Chloroplast ribonucleoprotein CP31A is required for editing and stability of specific chloroplast mRNAs

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Chloroplast ribonucleoproteins (cpRNPs) are nuclear-encoded, highly abundant, and light-regulated RNA binding proteins. They have been shown to be involved in chloroplast RNA processing and stabilization in vitro and are phylogenetically related to the well-described heterogeneous nuclear ribonucleoproteins (hnRNPs). cpRNPs have been found associated with mRNAs present in chloroplasts and have been regarded as nonspecific stabilizers of chloroplast transcripts. Here, we demonstrate that null mutants of the cpRNP family member *CP31A* exhibit highly specific and diverse defects in chloroplast RNA metabolism. First, analysis of *cp31a* and *cp31a*/*cp31b* double mutants uncovers that these 2 paralogous genes participate nonredundantly in a combinatorial fashion in processing a subset of chloroplast editing sites in vivo. Second, a genome-wide analysis of chloroplast transcript accumulation in *cp31a* mutants detected a virtually complete loss of the chloroplast *ndhF* mRNA and lesser reductions for specific other mRNAs. Fluorescence analyses show that the activity of the NADH dehydrogenase complex, which also includes the NdhF subunit, is defective in *cp31a* mutants. This indicates that cpRNPs are important in vivo for calibrating the expression levels of specific chloroplast mRNAs and impact chloroplast physiology. Taken together, the specificity and combinatorial aspects of cpRNP functions uncovered suggest that these chloroplast proteins are functional equivalents of nucleocytosolic hnRNPs.

Arabidopsis | RNA binding | RNA editing

Chloroplasts are derived cyanobacterial endosymbionts that carry their own genome. In seed plants, the chloroplast chromosome has an average size of 150 kbp and codes mostly for components of the photosynthetic and genetic apparatus. Expression of chloroplast genes is regulated at the transcriptional, post-transcriptional, and translational levels (1). Although similarities to prokaryotic gene expression systems exist, the organelle exhibits multiple derived features as well. For instance, in contrast to cyanobacterial RNAs, chloroplast transcripts are heavily processed, including splicing of group II introns, extensive endonucleolytic as well as exonucleolytic trimming, and C-to-U RNA editing.

RNA editing occurs both in chloroplast and mitochondrial transcripts in all vascular plants but with differing frequencies (2). With few exceptions, editing events restore conserved and essential codons in chloroplast mRNAs, and are thus vital for chloroplast development. Sequence elements immediately upstream of the edited C have been shown to be required for RNA editing, likely as docking sites for the nuclear-encoded RNA binding proteins (2). Recently, pentatricopeptide repeat (PPR) proteins have been identified as essential for processing specific editing sites (3–5). Other than that, the composition of the editing machinery remains largely unknown (6). In a tobacco in vitro editing system, immunological depletion of the RNA binding protein CP31 led to defects in RNA editing of 2 sites in the *psbL* and *ndhB* mRNAs (7).

CP31 belongs to a small family of chloroplast ribonucleoproteins (or short cpRNPs) that are characterized by a twin RNA recognition motif (RRM) (8, 9). These proteins are intriguing from a phylogenetic point of view, because their closest relatives are not cyanobacterial RRM proteins but belong to the eukaryotic heterogeneous nuclear ribonucleoprotein (hnRNP) family (10). Many hnRNPs are abundant proteins and participate in a variety of different tasks in nucleocytoplasmic RNA metabolism (11). Whether cpRNPs confer a touch of eukaryotic gene expression to the prokaryotic in origin organelle remains an interesting prospect. The cpRNPs are extremely abundant, exceeding the sum of all chloroplast mRNAs (12). In vitro, the cpRNPs bind various chloroplast mRNAs and have also been found to stick to the polyribonucleotide homopolymers poly(G) and poly(U) (13, 14). When mRNA probes are UV cross-linked in chloroplast extracts, a subset of proteins of approximately 30 kDa, including the cpRNPs, is usually detected (15, 16). Also, various RNA species are coenriched in immunoprecipitations of cpRNPs from stromal extracts (17, 18). All this has been taken as evidence that cpRNPs have no distinct binding sites on RNAs but rather associate nonspecifically with any ribosome-free RNA in the chloroplast (17). Specific members of the cpRNP family were implied in vitro in 3′-end processing of chloroplast mRNAs (19, 20), in general mRNA stability (12), and in RNA editing (7). Only *CP29A* from *Arabidopsis* has been analyzed genetically (21). However, in this latter study, no macroscopic defects were found in null mutants of *CP29A* and an investigation of molecular defects was not undertaken.

Here, we investigated the function of 2 *Arabidopsis* paralogues of tobacco *CP31*, which we have named *CP31A* (At4g24770) and *CP31B* (At5g50250). Genetic analysis of *CP31A* and *CP31B* uncovered that cpRNPs are required for specific RNA editing events and stabilize specific chloroplast mRNAs.

Results

Null Mutants of *Arabidopsis CP31A* Exhibit Multiple Specific Editing Defects in Chloroplast Transcripts. Starting from the finding that tobacco CP31 is required for editing of 2 selected sites in vitro (7), we decided to investigate the general impact of cpRNPs on chloroplast RNA editing in vivo. A close relative of *CP31* in *Arabidopsis* has been identified previously (8) and was called *CP31A*. We extended this phylogenetic study by including recently identified cpRNPs (9). Our analysis uncovers CP31A and CP31B as the 2 *Arabidopsis* cpRNPs most closely related to tobacco CP31 [supporting information (SI) Fig. S1]. CP31A has the longest acidic

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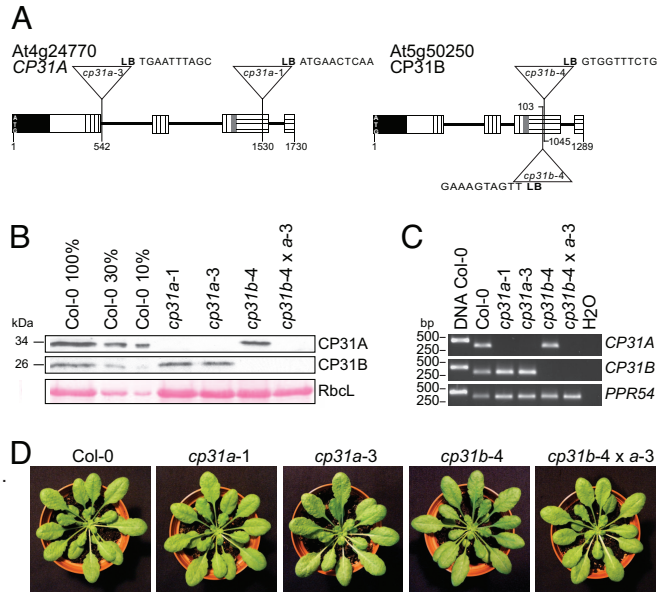

Fig. 1. Isolation of *cp31a*, *cp31b*, and *a*/*b* double mutants. (*A*) Schematic representation of gene organization and location of T-DNA insertions in CP31A (*Left*) and CP31B (*Right*) genes (drawn to scale). Insertion sites of T-DNAs were determined by sequencing PCR products generated with a left border (LB) and a gene-specific primer. The T-DNA in *cp31a*-1 plants is inserted before base pair 1530 and that in *cp31a*-3 plants is inserted before base pair 542. For line *cp31b*-4, we found a back-to-back insertion of 2 T-DNAs at positions 1036 and 1045, respectively. Gene-specific 10-mers adjacent to LB are shown for each T-DNA. Black, chloroplast targeting peptide; striped, RRM domains 1 (vertical striping) and 2 (horizontal striping); white, acidic domain; gray, spacer; triangles, insertion sites for T-DNAs. Numbers refer to positions relative to the ATG (A = 1). Solid horizontal lines represent introns. (*B*) Loss of CP31A and CP31B proteins in *cp31a* and *cp31b* single and double mutants. Immunoblot analysis of CP31A/B accumulation in mutant and WT tissue. Equal amounts or indicated dilutions of WT Col-0 total leaf proteins were analyzed by probing immunoblots with anti-tobacco CP31 antisera. Observed sizes match *in silico* predictions after removal of signal peptides and those determined previously by organelle import for CP31A (8). The same filter was stained with Ponceau S to visualize RbcL, the large subunit of Rubisco, as loading control. (*C*) CP31A and CP31B mRNA is undetectable in mutants. RT-PCR was used to assay mRNA levels in *cp31a* or *cp31b* mutants, respectively, using primer combinations cp31.ex4.for/cp31.ex7.rev and cp31B.revT/cp31B.for. The bottom panel shows RT-PCR amplification of PPR54 mRNA in the same RNA samples (PARAex1for/PARAex2rev). Comparison of DNA-based amplification products (lane DNA Col-0) with cDNA-derived amplification products shows that no intron-containing amplification products were detected, demonstrating that the bands observed arose from RNA templates. Expected sizes: CP31B DNA = 429 bp, cDNA = 332 bp; CP31B DNA = 372 bp, cDNA = 285 bp; PPR54 DNA = 322 bp, cDNA = 402 bp. (*D*) Phenotypes of *cp31a*-, *cp31b*- and double-mutant seedlings grown on soil in growth chambers

domain of all cpRNPs (73 aa) and is imported into chloroplasts (8). Two T-DNA insertion lines for *CP31A* were obtained from the SALK and SAIL collections, respectively (22). The locations of T-DNAs were confirmed in intron 1 and exon 3 (Fig. 1*A*). These insertion lines were designated *cp31a*-3 and *cp31a*-1, respectively. Both insertions abolish expression of *CP31A*, as demonstrated by the lack of protein and RNA accumulation (Fig. 1 *B* and *C*). Thus, both insertion lines are null alleles in regard to *CP31A* function, which does, however, not affect their macroscopic appearance relative to WT under standard growth conditions (Fig. 1*D*).

We next analyzed processing of all 34 known editing sites (23, 24) in the 2 *cp31a* mutant lines by direct sequencing of amplified cDNA. We found diminished editing efficiency for 13 sites relative to WT controls (see column *cp31a* in Table 1 and Fig. S2). To confirm these defects and to quantify reductions in editing efficiency, we cloned RT-PCR products for selected sites. We chose a site in *ndhF* as well as sites in *rps14* and *ndhD*, because cDNAs of these transcripts encompass both affected and unaffected editing sites. The unaffected fully edited sites furthermore serve as additional controls to verify that genomic DNA contaminations were absent from our RNA preparations. Of the 40 *ndhD* clones analyzed, on average, only 64% are edited for sites 3, 4, and 5 in *cp31a* plants (Table 2 and Table S1). By contrast, site 2 is not affected and is edited in almost all clones, as are all 4 sites in WT plants. Various combinations of editing defects were observed in individual clones: those with only 1 defect at site 3, 4, or 5 or others with a mixture of double defects. Similar to *ndhD*, editing of site *rps14*-2 is reduced to 68% in *cp31a* plants, whereas almost all clones are edited at site *rps14*-1. In WT, both sites are fully edited (Table 2 and Table S1). Finally, editing of site *ndhF*-1 is reduced to 50% of WT levels in clones of *cp31a-1* mutants. This demonstrates that loss of CP31A independently affects editing of individual cytidines in transcripts with multiple editing sites.

As an additional assay to estimate editing defects quantitatively, we checked selected sites by pyrosequencing (25). Editing frequencies determined with this method for editing sites *rps14*-1 and *rps14*-2 match those determined via cloning. Average editing efficiency for *rps14*-2 in *cp31a* plants is 74.8% by pyrosequencing (Table S2 and Fig. S3), in comparison to 68% determined by sequencing of cloned cDNAs. These frequencies are consistent with the ratios of C- and T-peaks in chromatograms generated by the classical Sanger sequencing method (Fig. S3). Thus, although not strictly quantitative, visual inspection of Sanger sequencing results obtained with the DYEnamic ET (GE Healthcare) chemistry is a good estimate of editing frequencies. Together, the 3 methods applied to determine editing efficiencies reveal an unexpected highly specific impact on chloroplast RNA editing in *cp31a* plants.

***Arabidopsis CP31B* Supports Editing of Specific *CP31A*-Dependent Sites.** Evolutionary investigation of known *Arabidopsis* cpRNPs discovered frequent paralogues in the cpRNP phylogenetic tree, probably attributable to partial genome duplications specific to the *Arabidopsis* lineage (refs. 9 and 26, Fig. S1). Functional redundancy of paralogues has been recognized as a frequent phenomenon in *Arabidopsis* (27) and could be the reason for the missing macroscopic defects in *cp31a* plants. If true, *cp31b* single mutants and particularly *cp31a*/*cp31b* double mutants should exhibit editing defects matching or exceeding those found in *cp31a* plants. We therefore isolated a *cp31b* T-DNA insertion line designated *cp31b*-4, confirmed loss of the encoded protein and RNA, and intercrossed it with *cp31a*-3 plants to generate the double mutant (Fig. 1 *A*–*C*). Both single and double mutants grew in soil under standard conditions like WT plants (Fig. 1*D*). Screening for editing defects in *cp31b* and double mutants identified various reductions in editing efficiency. We found 4 classes of editing defects (Table 1 and Fig. S2). Class I contains sites without any alteration relative to WT—neither in single mutants nor in the double mutant. Class II is represented by 3 sites; here, only the double mutant shows a strong reduction in RNA editing, implying that both cpRNPs are redundantly involved in processing these sites. Class III contains 8 sites, for which reductions in editing efficiency are similar between double mutants and *cp31a*, whereas *cp31b* looks like WT. Finally, class IV holds 5 sites in total, which show a clear editing defect in *cp31a* mutants and an even stronger defect in double mutants. The increased defect in double mutants suggests that *CP31B* does play a role for editing of these sites as well. In fact, there are mild defects visible for some of the sites in class IV such as, for instance, for *ndhD*-4 or possibly *ndhD*-5 (Fig. S2). However, for *rpoB*-3 and other sites with increased defects in double mutants, *cp31b* editing efficiency is hardly distinguishable from WT in electropherograms.

We next searched for sequence motifs that are present in cpRNP-dependent editing sites but are absent from the remaining sites. Using a simple algorithm, all possible nucleotide hexamers, including all possible degenerations (A/C/G/T/R/Y/S/W/K/M/B/D/H/V/N), were searched. Although no completely conserved motifs

Table 1. Processing of specific RNA editing sites depends on CP31A and CP31B

Editing sites			Editing defects*			
Genome position†	Gene	Editing site	*cp31b*	*cp31a*	*cp31b X cp31a*	Class‡
95644	*ndhB*	9	-	-	+	II
94999	*ndhB*	12	-	-	+	
21806	*rpoC1*	2	-	-	+	
2931	*matK*	2	-	+	+	III
96698	*ndhB*	2	-	+	+	
95225	*ndhB*	10	-	+	+	
116494	*ndhD*	3	-	+	+	
112349	*ndhF*	2	-	+	+	
65716	*petL*	2	-	+	+	
25992	*rpoB*	1	-	+	+	
37092	*rps14*	2	-	+	+	
35800	*psbZ*	1	-	+	++	IV
25779	*rpoB*	3	-	+	++	
23898	*rpoB*	7	-	+	++	
116290	*ndhD*	4	+/-	+	++	
116281	*ndhD*	5	+/-	+	++	

*Editing efficiency as in wild-type: - ; progressively increasing editing defects: +/++ . These evaluations of editing efficiencies are based on sequencing analyses presented fully in Fig. S2 and were confirmed by clonal (Table S1) and pyrosequencing (Table S2 and Fig. S3) analyses for selected editing sites.

†Nucleotide positions are given according to the *Arabidopsis* chloroplast genome annotation (GenBank accession no. AP000423).

‡Editing sites were classified according to defects in *cp31a*, *cp31b*, and double mutants.

were found with a biased distribution, we identified the degenerate hexamer KTyGST (lowercase Y indicates 1 optional deletion in this sequence pattern; Fig. S4) 13 times in the vicinity of cpRNP-dependent editing sites but only twice in front of other editing sites. This demonstrates that direct binding of a large subset of editing sites by cpRNPs can be envisioned if sufficiently degenerated target sequences are recognized. The biological relevance of these sequence elements can be tested experimentally by in vitro binding studies.

In summary, these results demonstrate that the impact of cpRNPs on RNA editing is highly site-specific and combinatorial. There are sites independent of *CP31A* and *CP31B* (class I), sites for which both cpRNPs are completely redundant (class II), other sites that depend solely on *CP31A* (class III), and also those editing sites for which both cpRNPs are required (class IV).

CP31A Is Essential for Accumulation of Specific mRNAs. Analyses of null mutants could indicate that CP31A and CP31B are editing factors but do not give clues as to whether the editing defects are truly primary or just repercussions of upstream events in the mutants. For example, it has previously been shown that an excess of RNA substrate can lead to a relative decrease of edited transcripts (28). Because cpRNPs had earlier been shown to be required for the stability of plastid mRNAs, we decided to determine transcript abundance for all mRNAs and ribosomal RNAs by a quantitative (q) RT-PCR screen (Fig. 2). This revealed that *cp31b* plants show little if any deviations in transcript accumulation from WT plants. By contrast, *cp31a* mutants show pronounced alterations concerning the *ndhF*, *ycf1*, and *rrn23* transcripts and minor alterations for several additional transcripts. mRNAs of *ndhF*, *ycf1*, and *rrn23* as well as mRNAs showing editing defects were selected for analysis by RNA gel blot hybridization. In the case of *ndhF* mRNA, we found that transcripts were reduced below the detection limit in *cp31a* mutants (Fig. 3). Although cpRNPs have been implicated in RNA stabilization, such radical effects in vivo were unexpected, given the similarity and thus potential redundancy among cpRNPs. Northern hybridization analysis of *ycf1* and *rrn23* did not reveal any striking differences in transcript abundance (Fig. 3). Because we hybridized RNA gel blots with strand-specific probes, it remains possible that antisense-RNA species account for the changed signals in qRT-PCR assays, which are not strand-specific. The *ycf1*-sequence is located downstream and on the opposite strand of *ndhF*, and the transcript derived from the *ndhF* promoter spans the region amplified with primers for *ycf1*. The *ycf1* transcripts are less abundant than *ndhF* as judged from Northern hybridization. Together, these observations suggest that the apparent decrease in *ycf1* expression in *cp31* mutants could result from the failure to accumulate *ndhF*-mRNA antisense to *ycf1*. For 23S rRNA, no straightforward explanation for the contradictory qRT-PCR results and Northern hybridization results can be presented at the moment. A direct function of cpRNPs for 23S rRNA seems unlikely, because it has been demonstrated that cpRNPs do not associate with 23S rRNA in tobacco (12).

Further RNA gel blot hybridizations showed that most RNAs accumulate to similar levels as in WT (Fig. 3). This includes 4 RNAs with editing defects in *cp31a* mutants (*ndhD*, *matK*, *psbZ*, and *rps14*). A decrease in transcript amounts in *cp31a* mutants is seen only for *petL* and *ndhB* (Fig. 3), which is in agreement with the corresponding qRT-PCR results (Fig. 2). No decreases in transcript accumulation were observed for *cp31b* mutants, again reflecting the qRT-PCR data. These findings suggest that only *CP31A* and not *CP31B* is required for stabilizing a limited number of transcripts and that *CP31A* is most important for *ndhF* mRNA.

Our data show that decreases in RNA stability and RNA editing are not generally correlated in *cp31* mutants. Still, it remained possible that independent of RNA steady-state levels, changes in transcription could secondarily affect RNA editing. To test this, we assayed transcription activity for selected genes by run-on analysis. No differences were found between *cp31a* plants and WT plants (Fig. S5*A*). Internal ratios between different probes of a single experiment (e.g., *ndhF* to *rrn16*) are similar between WT and *cp31a* plants. This demonstrates that *CP31A* does not affect transcription initiation of the assayed genes. Therefore, neither transcription defects nor steady-state RNA accumulation defects correlate with editing defects, which argues against the idea that changes in RNA synthesis and degradation are the primary cause for editing defects.

***cp31a* Mutants Are Defective in Postillumination Reduction of the Plastoquinone Pool.** With *ndhF* being a major target of *CP31A*, we decided to investigate whether the NADH dehydrogenase (NDH)

Table 2. Editing efficiency in *cp31a* mutants for selected sites as determined by cloning of amplified cDNA

	Col-0*	*cp31a*-1*	*cp31a*-3*
ndhD-2	95% (38/40)	97.5% (39/40)	100% (40/40)
ndhD-3	100% (40/40)	60% (24/40)	65% (26/40)
ndhD-4	100% (40/40)	60% (24/40)	67.5% (27/40)
ndhD-5	97.5% (39/40)	60% (24/40)	72.5% (29/40)
ndhF-1	100% (20/20)	50% (10/20)	n.d.
rps14-1	96% (48/50)	100% (100/50)	92% (46/50)
rps14-2	94% (47/50)	68% (24/50)	68% (24/50)

*Editing efficiency is calculated from the number of clones that do show editing at the respective site vs. the total number of clones analyzed (in parentheses). Reduced editing efficiencies are indicated in bold. Full data set is found in Table S1. n.d., not determined.

complex shows a decline in activity in *cp31a* mutants. The chloroplast NDH complex is believed to catalyze electron transfer from the stromal pool of reductants to plastoquinone (PQ), which leads to a transient increase in chlorophyll *a* fluorescence after the offset of actinic light (29–31). This increase was observed in WT plants and also in *cp31b* mutants (Fig. 4). By contrast, *cp31a* mutants behaved like *crr2*-2 control plants, because they did not exhibit this characteristic rise in postillumination fluorescence. CRR2 is an RNA-binding protein required for the expression of the NDH complex (32). Identical fluorescence phenotypes were also reported in direct knockout lines of different *ndh* genes (29, 30, 33). These results suggest that cpRNPs are required for NDH complex activity, likely via stabilization of *ndhF* mRNA and editing of *ndhF*, *ndhB*, and *ndhD* mRNAs.

Other fluorescence parameters were assessed as well. However, neither maximum nor effective quantum yield nor non-photochemical quenching (qP) was affected in *cp31a* or *cp31b* mutants, which indicates that there is no impairment of electron transport in these plants (Table S5). This is also supported by the normal accumulation of chloroplast-encoded subunits of the 4 major thylakoid membrane complexes. AtpB, PetD, PsaD, and D1 proteins accumulate to levels similar to WT controls in *cp31* mutants (Fig. S5*B*).

Discussion

We show here that cpRNPs are involved in chloroplast RNA editing and are required for transcript accumulation in vivo. The most pronounced defect observed on a transcriptome-wide scale was the loss of *ndhF* transcripts in *cp31a* plants. *ndhF* codes for an essential subunit of the NDH complex. The loss of *ndhF* mRNA suggests that *cp31a* mutants lack the NdhF protein, and thus NDH complex activity. This assumption is supported by fluorescence analysis. As shown in numerous previous studies, the postillumination reduction of the PQ pool no longer occurs if the NDH complex is disrupted. This is what is observed in *cp31a* mutants. As with other *ndh* mutants described to date, no visible developmental phenotype is observed (29). The function of the NDH complex is still under debate, but roles in cyclical electron transport processes in the thylakoid membrane and in stress tolerance have been postulated (29, 31, 34, 35). Future experiments are required to determine whether *cp31A* functions in adjusting *ndhF* expression, and thus the entire NDH complex, under different conditions. Such a specific function is highly surprising, given that cpRNPs have been thought to have general functions in RNA metabolism such as a universal protective role against degradation (17). Because cpRNPs are regulated in a light-dependent manner (17), it will be of great interest to determine whether they adjust the levels of the chloroplast NDH complex to environmental cues.

The plant organellar RNA editing machinery is still largely unknown. Our data show that cpRNPs facilitate RNA editing at selected sites. Two lines of evidence support that this role in RNA

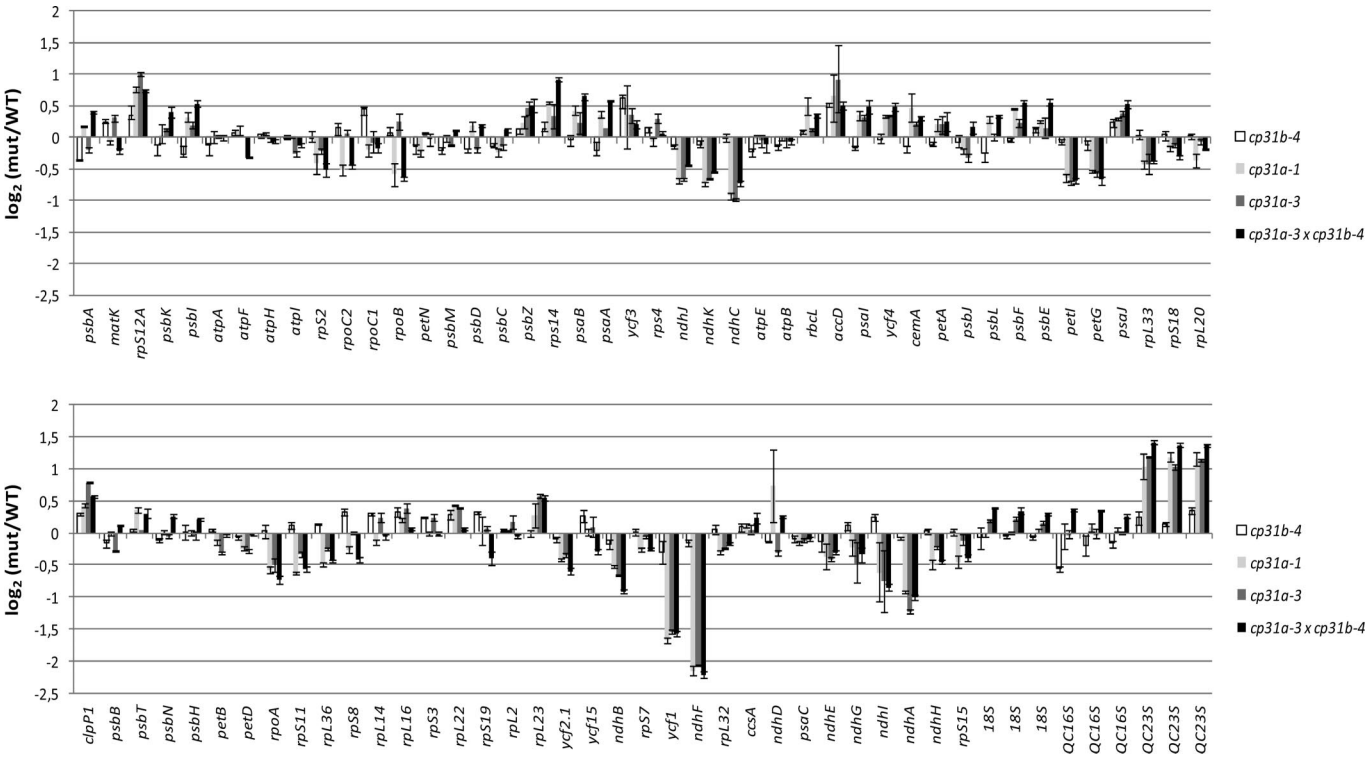

Fig. 2. Levels of plastid transcripts in *cp31* mutants. All chloroplast mRNA and rRNA transcripts were measured from *cp31* mutants and WT plants by qRT-PCR. The graph depicts the $\log_2$ ratio of transcript levels in the mutants compared with levels in the WT plants. The strongest decrease in transcript level was seen for the *ndhF* mRNA. The genes are sorted according to their physical location on the chloroplast chromosome. The full data set is given in Table S3.

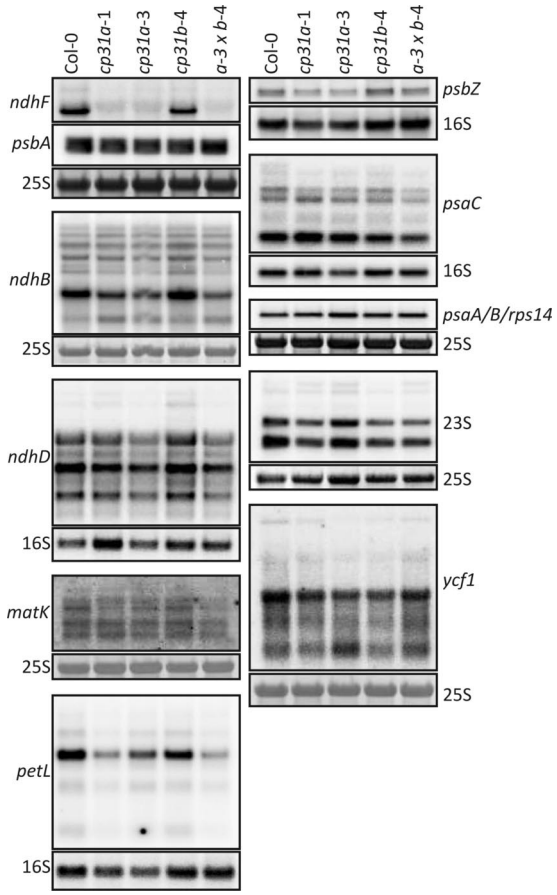

Fig. 3. RNA gel blot hybridizations showing plastid RNA accumulation for selected genes in *cp31* mutants. Total leaf RNA was fractionated on 1.2% agarose gels and analyzed by hybridization to probes for the plastid RNAs indicated. Equal loading was controlled by subsequently hybridizing filters to a probe for 16S rRNA and/or by staining the cytosolic 25S rRNA (25S) with methylene blue. Note that the filter probed for *ndhF* was subsequently also probed for *psbA*, which, together with the methylene blue stain of the cytosolic 25S rRNA, provides 2 internal loading controls. In the case of *rps14*, the tricistronic *psaA/psaB/rps14* mRNA is shown, because we were unable to detect a monocistronic *rps14* mRNA.

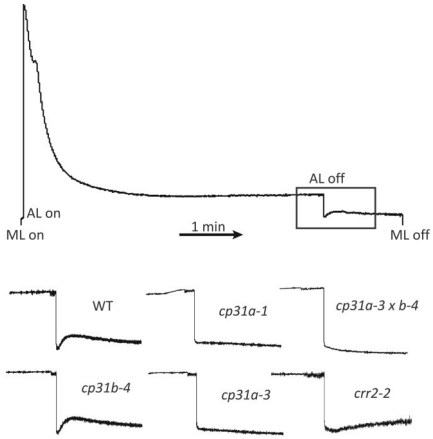

Fig. 4. Monitoring of NDH activity by chlorophyll *a* fluorescence analysis. (*Upper*) The curve shows a typical trace of chlorophyll *a* fluorescence in WT seedlings (WT: Col-0). Leaves were exposed to actinic light (AL; 50 μmol photons $m^{-2}\,s^{-1}$) for 5 min. AL was turned off, and the subsequent change in fluorescence level was monitored. (*Lower*) The postillumination chlorophyll *a* fluorescence curve is a characteristic feature of NDH activity, and was therefore magnified from the boxed area for all genotypes analyzed. ML, measuring light.

editing is a direct one. First, RNA editing defects are site-specific; only 13 of the 34 known sites are affected in *cp31a* plants. Importantly, in transcripts with multiple editing sites, only a subset shows defects. This was observed for *ndhB* (5 of 9 sites), *ndhD* (3 of 5 sites), and *rps14* (1 of 2 sites). Such specificity is difficult to explain by general effects on transcript accumulation and stability. Second, RNA editing defects do not correlate with altered transcript levels in *cp31* mutants. It is known that RNA editing may be decreased if transcript levels increase high enough to saturate the editing machinery (28, 36) or if RNA degradation reduces the time available for the RNA targets to be bound and edited (37). The latter explanation might hold for those transcripts reduced in *cp31a* such as *ndhF*, *petL*, and *ndhB*. However, given that levels for most RNAs with reductions in editing remain constant in *cp31* mutants and that transcription remains stable for RNAs analyzed here, it seems unlikely that increased RNA decay accounts indirectly for all observed editing defects.

Taken together, the data presented here suggest that cpRNPs have a direct role in RNA editing. This role is almost certainly not a catalytic one, because the small cpRNP proteins almost entirely consist of 2 RNA binding domains and a short acidic domain (8). This leaves little room for hitherto unrecognized domains capable of catalyzing C-to-U transitions. A more likely role for cpRNPs would lie in supporting recognition of editing site *cis*-elements by other factors, for example, by PPR proteins (3–5). This could be achieved by binding in the vicinity of editing sites, and thus preparing the RNA for entry of additional factors. The short sequence elements found here to be enriched close to CP31-dependent editing sites could be part of a consensus binding site for the CP31A and/or CP31B proteins.

The wide target range, combinatorial editing effects of *CP31A* and *CP31B*, and their variable impact on diverse gene expression steps are first indications that cpRNPs could act in a way similar to the hnRNP protein family. hnRNPs, like cpRNPs, are abundant proteins that bind to various precursor RNAs and participate in multiple RNA processing events. hnRNPs do act in large heterogenous complexes (as do cpRNPs, ref. 12) and are arranged in a unique combinatorial way on each mRNA (11). In the end, the fate of the mRNA (its processing and localization) is determined in a combinatorial fashion depending on which hnRNPs are present on a specific RNA (11). An important future experiment will be to analyze the composition of cpRNP complexes; in particular, it will be of great interest to determine whether cpRNPs interact with PPR proteins involved in RNA editing and/or whether they block or recruit RNases involved in transcript degradation. Similar questions pertain to nuclearly encoded RRM proteins targeted to mitochondria called mitochondrial RNA binding proteins (mRBPs). Here too, multiple roles for these proteins in organellar RNA metabolism have been predicted (38). The prospect that the eukaryotic cell has applied typical eukaryotic features of gene expression to control endosymbiontic prokaryotic RNA processing opens up exciting inroads into understanding gene regulation in chloroplasts.

Materials and Methods

Plant Materials and Growth Conditions. T-DNA insertion lines for *Arabidopsis* accessions used in this study (*cp31a*-1: SALK_109613, *cp31a*-3: SAIL_258_h02, and *cp31b*-4: WiscDsLox383H9) were obtained from the European Arabidopsis Stock Centre in Nottingham (22). WT *Arabidopsis thaliana* (ecotype Columbia) and T-DNA insertion mutants in the Columbia background were grown on soil in a growth chamber under short-day conditions (8-h day/16-h night) at 23 ± 1 °C.

Chlorophyll Fluorescence Analysis. In vivo chlorophyll *a* fluorescence of single leaves was monitored using the Dual-PAM-100 fluorometer (Walz) in ambient air at room temperature. Plants were dark-adapted for 30 min, and minimum fluorescence (F_0) excited by weak measuring light (settings: measuring light, 5) at

open photosystem (PSII) centers was measured. Then, pulses (0.8 s) of white light (5000 μmol photons $m^{-2} s^{-1}$) were used to determine the maximum fluorescence (F_m) at closed PSII centers, and the ratio $(F_m - F_0)/F_m = F_v/F_m$ (maximum quantum yield of PSII) was calculated. A 15-min illumination with actinic light of 80 μmol photons $m^{-2} s^{-1}$ (settings: actinic light, 5) was supplied to drive electron transport between PSII and PSI. Then, steady-state fluorescence (F_s) and the maximum fluorescence (F'_m) under actinic light illumination were determined. The effective quantum yield of PSII (Φ_{II}) was calculated as $(F'_m - F_s)/F'_m$, and the qP was calculated as $(F'_m - F_s)/(F'_m - F_0)$. The transient increase in chlorophyll fluorescence after actinic light had been turned off was monitored as described (31).

Genotyping of T-DNA Insertion Lines. Total cellular DNA was isolated and amplified using the Extract-N-Amp Plant PCR Kit (Sigma) according to the manufacturer's protocol. Plants were genotyped for homozygous lines by PCR, and integration of the T-DNA within the respective gene was confirmed by sequencing with both a gene-specific and a T-DNA–specific left border primer.

Western Blot Analyses. Total cellular proteins were isolated from leaf tissue as described (39). Standard Western blot analysis (40) was carried out to confirm null alleles using tobacco anti-CP31 antisera, as described previously (18).

Northern Hybridization Analyses. Total RNA was separated on a 1.2% (w/v) MOPS-formaldehyde agarose gel and transferred onto Hybond N membranes (GE Healthcare). Templates for riboprobes were amplified by PCR from *Arabidopsis* total cellular DNA or cDNA (see Table S4 for primers). In vitro transcription was performed with the Maxiscript Kit (Ambion) using 150 ng of PCR product as template in the presence of ^{32}P-UTP. Prehybridization of membranes was carried out for 30 min in Church hybridization buffer [0.5 M NaPO4 (pH 7.2)/7% wt/vol SDS]. Hybridization with the riboprobes was carried out in the same buffer overnight at 65 °C, followed by washes at the same temperature for 1 × 10 min in 0.5× SSC/0.1% SDS (1× SSC = 0.15 M sodium chloride/0. 015 M sodium citrate), 1 × 10 min in 0.2× SSC/0.1% SDS, 1 × 10 min in 0.1× SSC/0.1% SDS, and 1 × 10 min in 0.05× SSC/0.1% SDS. See *SI Text*.

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