An Src Homology 3 Domain-Like Fold Protein Forms a Ferredoxin Binding Site for the Chloroplast NADH Dehydrogenase-Like Complex in Arabidopsis

Hiroshi Yamamoto, a Lianwei Peng, a Yoichiro Fukao, b and Toshiharu Shikanaia,1

a Department of Botany, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502, Japan
b Plant Global Educational Project, Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma 630-0101, Japan

Some subunits of chloroplast NAD(P)H dehydrogenase (NDH) are related to those of the respiratory complex I, and NDH mediates photosystem I (PSI) cyclic electron flow. Despite extensive surveys, the electron donor and its binding subunits have not been identified. Here, we identified three novel components required for NDH activity. CRRJ and CRRL are J- and J-like proteins, respectively, and are components of NDH subcomplex A. CRR31 is an Src homology 3 domain-like fold protein, and its C-terminal region may form a tertiary structure similar to that of PsaE, a ferredoxin (Fd) binding subunit of PSI, although the sequences are not conserved between CRR31 and PsaE. Although CRR31 can accumulate in thylakoids independently of NDH, its accumulation requires CRRJ, and CRRL accumulation depends on CRRJ and NDH. CRR31 was essential for the efficient operation of Fd-dependent plastoquinone reduction in vitro. The phenotype of crr31 pgr5 mutants specifically defective in PSI cyclic electron transport (Arnon et al., 1954), its physiological significance has been unclear. Arabidopsis thaliana mutants specifically defective in PSI cyclic electron transport were identified, and the mutant phenotypes suggested that the electron transport functioned in both photosynthesis and photoprotection. In Arabidopsis, PSI cyclic electron transport is mediated by two pathways that depend on the PROTON GRADIENT REGULATION5 (PGR5)-PGRL1 complex and NAD(P)H dehydrogenase (NDH) (Munekage et al., 2004; Shikanai, 2007a; DalCorso et al., 2008). In C3 plants, the main pathway depends on the PGR5-PGRL1 complex, whereas NDH functions in stress resistance (Endo et al., 1999; Munekage et al., 2004; Wang et al., 2006).

Eleven plastid genes, ndhA-ndhK, encoding subunits of chloroplast NDH, were identified based on their similarity to genes encoding subunits of mitochondrial NADH dehydrogenase (Complex I) (Matsubayashi et al., 1987). However, chloroplast NDH is more similar to cyano bacterial NDH-1, which is believed to be an origin of chloroplast NDH (Friedrich et al., 1995; Shikanai, 2007b). By extensive surveys using proteomic, genetic, and bioinformatic approaches, over 15 nucleus-encoded subunits were identified (Shikanai, 2007b; Majeran et al., 2008; Peng et al., 2009; Suorsa et al., 2009; Takabayashi et al., 2009). Chloroplast NDH consists of four parts, A, B, membrane, and lumen subcomplexes, and it further associates with PSI to form the NDH-PSI supercomplex (see Supplemental Figure 1 online; Peng et al., 2008, 2009). Subunits included in A and membrane subcomplexes are also conserved in the cyanobacterial NDH-1 complex, but the B and lumen subcomplexes are specific to chloroplasts (Peng et al., 2009). On the other hand, the cyano bacterial NDH-1MS complex contains the specific subunits (CupA, CupB, and CupS) that are involved in the CO2-concentrating mechanism (Battchikova and Aro, 2007).

Recently, the three-dimensional structure of L-shaped complex I from the thermophilic bacterium Thermus thermophilus was reported (Sazanov and Hinchliffe, 2006; Efremov et al., 2010). The peripheral arm contains the NDH binding site; flavin mononucleotide (FMN), which primarily accepts electrons; and eight or nine iron-sulfur clusters (Sazanov and Hinchliffe, 2006; Efremov et al., 2010). Nqo1-Nqo6, Nqo9, and Nqo15 form the arm, whereas Nqo1-Nqo3 form the dehydrogenase domain for accepting two electrons from NDH (see Supplemental Figure 1A online). Electrons are transferred to menaquinone at its
binding site in a membrane-embedded subunit through iron-sulfur clusters in the peripheral arm.

NAD(P)H-oxidizing subunits corresponding to Nqo1-Nqo3 have not been found in chloroplasts or cyanobacteria even after the recent progress in understanding their structure. The identities of electron donors to NDH are also still debated (Friedrich et al., 1995; Friedrich and Weiss, 1997; Shikanai, 2007b). Identification of novel peripheral subunits of NDH is necessary for the clarification of the mechanism of electron input from PSI to NDH. Recently, we identified novel NDH subunits in NDH-PSI by shotgun proteomics and reverse genetics (Peng et al., 2009). Here, we focus on three proteins detected in wild-type NDH-PSI but not in its intermediate complex-lacking subcomplex A in the ndhl mutant, as candidate peripheral subunits of NDH (Peng et al., 2009; see Supplemental Data Set 1 online). CRR31 contributes to the formation of anFd binding site of NDH and probably interacts with NDH via J-protein, CRRJ, and J-like protein, CRRL, which are novel subunits of NDH subcomplex A.

A complex structure of NDH-PSI is summarized in Supplemental Figure 1B online; this includes the information reported in this article. We propose that chloroplast NDH accepts electrons from Fd and functions as PGR5-PGRL1 complex-independent Fd:PQ oxidoreductase in Arabidopsis.

RESULTS

The crr31, crrj, and crrl Mutants Are Specifically Impaired in Chloroplast NDH Activity

In the absence of subcomplex A, other parts of NDH-PSI accumulate stably and are detected as band II in blue-native (BN) gels, whereas full-size NDH-PSI is detected as band I (see Supplemental Figure 1 online; Peng et al., 2009). Because the putative subunits involved in electron donor binding are likely to associate with subcomplex A, we focused on unknown proteins At4g23980, At4g09350, and At5g21430, which were detected in band I but were absent from band II in our mass analysis (Peng et al., 2009; see Supplemental Data Set 1 online). They are shown to be components essential for NDH activity in this work; thus, we refer to them as CRR31, CRRJ, and CRRL. CRR31 does not contain any introns; T-DNA was inserted into the coding region (crr31-1) and 5′ untranslated region (crr31-2) (Figure 1A). RT-PCR showed leaky accumulation of transcript in crr31-2, whereas crr31-1 is a knockout allele (Figure 1B). CRRJ and CRRL consist of two and five exons, respectively, and T-DNA was inserted into the second exon of CRRJ (crrj-1) and the fourth exon of CRRL (crrl-1) (Figure 1A). RT-PCR did not detect any transcript in either mutant (Figure 1C).

Chloroplast NDH mediates electron flow from stromal reducers to PQ (Figure 1D). After actinic light (AL) illumination, NDH still donates electrons to PQ in the dark, and this PQ reduction can be monitored as a transient increase in chlorophyll fluorescence (Burrows et al., 1998; Shikanai et al., 1998) (Figure 1D). The crr23 mutant is defective in NdL, a subunit of subcomplex A (Shimizu et al., 2008), and this postillumination increase in chlorophyll fluorescence was arrested in this mutant (Figure 1E). In crr31-1, crr31-2, crrj-1, and crrl-1, the transient increase in chlorophyll fluorescence was absent, as in crr23, indicating that CRR31, CRRJ, and CRRL are required for NDH activity.

The wild-type genomic sequences of CRR31, CRRJ, and CRRL were introduced into the corresponding mutants (Figures 1B and 1C). All the transformations fully complemented the postillumination increase in chlorophyll fluorescence, confirming that each T-DNA insertion resulted in the absence of NDH activity (Figure 1E). We also analyzed several chlorophyll fluorescence parameters that reflect even subtle alterations of photosynthetic electron transport (see Supplemental Figure 2 online). The results are consistent with the phenotypes of other crr mutants specifically defective in NDH (Shimizu et al., 2008; Peng et al., 2009), reflecting its minor contribution to photosynthesis in growth chamber conditions.

CRR31 Is a Peripheral Thylakoid Membrane Protein That Accumulates Independently of the Other Parts of NDH

Localization of CRR31 was analyzed in protein blots with antibodies raised against mature CRR31 (Figure 2A). Chloroplasts were isolated from the wild type, crr31 alleles, and crr31-1 complemented by the introduction of the wild-type genomic CRR31 (crr31-1+CRR31) and further fractionated into the stroma and the membrane fraction containing thylakoids and envelopes. In the wild type, the antibody detected a protein of ~27 kD in the membrane fraction. This signal was absent in crr31-1 but was detected in crr31-1+CRR31. Consistent with the low-level accumulation of transcript (Figure 1B), a trace amount of CRR31 was detected in crr31-2. To clarify whether CRR31 is a peripheral or integral thylakoid protein, chloroplast membranes isolated from wild-type plants were incubated in 1 M NaCl, 0.1 M Na2CO3, 1 M CaCl2, and 6 M urea to release membrane-associated proteins (Figure 2B). CRR31 was significantly released from thylakoids under alkaline pH (0.1 M Na2CO3), saline (1 M CaCl2), and denaturing (6 M urea) conditions. During the treatments, an integral protein, Cytf, was retained in the membranes, while a stroma-side peripheral protein, Fd-NADP+ reductase 1 (FNR1), was released only in the presence of 6 M urea. These results indicate that CRR31 is a peripheral thylakoid protein.

To analyze the localization of CRR31 in thylakoid membranes, high molecular weight protein complexes were solubilized in 1% dodecyl-maltoside and separated in BN gels (Figure 2C; see Supplemental Figure 3 online). As previously reported (Peng et al., 2009), a high molecular weight green band (band I) was detected in crr4-3, where NDH-PSI was destabilized in the absence of a membrane subunit, NdHd (Kotera et al., 2005). In crr33, which is defective in NdL, band I was replaced by band II, corresponding to NDH-PSI lacking subcomplex A. However, band I was detected in crr31-1, whereas band II was not, suggesting that CRR31 is not required for stabilizing NDH-PSI (Figures 2C and 2D).
FKBP16-2 in crr23 (Figure 2E). By contrast, levels of any subunits were not affected in crr31-1. This observation is consistent with the results of the BN gels (Figure 2C), indicating that CRR31 is not required for the accumulation of the other parts of NDH-PSI. Furthermore, accumulation of CRR31 was not affected in the mutants defective in NDH subunits crr23, ndf2, ppl2, and crr4-3 (Figure 2E).

Although CRR31 and NDH-PSI accumulate independently of each other, they may interact in vivo because CRR31 was discovered in band I in a BN gel. To test this possibility, the BN gel was further separated by two-dimensional (2D) SDS-PAGE (Figure 2F). As previously reported (Peng et al., 2009), band I included both PsA and NdhL. Consistent with the fact that CRR31 was discovered in band I (see Supplemental Data Set 1 online), a trace level of CRR31 was detected in band I.

However, the majority of CRR31 was discovered in a putative free form, and a small amount of CRR31 was also detected in a 300-kD complex (Figure 2F). The 300-kD CRR31 complex does not contain NdhL or PsA, and it accumulated in crr4-3 (Figure 2G). Due to its fragile nature, we could not conclude that CRR31 associates with NDH-PSI simply based on these biochemical analyses.

**CRR31 Is a Novel Src Homology 3 Domain-Like Fold Protein Conserved in Phototrophs**

CRR31 encodes a protein consisting of 250 amino acids, and the first 48 amino acids are predicted to be a plastid-targeting signal (http://www.cbs.dtu.dk/services/TargetP/) (Figure 3A). Proteins homologous to CRR31 are found in other terrestrial plants and...
also in cyanobacteria (see Supplemental Figure 4 online) but not in nonphototrophs. The green alga *Chlamydomonas reinhardtii*, which does not have chloroplast NDH, does not have the CRR31 homolog (http://genome.jgi-psf.org/Chlre4/Chlre4.home.html). Consistent with the analysis of salt-washed thylakoids (Figure 2B) suggesting that CRR31 is a peripheral thylakoid protein, CRR31 does not have any transmembrane domains. The C-terminal region of CRR31 is highly conserved among plants, as well as among cyanobacteria, but CRR31 proteins of terrestrial plants have a long N-terminal extension, which includes a tandem repeat sequence (Figure 3A; see Supplemental Figure 4 online). Eukaryotic phototroph-specific N-terminal extension is also observed in PsaE and PsaD, which are stroma-side peripheral subunits of PSI (Varotto et al., 2000; Ihnatowicz et al., 2004).

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**Figure 2.** Characterization of crr31.

(A) Immunodetection of CRR31. Chloroplasts were isolated from the wild type (WT), crr31 alleles, and transgenic lines as indicated. Stroma and membrane protein extract corresponding to 2 μg chlorophyll were loaded. Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RbcL) and cytochrome f (Cyt f) were detected as a loading control of stroma and thylakoid proteins, respectively.

(B) Immunodetection of CRR31 in chloroplast membranes washed with salt or alkaline solutions. Chloroplast membranes isolated from the wild type were incubated with 1 M NaCl, 0.1 M Na2CO3, 1 M CaCl2, or 6 M urea for 30 min on ice and then the membranes were collected by ultracentrifugation. Membrane proteins corresponding to 2 μg chlorophyll were loaded. FNR1 and Cyt f were detected as controls of peripheral membrane protein and integral membrane protein, respectively.

(C) Analysis of the NDH-PSI supercomplex by BN-PAGE. Magnification of the top part of the BN-PAGE gel in Supplemental Figure 3 is shown. The BN-PAGE gel (top panel) was stained with Coomassie Brilliant Blue (CBB) (bottom panel). The positions of bands I and II are indicated based on Peng et al. (2009). Asterisks indicate bands probably corresponding to aggregated proteins.

(D) Immunodetection of CRRJ, CRRL, and NDH-PSI in 2D-BN-SDS-PAGE. Protein complexes isolated from crr31-1 were separated by BN-PAGE and further subjected to 12.5% SDS-PAGE. The position of band I is indicated by an arrowhead.

(E) Immunodetection of CRR31, CRRJ, CRRL, and NDH subunits in various genotypes as indicated across the top. Membrane protein extract corresponding to 2 μg chlorophyll was loaded onto each lane, as well as a dilution series of wild-type proteins. Antibodies used are indicated on the right. Cyt f was detected as a loading control.

(F) and (G) Immunodetection of CRR31, CRRJ, CRRL, and NDH-PSI in 2D-BN-SDS-PAGE. Protein complexes isolated from the wild type (F) and crr4-3 (G) were separated as in (E). The position of band I is indicated by an arrowhead. A red asterisk indicates a nonspecific signal.
To assess the function of this N-terminal extension, the \textit{CRR31\textsubscript{DE57-L168}} gene encoding the transit peptide fused to the C-terminal domain of CRR31 was introduced into \textit{crr31-1} (Figure 1B). The transformation complemented the postillumination increase in chlorophyll fluorescence, although the extent was slightly lower than that in the wild type (Figure 1E, \textit{crr31-1} + \textit{CRR31\textsubscript{DE57-L168}}). This result suggests that the N-terminal extension of CRR31 is not essential for NDH activity, but it might be required for the maximum activity or the stability of CRR31, which we could not evaluate in vivo due to the nature of the antibody (Figure 2A).

SSL0352 of \textit{Synechocystis} sp PCC 6803 is homologous to the C-terminal region of \textit{Arabidopsis} CRR31, and its tertiary structure was resolved by x-ray crystallography (PDB ID: 3C4S). SSL0352 is composed of five \(\beta\)-sheets and forms an Src homology 3 (SH3) domain-like structure (Figure 3B), which has been reported to be involved in protein–protein interactions (Kishan and Agrawal, 2005). Originally, the SH3 domain was identified as a conserved sequence of src (sarcoma) protein Tyr kinases, which are encoded in viral oncogenes (Mayer et al., 1988; Musacchio et al., 1992). Furthermore, the overall structure of SSL0352 was predicted to be similar to that of PsaE, a stroma-side peripheral subunit of PSI, by the VAST program. PsaE forms the Fd-docking site of PSI with PsaC and PsaD. The SH3 domain-like structure in PsaE is involved in interactions with Fd and PsaC, which contain Fe-S clusters (Se´ tif et al., 2002; Amunts and Nelson, 2009). CRR31 is an oxygenic phototroph-specific SH3 domain-like fold protein required for NDH activity and may form the Fd-docking site for NDH.

\textbf{J-Protein CRRJ and J-Like Protein CRRL Are Thylakoid Proteins Essential for NDH Activity}

\textit{CRRJ} and \textit{CRRL} encode proteins composed of 249 and 218 amino acids, respectively (Figure 4A; see Supplemental Figure 5 online). The first 45 and 53 residues, respectively, were predicted by TargetP to be plastid-targeting signals. TMHMM and SOSUI programs predicted one transmembrane domain at the C-terminal regions of both proteins. Furthermore, SMART (http://smart.embl-heidelberg.de/) and Pfam 24.0 (http://pfam.sanger.ac.uk/) analyses predicted that CRRJ and CRRL have J-domain or J-domain-like structure in the middle of their mature forms. No homologs of CRRJ and CRRL were identified in cyanobacteria or in \textit{Chlamydomonas} by BLAST.

J-proteins are members of molecular chaperone DnaJ/heat shock protein 40 (Hsp40) and are ubiquitously conserved in organisms. J-protein assists protein folding, disassembly, and translocation across membranes by collaborating with DnaK/Hsp70 (Walsh et al., 2004). J-protein stimulates ATPase activity of DnaK/Hsp70 and stabilizes the interaction between Hsp70 and its substrates. J-domains are critical for the interaction with the ATPase domain of DnaK/Hsp70 (Walsh et al., 2004). The J-protein family is classified into four types on the basis of their domain structure and the presence or absence of an HPD (His, Pro, and Asp) motif in their J-domains (Walsh et al., 2004; Rajan and D’Silva, 2009). In CRRJ, the J-domain is highly conserved, including the HPD motif. By contrast, the conservation is low in the corresponding region of CRRL (J-like domain), and the HPD motif is absent (Figure 4B). Neither protein has a G/F region, zinc-finger, or C-terminal domain (Figure 4A); thus, CRRJ and CRRL are classified as type III and IV J-proteins, respectively. By homology modeling based on the tertiary structure of \textit{Escherichia coli} DnaJ (Pellecchia et al., 1996), the J-domain of CRRJ forms four \(\alpha\)-helices, and the HPD motif was predicted to be located between helices II and III, as in other J-proteins (Figure 4C).

Specific antibodies raised against mature CRRJ and CRRL detected proteins whose molecular masses were similar to those of predicted mature forms of CRRJ (23.4 kD) and CRRL (18.7 kD). Consistent with the fact that CRRJ and CRRL each contain a

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\includegraphics[width=\textwidth]{Figure3.png}
\caption{Structures of CRR31, SSL0352, and PsaE.}
\end{figure}
Figure 4. Characterization of crrj-1 and crrl-1.

(A) Schematic structures of CRRJ, CRRL, and E. coli DnaJ. Boxes indicate the positions of plastid targeting signal (PTS), J- and J-like domains (J and JL), transmembrane domains (TM), Gly/Phe-rich motif (G/F), zinc-finger, and DnaJ C-terminal domains (CTD).

(B) Alignment of amino acid sequences of J- and J-like domains in E. coli DnaJ, CRRJ, and CRRL. The positions of helices I to IV in DnaJ are indicated by gray bars above sequences. Symbols above sequences indicate residues believed to be important in E. coli DnaJ for maintaining J-domain structure (X), for binding to Hsp70 chaperones (#), and for the specificity of this interaction (*) (Hennessy et al., 2000). The HPD motif is indicated by red #. The sequences were aligned by ClustalW2.

(C) Homology modeling of the tertiary structures of the J-domain in CRRJ and J-like domain in CRRL. The homology modeling was done by SwissModel using the J-domain of E. coli DnaJ (PDB ID: 1bq0) as a template. The models were constructed based on the amino acid sequences from Ser-103 to Gln-180 in CRRJ and from Thr-92 to Ile-176 in CRRL.

(D) Immunodetection of CRRJ, CRRL, CRR31, and NDH subunits. Chloroplasts were isolated from various genotypes as indicated across the top and further fractionated to stroma and membrane fractions. Protein extracts corresponding to 2 μg chlorophyll were loaded onto each lane as well as a dilution series in wild-type (WT) protein. Antibodies used are indicated on the right. RbcL (stroma) and Cytf (thylakoids) were detected as loading controls. An asterisk indicates nonspecific signal.

(E) Analysis of thylakoid protein complexes isolated by BN-PAGE. Chloroplasts were isolated from various genotypes as indicated across the top. Thylakoid protein extract corresponding to 10 μg chlorophyll was loaded onto each lane. The positions of bands I and II are indicated. Asterisks indicate bands probably corresponding to aggregated proteins.
transmembrane domain, they are localized to the membrane fraction of chloroplasts (Figure 4D). The crrl-1 mutant does not accumulate CRRL, and the introduction of the wild-type genomic CRRL complemented the protein accumulation (see Supplemental Figure 6A online), as observed in the complementation in NDH activity (Figure 1E). Although the CRRJ level was higher in crrl-1 than in the wild type (Figure 4D), the increased CRRJ did not complement the CRRL function because crrl-1 lacked NDH activity (Figure 1E). By contrast, neither CRRJ nor CRRL accumulated in crrj-1 (Figure 4D). Introduction of the wild-type genomic CRRJ into crrj-1 restored the levels of both CRRJ and CRRL (see Supplemental Figure 6A online). We conclude that CRRJ is required for the accumulation of CRRL, but CRRJ can accumulate independently of CRRL.

The J-domain is highly conserved in CRRJ. To assess the function of the J-domain, a mutation was introduced into the HPD motif of CRRJ, and this CRRJ_H134Q was introduced into crrj-1 (see Supplemental Figure 6 online). The mutation did not affect the accumulation of CRRJ, but the level of CRRL was not complemented by the transformation (see Supplemental Figure 6A online). NDH activity was not complemented by the transformation either (see Supplemental Figure 6B online), indicating that the HPD motif is essential for CRRJ function.

CRRJ Is Required for the Accumulation of Both the Subcomplex A of NDH and CRR31

Like CRR31, CRRJ and CRRL were discovered in band I (NDH-PSI) in the BN gel (see Supplemental Data Set 1 online). To confirm their localization, protein blots of 2D-BN-SDS-PAGE were probed with antibodies. As was the case for NdhL, both CRRJ and CRRL were specifically detected in the position of band I, suggesting that both proteins are components of NDH-PSI (Figure 2F).

Subsequently, we analyzed the accumulation of CRRJ and CRRL in the mutants lacking NDH. Although accumulation of CRRJ was not affected in any mutant background, CRRL was destabilized in the absence of NDH (Figure 2E), suggesting that CRRL interacts with NDH in thylakoid membranes.

If CRRJ and CRRL are subunits of NDH, their absence may destabilize NDH-PSI. To test this possibility, the levels of NDH subunits were analyzed in crrj-1 and crrl-1. crrl-1 accumulates the wild-type level of NDH subunits, indicating that CRRL is not required for stabilizing NDH (Figure 4D). By contrast, the levels of NdhH and NdhL were reduced to 25 to 50% of the wild-type levels in crrj-1 (Figure 4D). In crrj-1 transformed with the mutant CRRJ_H134Q, the levels of NdhH and NdhL were similarly affected (see Supplemental Figure 6 online). The levels of NDF1 and NDF2 (subcomplex B) and FKBP16-2 (lumen subcomplex) were not affected (Figure 4D); these results suggest that CRRJ is required for the accumulation of subcomplex A of NDH. Although CRRL is not required for stabilizing NDH subunits (Figure 4D), both bands I and II were detected in a BN gel in the crrl-1 and crrj-1 mutants (Figures 4E and 4F). Subcomplex A may dissociate from NDH-PSI in a BN gel, possibly in a putative partially stable subsupercomplex that was reported previously (Peng et al., 2009; Figure 2G). All the results support the hypothesis that CRRJ interacts with subcomplex A.

CRR31 Is Required for the Efficient Operation of Fd-Dependent PQ Reduction in Vitro

In PSI cyclic electron transport, electrons are recycled to PQ via two pathways that depend on the PGR5-PGRL1 complex and NDH. The activity can be monitored as Fd-dependent PQ reduction in the ruptured chloroplast system (Munekage et al., 2004), where NADPH is needed to reduce Fd via the reverse reaction of FNR (Miyake and Asada, 1994; Figure 5A). To monitor NDH-dependent PQ reduction specifically, antimycin A (AA) was added to inhibit PGR5-PGRL1-dependent PQ reduction in all of the assays except for pgr5. The addition of NADPH did not reduce PQ (Figure 5B); this is also true for NADH (Okegawa et al., 2008), implying that NDH does not accept electrons from NAD(P)H. In pgr5, PQ reduction activity was similar to that in the wild type in the presence of AA. In crr23, PQ reduction activity was almost completely inhibited. Although PQ reduction activity was higher in crr31-1 than in crr23, it was significantly lower than that in the wild type (Figure 5B). Since the Fd-dependent PQ reduction rate was slow in this system, the final PQ reduction level was lower in crr31-1 than in the wild type due to the competition with unknown PQ oxidizing reactions. Transformation of crr31-1 with wild-type genomic CRR31 fully complemented PQ reduction activity, whereas the PQ reduction activity was slightly lower in crr31-1 transformed with CRR31 D577 L168 (Figure 5B). This result is consistent with the observation that the postillumination increase in chlorophyll fluorescence was slightly lower in crr31-1 complemented by CRR31 D577 L168 (Figure 1E). We conclude that NDH activity monitored in ruptured chloroplasts was greatly but not completely inhibited in crr31-1.
Because NDH-PSI, which includes CRRJ and CRRL, is stable in crr31 (Figures 2D and 2F), it may be possible to reconstruct the active supercomplex by adding CRR31 to ruptured chloroplasts isolated from crr31. To test this possibility, recombinant CRR31 was expressed as a fusion protein with glutathione S-transferase (GST) in E. coli and was affinity purified. In the presence of AA, the addition of GST-CRR31 into the ruptured chloroplasts of crr31-1 restored PQ reduction activity to the wild-type level (Figure 5C).

The addition of GST alone did not affect the activity, indicating that the complementation depended on CRR31. These results suggest that all the components required for NDH-dependent PQ reduction, except for CRR31, are present in crr31-1, and the addition of CRR31 can complement NDH activity.

To confirm that the addition of CRR31 activates PQ reduction activity via NDH, we added GST-CRR31 to ruptured chloroplasts isolated from crr23, which does not accumulate subcomplex A.
The addition of CRR31 did not increase PQ reduction activity, indicating that CRR31 activates PQ reduction activity via NDH (Figure 5D).

We also added GST-CRR31 to ruptured chloroplasts isolated from crlj-1 and ctrl-1 (Figure 5E). Accumulation of NDH-PSI was partially affected in crlj-1, whereas ctrl-1 accumulated the wild-type level of other NDH subunits (Figure 2E). Despite the presence of NDH-PSI in crlj-1 and ctrl-1, the addition of GST-CRR31 did not activate PQ reduction activity as in cr23 (Figure 5E). This result indicates that both CRRJ and CRRL are required for NDH activity in ruptured chloroplasts, consistent with the in vivo result (Figure 1E). These results suggest that CRRJ and CRRL are essential components of NDH activity, but CRR31 is an activator of the activity.

The predicted tertiary structure of CRR31 is similar to that of PsaE, a PSI subunit forming the Fd binding site (Se´ tif et al., 2002). Our assay system using ruptured chloroplasts implies that Fd, rather than NAD(P)H, is an electron donor to NDH (Munekage et al., 2004). Based on these results, we hypothesize that CRR31 forms the Fd binding site in NDH-PSI and increases the affinity of NDH for Fd. To test this possibility, the effect of Fd concentration on PQ reduction activity was analyzed. In the wild type, 5 µM Fd was enough to saturate the reduction activity of PQ (Figure 5F). In cr31-1, however, even in the presence of 20 µM Fd, PQ reduction activity was comparable to that in the wild type with 1 µM Fd (Figure 5G). The addition of 1 µM GST-CRR31 to the ruptured chloroplasts of cr31-1 complemented PQ reduction activity in the presence of 3 µM Fd even more than did 20 µM Fd (Figure 5H). Taking all of these results together, we conclude that CRR31 is a factor required for the efficient operation of Fd-dependent PQ reduction in vitro and also that Fd is an electron donor to chloroplast NDH.

**CRR31 Is Required for NDH Function in Vivo**

CRR31 is not essential for NDH activity, but its efficient operation required higher concentrations of Fd in the absence of CRR31 (Figure 5). To analyze the contribution of CRR31 to NDH activity in vivo, we created the double mutants cr31-1 pgr5, crlj-1 pgr5, and ctrl-1 pgr5 (Figure 6). Because NDH is the machinery for stress resistance, the cr mutants specifically defective in NDH do not show particular phenotypes except for the minor alteration in chlorophyll fluorescence under growth chamber conditions (Shimizu et al., 2008). However, NDH is indispensable in pgr5, which is defective in the main pathway of PSI cyclic electron transport (Munekage et al., 2004), and the double mutant cr4-2 pgr5 showed severe reduction in growth and also showed high chlorophyll fluorescence (Figures 6A and 6B). Consistent with these phenotypes, electron transport is also impaired in cr4-2 pgr5 (Figure 6C). As in other single cr mutants, cr31-1, crlj-1, and ctrl-1 did not exhibit any phenotype (Figure 6; see Supplemental Figure 7 online). Consistent with the fact that CRRJ and CRRL are required for NDH activity, the double mutants crlj-1 pgr5 and ctrl-1 pgr5 showed definite phenotypes, as did cr4-2 pgr5 (Figure 6; see Supplemental Figure 7 online). Although the growth of cr31-1 pgr5 was comparable to that of the wild type, this double mutant showed high chlorophyll fluorescence (Figures 6A and 6B). Electron transport was also affected in cr31-1 pgr5, although the phenotype was milder than...
in the other double mutants (Figure 6C). These results suggest that CRR31 function is required for NDH activity in vivo.

**DISCUSSION**

Three novel components (CRR31, CRRJ, and CRRL) are required for NDH activity. Consistent with their specific function in NDH activity, their putative orthologs are highly expressed in maize (Zea mays) bundle sheath cells, which accumulate high levels of NDH (Friso et al., 2010). Based on the following experimental evidence, we conclude that CRRJ and CRRL are NDH subunits included in subcomplex A. (1) CRRJ and CRRL are detected in band I but not in band II when NDH-PSI was analyzed on a BN gel (see Supplemental Data Set 1 online). (2) CRRJ comigrates with NdhL as a putative partially stable complex in crr4-3 (Figure 2G). (3) The accumulation of CRRL depends on NDH (Figure 2E). (4) CRRJ is required for the accumulation of CRRL and NDH (Figure 4D). (5) The NDH-PSI level is lower in crrl-1 than in the wild type (Figure 4F). However, we do not eliminate the possibility that CRRJ assists the protein folding of subcomplex A subunits or CRR31 by interacting with DnaK/Hsp70 via its J-domain. The Arabidopsis genome encodes two copies of plastid-targeting Hsp70, and the conserved positive charges in the C-terminal region of CRR31 might be involved in the interaction with negatively charged Fd, as in Psae (Barth et al., 2000; see Supplemental Figure 4 online). In contrast with CRRJ and CRRL, CRR31 is mainly present as free protein and also in the putative 300-kD complex in the BN gel (Figure 2F). Furthermore, CRR31 is stable in the absence of NDH, and NDH accumulates in the absence of CRR31 (Figure 2E). From these results, we do not eliminate the possibility that CRR31 or the putative 300-kD CRR31 complex weakly interacts with NDH in thylakoid membranes. In any case, the accumulation of CRR31 partially depends on CRRJ (Figure 4), and a trace level of CRR31 was detected in band I (Figure 2F). Although further biochemical evidence is needed to conclude its localization and binding stability with NDH in vivo, we illustrate CRR31 on CRRJ to form theFd binding site of NDH (see Supplemental Figure 1B online). However, the site of Fd oxidation and the route of electrons to the electron carriers present in subcomplex A are still unclear. Since information is still lacking on the active part of Fd oxidation, we do not completely eliminate the possibility that other electron donors exist. Future work will focus on the most fragile part of NDH, which is possibly included in the putative 300-kD CRR31 complex (see Supplemental Figure 1B online).

Why did plants alter the electron donor for NDH from NAD(P)H to Fd? In mitochondria, the major site of reactive oxygen generation is FMN in the NADH-oxidizing subunits (Hirst et al., 2008). If chloroplast NDH is the machinery that counteracts oxidative stress in chloroplasts, the presence of FMN to oxidize NAD(P)H would have been counterproductive, as FMN is the site of reactive oxygen generation.

**METHODS**

**Plant Material and Growth Conditions**

Arabidopsis thaliana (Columbia) was grown in soil in a growth chamber (50 μmol photons m⁻² s⁻¹, 16 h photoperiod, 23°C) for 3 to 4 weeks. The Signal T-DNA express database (http://signal.salk.edu/cgi-bin/tdnaexpress) was used to find T-DNA insertion mutants for crr31, crrj-1, and crrl-1. The T-DNA insertion lines SALK_067869 (crr31-2) and SALK_111394 (crrj-1) were provided by the Salk Institute Genomic Analysis Laboratory. GABI_147F12 (crrj-1) and GABI_023D06 (crrl-1) were provided by GABI-Kat (http://www.gabi-kat.de).

**In Vivo Chlorophyll Fluorescence Analysis**

Three to four plants of each genotype were analyzed, and average values and standard deviations were calculated. Chlorophyll fluorescence was...
measured using a MINI-pulse-amplitude modulation portable chlorophyll fluorometer (MINI-PAM; Walz). Minimal fluorescence at open PSI centers in the dark-adapted state (F₀) was excited by a weak measuring light (650 nm) at a PFD of 0.05 to 0.1 µmol photons m⁻² s⁻¹. A saturating pulse of white light (800 nm, 8000 µmol photons m⁻² s⁻¹) was applied to determine the maximal fluorescence at closed PSI centers in the dark-adapted state (F₉₊₊). During AL illumination (F₉₋₋). The steady state fluorescence level (Fₛ) was recorded during AL illumination (5 to 1000 µmol photons m⁻² s⁻¹). Maximum quantum yield of PSI was calculated as F₉₋₋/F₉₊₋. NPQ was calculated as (F₉₋₋/F₉₊₊) - Fₛ. Quantum yield of PSI (ΦPSII) was calculated as (F₉₋₋/Fₛ)/(F₉₊₊/Fₛ) - 1. The quantum yield of PSI (ΦPSII) was calculated as (F₉₋₋/Fₛ)/(F₉₊₊/Fₛ) - 1 (Genty et al., 1989). ETR was calculated as ΦPSII × light intensity (µmol photons m⁻² s⁻¹). ETR, the fraction of open PSI center, was calculated as (ΦPSII/(1 - ΦPSII)) × [(1 - Fₛ/F₉₊₊)/(Fₛ/F₉₊₊)] × (NPQ + 1) (Myoke et al., 2005). The transient increase in chlorophyll fluorescence after turning off AL was monitored as described in the legend for Figure 1 (Shikanai et al., 1998).

**RT-PCR Analysis**

Total RNA was prepared from rosette leaves using an RNeasy plant mini kit (Qiagen). Contaminating DNA was digested with DNase I. Total RNA (2 µg) was reverse transcribed with random hexamers in a PrimeScript first-strand cDNA synthesis kit (TaKaRa Bio) in a total volume of 20 µL. After 10-fold dilution of the reaction mixture, a 1-µL aliquot containing cDNA was used in a subsequent PCR with TaKaRa Ex Taq DNA polymerase (TaKaRa Bio). The PCRs were performed in a final volume of 50 µL containing 1.25 units of DNA polymerase and 10 pmol of each primer. PCR primers used for amplification of CRR31, CRRJ, CRRL, and ACT8 are listed in Supplemental Table 1 online. cDNA was amplified so as to include at least one intron to distinguish cDNA from genomic DNA. PCRs consisted of 30-s denaturation at 94°C, 20-s annealing at 54°C, and 1-min extension at 72°C. RT-PCR products were separated on a 1.2% agarose gel and detected by ethidium bromide staining. The number of cycles was optimized so that the abundance of products could be compared within the linear phase of amplification.

**Vector Construction and Plant Transformation**

For complementation, the wild-type genomic sequences containing CRR31, CRRJ, and CRRL were amplified by PCR with primers listed in Supplemental Table 1 online and cloned into pDONR207-LNPS and pCRJ1340 by LR clonase reaction (Invitrogen). The resultant vectors were used as a control. These suspensions were incubated on ice for 30 min with gentle mixing. After the treatment, the membranes were centrifuged at 10,000 g for 20 min and the supernatant was removed. The membrane fraction without supplements was used as a control. These suspensions were incubated on ice for 30 min with gentle mixing. After the treatment, the membranes were centrifuged at 10,000 g for 10 min, and then the supernatant was centrifuged at 48,000 g for 1 h at 4°C. The resulting supernatant was filtered through a 0.4-µm filter and loaded onto a 1-mL HisTrap FF crude column (GE Healthcare) equilibrated with binding buffer. After the column was washed with 20 bed volumes of binding buffer, bound His-tagged fusion proteins were eluted from the column with 5 bed volumes of elution buffer (20 mM potassium phosphate buffer, pH 7.4, containing 40 mM imidazole, 500 mM NaCl, 4 M urea, and Complete EDTA-free protease inhibitor cocktail (Roche)). The lysate was centrifuged at 1800g for 10 min, and then the supernatant was centrifuged at 48,000 g for 1 h at 4°C. The resulting supernatant was filtered through a 0.4-µm filter and loaded onto a 1-mL HisTrap FF crude column (GE Healthcare) equilibrated with binding buffer. After the column was washed with 20 bed volumes of binding buffer, bound His-tagged fusion proteins were eluted from the column with 5 bed volumes of elution buffer (20 mM potassium phosphate buffer, pH 7.4, containing 500 mM imidazole, 500 mM NaCl, and 4 M urea). The purity of the fusion proteins was examined by SDS-PAGE, and the proteins were used as antigens.

**Purification of GST-CRR31 Fusion Protein**

A cDNA encoding the mature form CRR31 was amplified by PCR using primers 5’-AAATTTCGATATGCGGACTATCAGTGCGG-3’ and 5’-AAATTTAAGCTTATGTCGCTCTCTCCA-3’. The amplified DNA was digested with Mulu and HindIII and then cloned into pET41b (Novagen) to express CRR31 as a fusion protein with GST at the N terminus in E. coli. The resulting plasmid, pET41b-CRR31, was introduced into the E. coli Rosetta (DE3) pLysS strain. The transformed E. coli cells were grown in 250 mL Terrific Broth (1.2% tryptone, 2.4% yeast extract, 0.4% glycerol, 72 mM K₂HPO₄, and 17 mM KH₂PO₄) supplemented with 100 µg mL⁻¹ kanamycin and 40 µg mL⁻¹ chloramphenicol at 37°C for 7 h in the presence of 1.5 mM isopropyl β-D-thiogalactopyranoside to induce the expression of GST-CRR31 fusion protein. The E. coli cells were disrupted in 20 mL of PBS (pH 7.3, 10 mM NaHPO₄, 1.8 mM KH₂PO₄, 140 mM NaCl, and 2.7 mM KCl) containing Complete protease inhibitor (Roche) by sonication. The lysate was centrifuged at 1800g for 10 min and then the supernatant was centrifuged at 48,000 g for 1 h at 4°C. The resulting supernatant was filtered with a 0.4-µm filter and loaded onto a 1-mL GSTTrap FF column (GE Healthcare) equilibrated with PBS buffer. After the column was washed with 20 bed volumes of PBS buffer, GST-CRR31 fusion protein was eluted from the column with 5 bed volumes of elution buffer (50 mM Tris-HCl, pH 8.0, containing 10 mM reduced glutathione). The eluate containing the fusion protein was applied to PD-10 (GE Healthcare) equilibrated with PBS buffer containing 20% (v/v) glycerol to remove glutathione. GST protein was also purified by the same procedure from E. coli cells harboring pET41b. The concentration of protein was determined as reported by Bradford (1976) using BSA as the standard. The purified GST-CRR31 fusion protein and GST were frozen in liquid nitrogen and stored at -80°C until used.

**Isolation of Intact Chloroplasts and Chloroplast Membranes**

Intact chloroplasts were purified from leaves of 3- to 4-week-old plants as previously described (Munekage et al., 2002). 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 stroma fraction by centrifugation for 5 min at 15,000g. The concentration of chlorophyll was determined as described previously (Porra et al., 1989). The salt washes of thylakoids were done as described previously with minor modifications (Peng et al., 2006). Isolated chloroplast membranes were resuspended to a final concentration of 100 µg chlorophyll ml⁻¹ in 10 mM HEPES-KOH, pH 8.0, containing 10 mM MgCl₂, 330 mM sorbitol, and 1 mM phenylmethylsulfonyl fluoride supplemented with 1 M NaCl, 100 mM Na₂CO₃, 1 M CaCl₂, or 6 M urea, respectively. The membrane fraction without supplements was used as a control. These suspensions were incubated on ice for 30 min with gentle mixing. After the treatment, the membranes were centrifuged at 100,000 g for 2 h at 4°C, washed with 20 mM HEPES-KOH, pH 7.6, containing 5 mM MgCl₂ and 2.5 mM EDTA, and then solubilized with...
Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Schematic Models of Bacterial Complex I and Chloroplast NDH-PSI.

Supplemental Figure 2. Chlorophyll Fluorescence Parameters of Wild-Type, crrl-1, crrl-2, crrj-1, and crrj-1 Leaves.

Supplemental Figure 3. Analysis of Thylakoid Protein Complexes in crrl-1.

Supplemental Figure 4. A Multiple Alignment of Amino Acid Sequences of CRR31 Homologs from Plants and Cyanobacteria.

Supplemental Figure 5. Multiple Alignments of Amino Acid Sequences of CRRJ and CRRL Homologs from Plants.

Supplemental Figure 6. The HPD Motif Is Essential for CRRJ Function.

Supplemental Figure 7. Chlorophyll Fluorescence Parameters of Wild-Type, crrl-1 pgr5, crrl-1 pgr5, and crrj-1 pgr5 Leaves.

Supplemental Table 1. Primer List and Sequences.

Supplemental Data Set 1. Proteins Identified in Band I but Not in Band II by LC-MS/MS Analysis.

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An Src Homology 3 Domain-Like Fold Protein Forms a Ferredoxin Binding Site for the Chloroplast NADH Dehydrogenase-Like Complex in *Arabidopsis*

Hiroshi Yamamoto, Lianwei Peng, Yoichiro Fukao and Toshiharu Shikanai

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