are consistent with those of chlorophyll fluorescence analyses, which indicated a specific loss of NDH activity in *crr6* (Figs. 1 and 2). We conclude that the accumulation of the NDH complex is specifically impaired in *crr6*.

CRR6 Encodes a Novel Protein Conserved in Phototrophs

The gene affected in *crr6* was identified by map-based cloning. The *crr6* mutant (Columbia *gl1* background) was crossed to a polymorphic wild-type strain (Landsberg *erecta*), and the mutation was mapped to the bottom of chromosome 2. Fine mapping using 294 F_2 plants identified a 124-kb region between the markers T30B22 and T9J23. The nucleotide sequences of candidate genes that encode the predicted plastid-targeting proteins were determined. Although TargetP (http://www.cbs.dtu.dk/services/TargetP) predicted that At2g47910 encodes a mitochondrial protein, this gene was included in the candidates because the homologs to At2g47910 are present in the cyanobacterial genomes. This is a criterion for candidates for novel subunits of the NDH complex because the chloroplast NDH complex is believed to have originated from the cyanobacterial complex. Finally, a sequence alteration was found in a single gene, At2g47910 in *crr6*.

Direct sequencing of the reverse transcription-PCR product showed that At2g47910 consists of two exons and one intron (Fig. 4A). A single-nucleotide substitution in *crr6* resulted in an amino acid alteration from well-conserved Gly to Arg in the C-terminal region of CRR6 (Fig. 4, A and B). To confirm that the *crr6* phenotype is ascribed to the mutation in At2g47910, the wild-type genomic sequence of At2g47910 was introduced into *crr6*. This complementation fully restored the transient increase in chlorophyll fluorescence after turning off AL illumination (Fig. 1B). The NdhH level was also complemented by the transformation (Fig. 3). We conclude that the *crr6* defect was due to the mutation in At2g47910 (*CRR6*).

The CRR6 protein consists of 246 amino acids and does not contain any known motifs (Fig. 4B). In addition to higher plants, CRR6 is conserved in cyanobacteria, but not in nonphototrophs. In higher plants, CRR6 has a short N-terminal extension that is absent in the cyanobacterial version (Fig. 4B). Interestingly, CRR6 is not conserved in *Chlamydomonas reinhardtii* in which the chloroplast NDH complex is absent

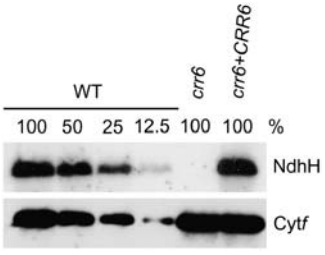

Figure 3. Protein-blot analysis of the NDH complex. Immunodetection of an NDH subunit, NdhH, and a subunit of the Cyt b_6f complex, Cyt *f*. Proteins were extracted from the thylakoid membrane fraction of the chloroplasts. Lanes were loaded with the protein samples corresponding to 0.2 μg chlorophyll for Cyt *f* and 5 μg chlorophyll for NdhH (100%) and the series of dilutions indicated. *crr6 + CRR6*, *crr6* transformed by wild-type genomic *CRR6*.

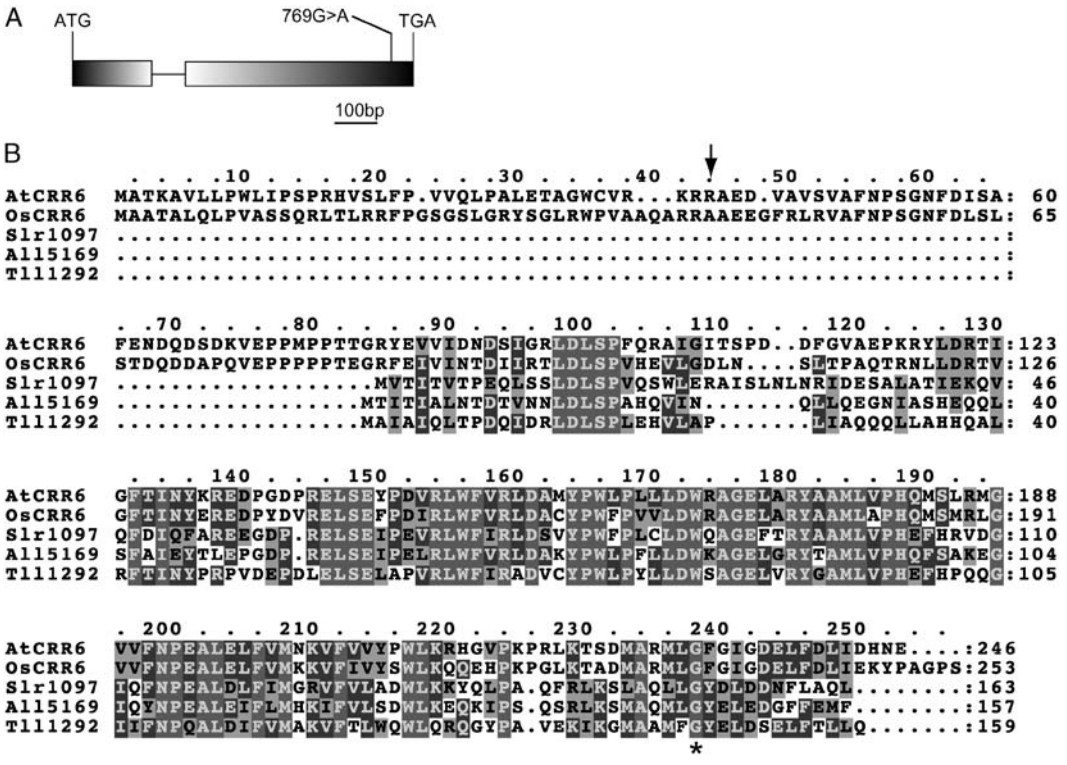

Figure 4. Positional cloning of *crr6*. A, Structure of *CRR6*. Exons (boxes) and an intron (horizontal thin line) were determined by direct sequencing of the reverse transcription-PCR products. The position of the *crr6* mutation is indicated. B, Alignment of CRR6 homolog sequences. The predicted cleavage site of the target signal (TargetP) is indicated by a vertical arrow. AtCRR6, Arabidopsis; OsCRR6, rice; Slr1097, *Synechocystis* sp. PCC 6803; All5169, *Anabaena* sp. PCC 7120; Tll1292, *Thermocynechococcus elongatus* BP-1. The position of the *crr6* mutation is indicated by an asterisk.

(http://genome.jgi-psf.org/chlre2/chlre2.home.html). This fact suggests that the function of CRR6 is specific to the NDH complex and includes the possibility that CRR6 is a novel subunit of the complex.

CRR6 Localizes to the Chloroplast

Although TargetP and Predotar (http://urgi.infobiogen.fr/predotar/predotar.html) predict mitochondrial localization of CRR6, the *crr6* phenotype strongly suggests that CRR6 is a chloroplast protein (Figs. 1–3). Predotar does, in fact, predict chloroplast localization for rice (*Oryza sativa*) CRR6. To assess this possibility, we determined the subcellular localization of CRR6 using antibodies. We constructed a chimeric gene in which the C terminus of CRR6 was fused with the influenza hemaglutinin protein (HA) epitope (CRR6-HA) and transcribed it under the control of the CRR6 promoter. The construct was then introduced into *crr6*. The transformation complemented the activity of the NDH complex, which was monitored as a transient increase in chlorophyll fluorescence after turning off AL (data not shown). The level of NdhH was also restored to that in the wild type (Fig. 5A). This result indicates that the fusion protein was functional and localized to the proper position.

From leaves of *crr6* transformed with CRR6-HA, chloroplasts were isolated and further fractionated into the stromal fraction and the thylakoid membrane fraction that possibly also contains the chloroplast envelopes. The monoclonal antibody against the HA tag detected a protein in the thylakoid membrane fraction isolated from the transgenic lines. The size of the protein is approximately consistent with that of CRR6 without the putative transit peptide (23.5 kD) plus the linker and the HA tag (1.6 kD). However, the protein was absent in both fractions isolated from the wild type that do not carry the transgene. Although CRR6 is unlikely to have any transmembrane domains (http://sosui.proteome.bio.tuat.ac.jp/sosuimenu0.html), it localized to the thylakoid membrane fraction of the chloroplast. We speculate that CRR6 interacts with other membrane proteins.

Because TargetP predicted the mitochondrial localization of CRR6, we assessed the possibility of whether CRR6 colocalizes in both the chloroplast and the mitochondria. The crude mitochondrial fraction containing a marker protein, alternative oxidase, did not include CRR6-HA protein (Fig. 5B). We conclude that CRR6 exclusively localizes to the chloroplast.

The NDH Complex Is Not Essential for Stabilizing CRR6

CRR6 is essential for the accumulation of the NDH complex (Figs. 1 and 3) and localizes to the thylakoid membrane fraction (Fig. 5). CRR6 is conserved in

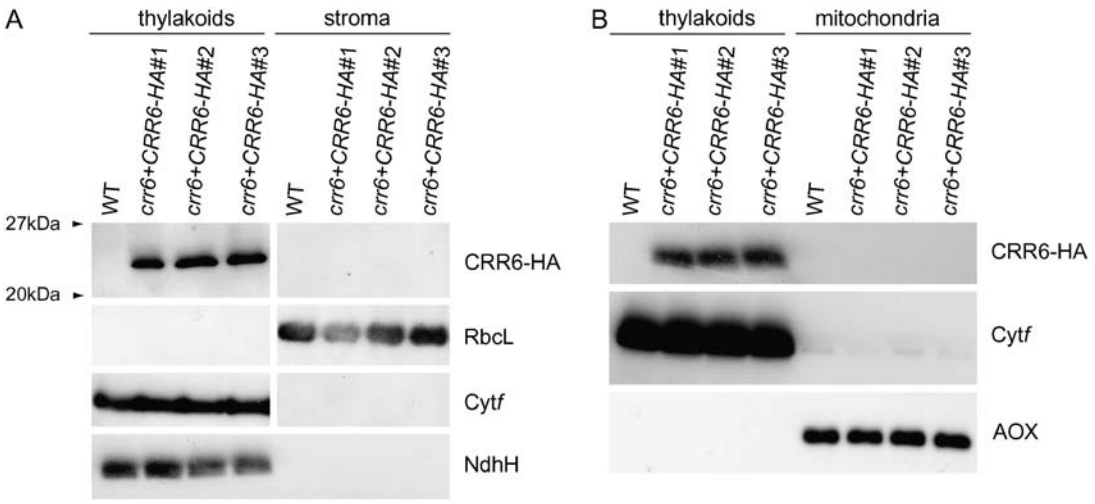

Figure 5. Protein-blot analysis of HA-tagged CRR6. A, Immunodetection of CRR6 protein using a monoclonal antibody against the HA tag. Chloroplast preparations were further fractionated to obtain a thylakoid membrane fraction and a stromal fraction. Large subunit of Rubisco (RbcL) and Cyt *f* were detected as the control for the fractionation. B, CRR6 protein was absent in the mitochondrial fraction. The lanes were loaded with protein samples corresponding to 5 μg (CRR6-HA), 5 μg (NdhH), 0.2 μg (Cyt *f*), and 0.01 μg (RbcL) chlorophyll. The crude mitochondrial protein was loaded so that it corresponded to 0.2 μg chlorophyll of the thylakoid fraction. Alternative oxidase was detected as a marker of the mitochondrial fraction. *crr6 + CRR6-HA*, *crr6* transformed by genomic *CRR6* fused to the HA epitope tag. Three independent lines (nos. 1–3) were analyzed.

phototrophs, but not in *C. reinhardtii* in which the chloroplast NDH complex is absent (Fig. 4). All the characters are identical to those of CRR7 (Munshi et al., 2005), NdhN, and NdhO (Rumeau et al., 2005), suggesting that CRR6 is a novel subunit of the NDH complex. To assess this possibility, we analyzed the stability of CRR6 under the mutant background lacking the NDH complex. If CRR6 were a subunit of the NDH complex, CRR6 would be unstable without the accumulation of the other subunits.

In the Arabidopsis mutant *crr2-2*, expression of the chloroplast *ndhB* gene encoding a subunit of the NDH complex is impaired due to lack of intergenic RNA cleavage between *rps7* and *ndhB* (Hashimoto et al., 2003). NdhB is essential for stabilizing other subunits (Hashimoto et al., 2003; Munshi et al., 2005). To assess the stability of CRR6, the CRR6-HA construct was introduced into the double mutant *crr2-2 crr6*. In the double mutant, *CRR6* function was complemented by the transgene (Fig. 5). Unexpectedly, the CRR6-HA protein was detected in the double-mutant lines (Fig. 6). To quantitatively assess the effect of the *crr2* mutant background on CRR6 accumulation, the double mutants were crossed with the single mutant of *crr6*. In the resulting F_1 plants, the *crr6* locus is homozygous for the mutant allele, whereas the *crr2* locus is heterozygous, indicating that *ndhB* expression had been restored. Seedlings carrying the transgene were used for chloroplast isolation and the CRR6 level was evaluated by protein-blot analysis (Fig. 6). The level of CRR6 was identical between seedlings carrying the homozygous *crr2/crr2* and the heterozygous *crr2/+*. This result indicates that the NDH complex is not essential for stabilizing CRR6 and does not influence the level of CRR6.

DISCUSSION

In *Synechocystis* sp. PCC 6803, four distinct protein complexes contain *ndh* gene products (Zhang et al., 2004). The largest NDH-1L complex includes at least 15 subunits and is essential for heterotrophic growth (Ohkawa et al., 2000; Zhang et al., 2004). In addition to 11 subunits, NdhA to NdhK, which are encoded by the chloroplast genome in higher plants, four subunits, NdhL to NdhO, have recently been identified (Ogawa,

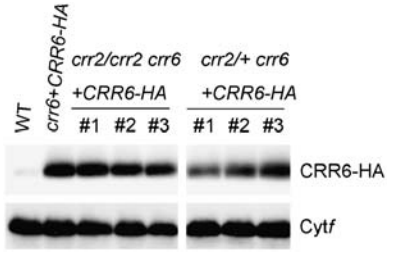

Figure 6. Immunodetection of CRR6-HA protein under the *crr2-2* mutant background. The lanes were loaded with the thylakoid membrane protein samples corresponding to 0.2 μg chlorophyll for Cyt *f* and 5 μg chlorophyll for CRR6-HA. *crr2-2 crr6 + CRR6-HA*, *crr2-2 crr6* transformed by the chimeric gene encoding CRR6-HA. Three independent lines (nos. 1–3) were analyzed and then used for crosses with *crr6* to generate *crr2-2/+ crr6 + CRR6-HA* (heterozygous for *crr2-2*, homozygous for *crr6*, and containing the transgene).

1992; Prommeenate et al., 2004; Battchikova et al., 2005). At least for NdhM, NdhN, and NdhO, the orthologs were discovered in the nuclear genome of higher plants (Rumeau et al., 2005). Although the NDH-1M complex lacks NdhD1 and NdhF1 subunits, it interacts with two versions of the NDH-1S complex and functions in CO_2 uptake (Zhang et al., 2004, 2005). Whereas the NDH-$1S_1$ complex consists of NdhD3, NdhF3, CupA, and Sll1735, forming the inducible high-affinity CO_2 transporter, the NDH-$1S_2$ complex consists of NdhD4, NdhF4, and CupB, forming the constitutive low-affinity CO_2 transporter (Ohkawa et al., 2000; Shibata et al., 2001; Zhang et al., 2004). In higher plants, *ndhD* and *ndhF* are single-copy genes and homologous to *ndhD1/D2* and *ndhF1* of *Synechocystis* sp. PCC 6803, respectively. This fact suggests that the chloroplast NDH complex is similar to the cyanobacterial NDH-1L complex. This is consistent with the results indicating that the chloroplast NDH complex is involved in PSI cyclic electron flow in the light and also in chlororespiration in the dark (Burrows et al., 1998; Shikanai et al., 1998). Despite extensive biochemical studies, the entire subunit composition of the NDH complex has remained unclear in both the cyanobacteria and the chloroplast.

Our genetic approach aims to complement the biochemical approach in which the fragility of the NDH complex makes it difficult to isolate the entire complex. To select the candidate genes for the novel subunits of the NDH complex from the *crr* mutant pool, our criteria are as follows: (1) the protein is essential for stabilizing the NDH complex; (2) conversely, the NDH complex is required for stabilizing this protein; and (3) the gene is specifically conserved in phototrophs, including cyanobacteria, but not in *C. reinhardtii*. CRR7 fits all these criteria and is therefore a candidate for biochemical confirmation such as by use of an ultraviolet cross-linking technique. Although CRR6 was also applicable to criteria 1 and 3, it was stable under the *crr2* mutant background. CRR6 is classified into a new group of CRR proteins.

The most straightforward interpretation of the results is that CRR6 is a nonsubunit factor involved in the accumulation of the NDH complex in the chloroplast. CRR6 may be a chaperone-like protein for assembly of the NDH complex. We cannot completely exclude the possibility that CRR6 is involved in chloroplast gene expression as CRR2 and CRR4, although the sequence information does not suggest this. In any case, characterization of the *crr6* phenotype (Figs. 1–3) indicates that the function of CRR6 is specific to the NDH complex. A defect in the more general machinery involved in the protein complex assembly is likely to result in a more severe phenotype. An Arabidopsis CE26-48 mutant was isolated based on its defective NDH activity and was shown to have an amino acid alteration in a putative α-subunit of the Cpn60 chaperone in the chloroplast (At2g28000). In contrast to typical *crr* mutants, CE26-48 also showed the pale-green leaf phenotype and a drastic reduction in electron transport activity, even though gene function was partially impaired in the mutant (T. Shikanai, unpublished data). The knockout of this gene results in a defect in embryogenesis (Apuya et al., 2001).

Our results do not suggest that CRR6 is a subunit of the chloroplast NDH complex, which is similar to the cyanobacterial NDH-1L complex. No subunits have thus far been shown to be stable under the mutant background of other subunits. However, it is still possible that CRR6 is a component of another protein complex that interacts with the chloroplast NDH complex. The cyanobacterial NDH-1S complexes are stable even under the mutant background of M55, in which both NDH-1L and NDH-1M complexes are absent (Zhang et al., 2004). Although the NDH-1L complex does not interact with the NDH-1S complexes in cyanobacteria, and higher plants are unlikely to contain the NDH-1S-like complex, the chloroplast NDH complex may be associated with an unknown peripheral complex that is essential for stabilizing the chloroplast NDH complex. Although CRR6 does not have any transmembrane domains, it localizes to the thylakoid membrane fraction of the chloroplast (Fig. 5), suggesting that CRR6 anchors to a membrane protein. Our genetic approach has identified two new pieces in the puzzle of the NDH complex: CRR6 and CRR7. It is essential to fit them into place and continue the survey of the entire complex using them as tags.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) seedlings were grown in soil under growth chamber conditions (50 μmol photons m^{-2} s^{-1}) for 3 to 4 weeks. *crr6* was mutagenized by ethyl methanesulfonate (Hashimoto et al., 2003).

Chlorophyll Fluorescence Analysis

Chlorophyll fluorescence was measured using a minipulse-amplitude modulation portable chlorophyll fluorometer (Walz). Minimal fluorescence at open PSII 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 μmol photons m^{-2} s^{-1}. A saturating pulse of white light (800 ms, 3,000 μmol photons m^{-2} s^{-1}) was applied to determine the maximal fluorescence at closed PSII centers in the dark-adapted state (F_m) and during AL illumination (F_m'). The steady-state fluorescence level (F_s) was recorded during AL illumination (15–1,000 μmol photons m^{-2} s^{-1}). NPQ was calculated as $(F_m - F_m')/F_m'$. The quantum yield of PSII (Φ_{PSII}) was calculated as $(F_m' - F_s)/F_m'$ (Genty et al., 1989). ETR was calculated as $\Phi_{PSII} \times$ light intensity (μmol photons m^{-2} s^{-1}). The transient increase in chlorophyll fluorescence after turning off AL was monitored as described previously (Shikanai et al., 1998).

Map-Based Cloning

The *crr6* mutation was mapped with molecular markers based on a cleaved amplified polymorphic sequence (Konieczny and Ausubel, 1993). Primer sequences and the restriction enzymes are 5′-CATAGACTTGAAGAACCGTGC-3′ and 5′-TGGCTCACTCTGTTTCATCTG-3′, *Bam*HI for T30B22 and 5′-CCTTTTTCTGCCACATTCCC-3′ and 5′-CATACTGAGCGGAATATTCTG-3′, *Rsa*I for T9J23. Genomic DNA was isolated from F_2 plants derived from a cross between *crr6* and the wild type (Landsberg *erecta*). The genomic sequences of the candidate genes were amplified by PCR using ExTaq DNA polymerase (Takara). The resulting PCR products were directly sequenced using a dye terminator cycle sequencing kit and an ABI PRISM 3100 sequencer (Applied Biosystems).

For complementation of the *crr6* mutation, the 2.1-kb wild-type genomic sequence surrounded by 5′-TCTAGAAATGTTGGAACTTC-3′ and 5′-TTTGGGTATTTGATACACAC-3′ was cloned in pBIN19 and introduced into *crr6* via *Agrobacterium tumefaciens* MP90.

Isolation of Chloroplasts and Mitochondria

Leaves of 4- to 5-week-old plants were homogenized in a medium containing 330 mM sorbitol, 20 mM Tricine/NaOH, pH 7.6, 5 mM EGTA, 5 mM EDTA, 10 mM $NaCO_3$, 0.1% (w/v) bovine serum albumin, and 1.87 mM ascorbate. After centrifugation for 5 min at 2,000*g*, the pellet was resuspended in 300 mM sorbitol, 20 mM HEPES/KOH, pH 7.6, 5 mM $MgCl_2$, and 2.5 mM EDTA. Intact chloroplasts were purified by passing through 40% Percoll. The purified chloroplasts were suspended in 20 mM HEPES/KOH, pH 7.6, 5 mM $MgCl_2$, and 2.5 mM EDTA. The insoluble fraction containing the thylakoids and the envelopes was separated from the stroma fraction by centrifugation for 5 min at 15,000*g*. The first low-speed centrifugation was followed by centrifugation for 10 min at 25,000*g* to pellet the crude mitochondrial fraction.

Protein Analysis

The genomic sequence used for the complementation was modified to carry the sequence encoding the HA tag (YPYDVPDYAG) so that the C terminus of CRR6 fused with the HA tag via a linker (PRGG). The fusion gene was cloned between the CRR6 promoter and the Nos terminator in pBIN19 and introduced into *crr6* via *A. tumefaciens* MP90. The CRR6-HA protein was detected using a monoclonal antibody against the HA tag (Santa Cruz Biotechnology). Proteins were separated by 12.5% SDS-PAGE and the protein blot was analyzed as previously described (Munekage et al., 2002).

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