

Amino acid sequence variations in *Nicotiana* CRR4 orthologs determine the species-specific efficiency of RNA editing in plastids

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ABSTRACT

In flowering plants, RNA editing is a posttranscriptional process that converts specific C to U in organelle mRNAs. *Nicotiana tabacum* is an allotetraploid species derived from the progenitors of *Nicotiana sylvestris* and *Nicotiana tomentosiformis*. These *Nicotiana* species have been used as a model for understanding the mechanism and evolution of RNA editing in plastids. In *Nicotiana* species, the *ndhD-1* site is edited to create the translational initiation codon of *ndhD* that encodes a subunit of the NAD(P)H dehydrogenase (NDH) complex. An analysis of this RNA editing revealed that editing efficiency in *N. tomentosiformis* is lower (15%) than that in *N. tabacum* (42%) and *N. sylvestris* (37%). However, this level of editing is sufficient for accumulating the NDH complex and its activity. The heterologous complementation of *Arabidopsis crr4-3* mutant, in which RNA editing of *ndhD-1* is completely impaired, with *CRR4* orthologous genes derived from *Nicotiana* species suggested that the reduction in editing efficiency in *N. tomentosiformis* is caused by amino acid variations accumulating in *CRR4*.

INTRODUCTION

RNA editing is a posttranscriptional process in which a specific C nucleotide is altered to U in an RNA molecule in the mitochondria and plastids of flowering plants (1–3). In contrast, U to C conversions occur frequently in both ferns and hornworts (4,5). In flowering plants, about 30 editing sites have been detected in plastid genomes and more than 400 editing sites in mitochondrial

genomes (6–9). In contrast to other RNA maturation steps in plant organelles including RNA splicing, intergenic RNA cleavage and RNA stabilization, RNA editing sites are highly divergent among species. Unlike introns, which are phylogenetically conserved in their positions, and structures in plastids, even closely related species exhibit distinct editing site patterns (10–13), suggesting the dynamic evolution of editing sites even in current establishments of species. An analysis of transplastomic lines suggested that the cognate editing factors corresponding to specific editing sites are co-evolving rapidly: spinach- and maize-specific sites introduced into the tobacco plastid genome remained unedited (14,15). In addition, a tobacco-specific editing site was not edited in a pea *in vitro* editing system (16). Thus, the RNA editing machinery in plastids appears to be phylogenetically dynamic.

Recent work employing plastid transformation and *in vitro* RNA editing system has shed some light on the molecular mechanisms of plastid RNA editing. For site-specific RNA editing in plastids, a *cis*-element is essential and consists of fewer than 30 nt surrounding the editing site and also a further upstream sequence in some cases (17–20). The case is similar for mitochondria (21). In addition, a genetic study of photosynthetic electron transport led to the discovery of nucleus-encoded factors responsible for specific RNA editing events. The *Arabidopsis crr4* (*chlororespiratory reduction*) and *crr21* mutants are defective in RNA editing for sites 1 (*ndhD-1*) and 2 (*ndhD-2*), respectively, in *ndhD* mRNA (22,23). The *ndhD* gene encodes a subunit of the chloroplast NAD(P)H dehydrogenase (NDH) complex, which is involved in the cyclic electron flow around photosystem I (24). *CRR4* and *CRR21* genes both encode members of the pentatricopeptide repeat (PPR) protein family (22,23). More recently, it was found that *Arabidopsis* PPR protein, CLB19, is involved in RNA editing of *rpoA* and

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clpP transcripts (25). PPR proteins form one of the very large protein families that are present in higher plant genomes, and which have 450 members in *Arabidopsis* and 477 in rice (26). The family members are defined by the tandem array of a PPR motif, which is a highly degenerate unit consisting of 35 amino acids (27). Current evidence indicates that PPR proteins are generally involved in almost all stages of gene expression, including transcription (28), splicing (29,30), RNA cleavage (31–33), RNA editing (22,23,25), translation (34,35) and RNA stabilization (36), in both plastids and mitochondria. The most probable explanation for these divergent roles is that a PPR protein is a sequence-specific RNA binding adaptor capable of directing an effector enzyme to the defined site on mRNA. Consistent with this idea, we showed that the recombinant CRR4 binds to the sequence surrounding its target editing site, confirming that a PPR protein is a *trans*-factor essential for recognizing the RNA editing site (37).

Nicotiana tabacum (tobacco) is a model plant in chloroplast molecular biology and its entire genome sequence was determined early in the history of plant molecular biology (38). The genome sequences of other *Nicotiana* species were also completely determined (39), and their RNA editing sites were determined in a systematic search (12,40). In addition, of the species whose plastids can be transformed (41), only in tobacco is the *in vitro* RNA editing system (18) also available. Therefore, *Nicotiana* species are the best choice for analyzing the detailed mechanism of RNA editing in chloroplasts, as well as *Arabidopsis* for a genetic approach. *Nicotiana tabacum* is a natural amphidiploid derived from two progenitors, which are likely to be ancestors of *Nicotiana sylvestris* (female parent) and *Nicotiana tomentosiformis* (male parent) (42). The chloroplast genome of *Nicotiana tabacum* is believed to have originated from *N. sylvestris* (39). Hence, a comparative analysis of the editing sites of these *Nicotiana* species is expected to provide clues for a better understanding of the evolution of editing events in plastids. Recently, Sasaki *et al.* (12) reported that the *ndhD-1* site is edited to create a translational initiation codon in *N. sylvestris* as well as in *N. tabacum* but not in *N. tomentosiformis*. On the basis of these results, the authors suggested that editing of this site is dispensable and *N. tomentosiformis* lost its site-specific *trans*-factor with editing activity. Zondueta-Criado and Bock (43) showed that unedited *ndhD* transcripts are associated with polysomes *in vivo*, suggesting that they are translated. However, this idea is inconsistent with our finding that RNA editing in this site is essential for accumulating the NDH complex in *Arabidopsis* (22). Based on the determined physiological function of the NDH complex (44), it is likely that *N. tomentosiformis* expresses the *ndhD* gene.

Here, we show that *N. tomentosiformis* has lower editing efficiency at the *ndhD-1* site compared with those of other *Nicotiana* species, but the level of editing is sufficient for the accumulation of the NDH complex. We also report the identification of *CRR4* orthologous genes in *Nicotiana* species. The heterologous complementation of an *Arabidopsis crr4* mutant by *Nicotiana CRR4* genes indicated that the lower editing efficiency at the *ndhD-1* site is

due to lower CRR4 activity or stability in *N. tomentosiformis*. On the basis of these findings, we discuss the difference between the editing efficiencies of the *ndhD-1* site for different *Nicotiana* species and its physiological and evolutionary meaning.

MATERIALS AND METHODS

Plant materials

Nicotiana tabacum (L. var. Xanthi), *N. sylvestris* and *N. tomentosiformis* leaves were harvested from 6-week-old plants grown in a growth chamber at 28°C under 16 h light/8 h dark conditions.

Chlorophyll fluorescence analysis

Chlorophyll fluorescence was measured by using a MINI-PAM (pulse amplitude modulation) portable chlorophyll fluorometer (Walz, Effeltrich, Germany). The transient increase in chlorophyll fluorescence after turning off the actinic light (AL) was monitored as previously described (45).

Analysis of RNA editing

Total RNA was isolated from green leaves of *Nicotiana* species by using an RNaseasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and treated with DNase I (Invitrogen, Carlsbad, CA, USA). DNA-free RNA (2.5 µg) was reverse-transcribed with random hexamers. The sequence including the *ndhD-1* editing site was amplified by PCR with primers *ndhD-1-FW* and *ndhD-1-RV* (Supplementary Data 1). The RT-PCR products were sequenced directly. To analyze the editing efficiency of *ndhD-1*, the sequence including the *ndhD-1* editing site was amplified by PCR using the primers *ndhD-1-FW2* and *ndhD-1-RV* (Supplementary Data 1). The RT-PCR products were cloned into the pTAC-1 vector (BioDynamics Laboratory, Tokyo, Japan) and transformed in *Escherichia coli*. PCR products were amplified by using primers *BD-FW* and *BD-RV* (Supplementary Data 1) from 100 independent clones, digested with *NlaIII* and analyzed with 8% polyacrylamide gel.

For analysis of the RNA editing efficiency of *ndhD-1* in *Arabidopsis*, cDNAs were isolated as previously described (22). The sequence including the *ndhD-1* editing site was amplified by PCR using the primers *AtndhD-1-FW* and *AtndhD-1-RV* (Supplementary Data 1). The RT-PCR products were cloned into the pTAC-1 vector (BioDynamics Laboratory) and transformed in *E. coli*. PCR products were amplified by using primers *BD-FW* and *BD-RV* (Supplementary Data 1) from 100 independent clones, digested with *NlaIII* and analyzed with 8% polyacrylamide gel. For further high-throughput quantitative analysis of editing efficiency, the RT-PCR products, which were digested with *NlaIII*, were analyzed with QIAxcel System (Qiagen). The efficiency of RNA editing was quantified by comparing the signal intensity of the sensitive and resistant DNA fragments to *NlaIII* using the software of Biocalculator (Qiagen).

Protein blot analysis

Chloroplasts were isolated from the leaves of 4-week-old plants as previously described (22). The protein samples were separated by 12.5% SDS-PAGE and used for immunodetection.

Isolation and sequence analysis of cDNAs

ESTs encoding for protein that shows a high sequence identity with *Arabidopsis thaliana* *CRR4* (*AtCRR4*) was sought by the RIKEN Transcriptome Analysis of BY-2, EST search database (<http://mrg.psc.riken.go.jp/struc/blast.html>). Total RNA was isolated from green leaves of *Nicotiana* species by using an RNaseasy Plant Mini Kit (Qiagen) and treated with DNase I (Invitrogen). DNA-free RNA (2.5 µg) was reverse-transcribed with oligo(dT)₂₀ primer. cDNA was amplified by PCR using appropriate primers designed from the EST sequence and the resulting DNAs were cloned into the pGEM-T vector (Promega, Madison, WI, USA), and its sequence was determined.

The 5'-terminal portion of the cDNA was isolated by 5'-rapid amplification of cDNA ends (5'-RACE) using primers designed from the sequences determined in this study. The procedure is as described in the instruction manual for the 5'-RACE System for Rapid Amplification of cDNA Ends, version 2.0 Kit (Invitrogen) and the resulting cDNAs were cloned into the pGEM-T vector (Promega), and their sequences were determined. The 3'-terminal portion of the cDNA was isolated by 3'-rapid amplification of cDNA ends (3'-RACE) using primers designed from the sequences determined in this study. The procedure is as described in the instruction manual for 3'-Full RACE Core Set (Takara, Kyoto, Japan), and the resulting cDNAs were cloned into the pGEM-T vector (Promega), and their sequences were determined.

Plant transformation

For heterologous complementation of *crr4-3*, the nucleotide sequences encoding the putative *CRR4* orthologous genes were amplified from the genomic DNA of *N. sylvestris* (*NsylCRR4*) and *N. tomentosiformis* (*NtomCRR4*) by PCR using primers NsylCRR4-FW and -RV and NtomCRR4-FW and -RV, respectively (Supplementary Data 1). The amplified DNA fragments were translationally fused with the promoter and 5'-UTR of *AtCRR4* PCR-amplified using primers ProCRR4-FW and ProCRR4-RV (Supplementary Data 1). The resultant DNA fragments were subcloned into the pBIN19 vector and introduced into *crr4-3* via *Agrobacterium tumefaciens* MP90.

Quantitative real-time RT-PCR

Total RNA was isolated from green leaves of *Arabidopsis* by using an RNaseasy Plant Mini Kit (Qiagen) and treated with RNase-free DNase Set (Qiagen). DNA-free RNA (1.0 µg) was reverse transcribed with oligo(dT)₂₀ primer. For quantitative RT-PCR, gene-specific primers were designed for *NsylCRR4*, *NtomCRR4*, and a potential control gene, *AtACT8* (Supplementary Data 1).

Primers were validated using serial dilutions of purified plasmids containing the corresponding genes. Standard curves were plotted and primers with amplification efficiencies of $100 \pm 10\%$ were selected for quantitative RT-PCR analysis. Dissociation curves were performed after the PCR reaction to confirm that single, specific products were produced in each reaction. The PCR amplification reaction was carried out with the Mx3000P QPCR system (Stratagene, LA Jolla, CA, USA). PCR reaction mixture (20 µl) contained 10 µl of 2 × Brilliant II SYBR Green QPCR Master Mix, 0.5 µM of each primers and 1 µl of a 20th diluted cDNA template. The PCR reaction was 95°C for 10 min, 40 cycles of 95°C for 30s and 60°C for 1 min and then the fluorescence was measured at each cycle at 60°C. Threshold cycle (C_t) values from triplicate samples were averaged and the SD value was calculated. The logarithmic average C_t value for each gene and the control gene was converted to a linear value using the 2^{-C_t} term (46). Converted values were normalized to the *AtACT8* gene by dividing average value for each gene by the average value of the control gene *AtACT8*.

RESULTS

NDH complex is active in *N. tomentosiformis*

The *ndhD-1* site is edited to create the translational initiation codon of *ndhD* in *N. tabacum* and *N. sylvestris*, but the site was reported to remain unedited in *N. tomentosiformis* (12). To investigate whether the NDH complex is functional in *N. tomentosiformis*, we analyzed NDH activity by using PAM fluorometry in *Nicotiana* species. The chloroplast NDH complex catalyzes electron donation to plastoquinone from the stromal electron pool and its activity can be monitored as a transient increase in chlorophyll fluorescence after turning off AL (45). The increase in fluorescence is due to the reduction of plastoquinone by the stromal electron pool, which accumulates during AL illumination. Figure 1 shows a typical trace of the chlorophyll fluorescence level in *N. tabacum*, in which a transient increase in chlorophyll fluorescence after AL illumination was detected. In tobacco *AndhB* mutant, in which the *ndhB* gene was disrupted by the insertion of an *aadA* cassette (45), this transient increase in fluorescence level was suppressed (Figure 1), indicating that NDH activity is impaired. In *N. tomentosiformis*, the transient increase in fluorescence level was detected as well as in *N. tabacum* and *N. sylvestris* (Figure 1), indicating that NDH complex is active in *N. tomentosiformis*.

RNA editing activity of *ndhD-1* site is reduced in *N. tomentosiformis*

The detection of NDH activity in *N. tomentosiformis* suggests that the *ndhD* mRNA would be translated. The *crr4* phenotype indicates that the translation is severely suppressed in the absence of this RNA editing (22). To investigate whether the site is also edited in *N. tomentosiformis*, RNA editing of the *ndhD-1* site was analyzed by directly sequencing RT-PCR products (Figure 2A).

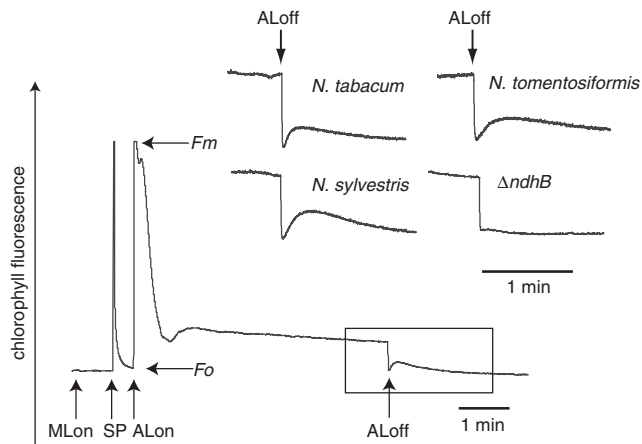


Figure 1. Monitoring of NDH activity using chlorophyll fluorescence analysis. The central pattern shows a typical trace of chlorophyll fluorescence in the wild-type tobacco leaves. The leaves were exposed to AL ($50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 5 min. The AL was turned off and the subsequent change in chlorophyll fluorescence level was monitored. The insets are magnified traces taken from the boxed area. The fluorescence levels were normalized by the maximum fluorescence at closed PSII centers in the dark (F_m) levels. ML, measuring light; SP, saturating pulse of white light.

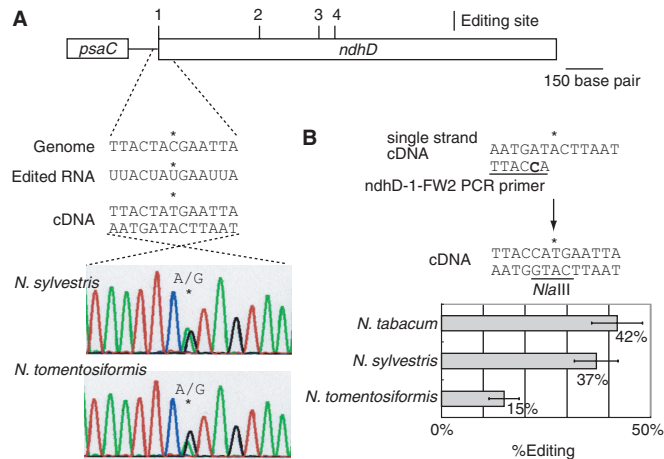


Figure 2. Analysis of RNA editing of *ndhD-1*. (A) Direct sequencing of RT-PCR products containing the *ndhD-1* editing site. The target C is shown by an asterisk. (B) Analysis of the extent of RNA editing in the *ndhD-1* site. The editing efficiency of *ndhD-1* was analyzed as described in Materials and methods section. The primer *ndhD-1-FW2* was used to create the restriction enzyme site of *NlaIII* that specifically cleaves cDNA derived from edited molecules. RT-PCR products were cloned in *E. coli* and were digested with *NlaIII*. The ratio of the clones that originated from the edited RNA molecule is indicated with gray bars. Results are expressed as the total averages of clone analyzes using three independent plants. Approximately 100 clones were analyzed in each plant.

Consistent with a previously reported result (12), the *ndhD-1* site was partially edited in leaves of both *N. tabacum* and *N. sylvestris* (Figure 2A). However, in contrast with the previous results, the *ndhD-1* site was also partially edited in leaves of *N. tomentosiformis* (Figure 2A).

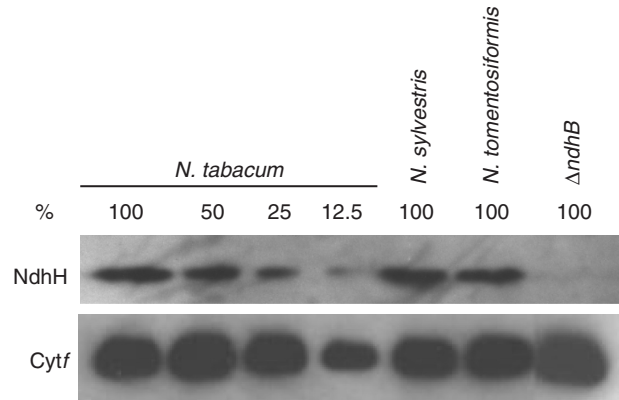


Figure 3. Protein blot analysis of the NDH complex. Immunodetection of NDH subunits, NdhH; and a subunit of the cytochrome b_6/f complex, Cytf (control). Proteins were extracted from the thylakoid membrane fractions. Lanes were loaded with protein samples corresponding to $0.5 \mu\text{g}$ chlorophyll for Cytf and $5 \mu\text{g}$ chlorophyll for NdhH (100%) and the series of dilutions indicated.

To estimate the efficiency of RNA editing more quantitatively, cDNA including the editing site was amplified by PCR and cloned in *E. coli*. One hundred independent clones were analyzed by digestion with *NlaIII* to detect cDNA originating from the edited RNA molecules. The experiment was repeated two more times with different plants. Of the RNA molecules, 42% and 37% were edited in *N. tabacum* and *N. sylvestris*, respectively (Figure 2B). The editing efficiency was significantly lower (15%) in *N. tomentosiformis* (Figure 2B). These results indicate that *N. tomentosiformis* does not lose the RNA-editing activity of *ndhD-1*, although the editing efficiency remains lower than that in *N. tabacum* and *N. sylvestris*.

Lower efficiency of RNA editing does not limit accumulation of sufficient NdhD

Nicotiana tomentosiformis shows lower *ndhD-1* editing efficiency than *N. tabacum* and *N. sylvestris* (Figure 2). This lower editing efficiency may affect the level of the NDH complex in *N. tomentosiformis*, although its activity was detected (Figure 1). To assess this possibility, protein blot experiments were performed using an antibody raised against NdhH. The NdhH subunit is unstable in the absence of the NdhD subunit and the antibody can be used to monitor the accumulation of NDH complex (22). In *N. tomentosiformis*, the NdhH level was comparable to that of *N. tabacum* and *N. sylvestris* (100%) (Figure 3). This result suggests that the lower level of RNA editing (15%) does not limit the level of the NDH complex because an adequate amount of the NdhD subunit evidently accumulates despite the absence of an AUG start codon on 85% of the transcripts.

Isolation of putative *CRR4* orthologous genes in *N. sylvestris* and *N. tomentosiformis*

What is the determinant for the lower editing efficiency of *ndhD-1* in *N. tomentosiformis*? The recognition of a *cis*-element by a *trans*-factor is essential for site-specific

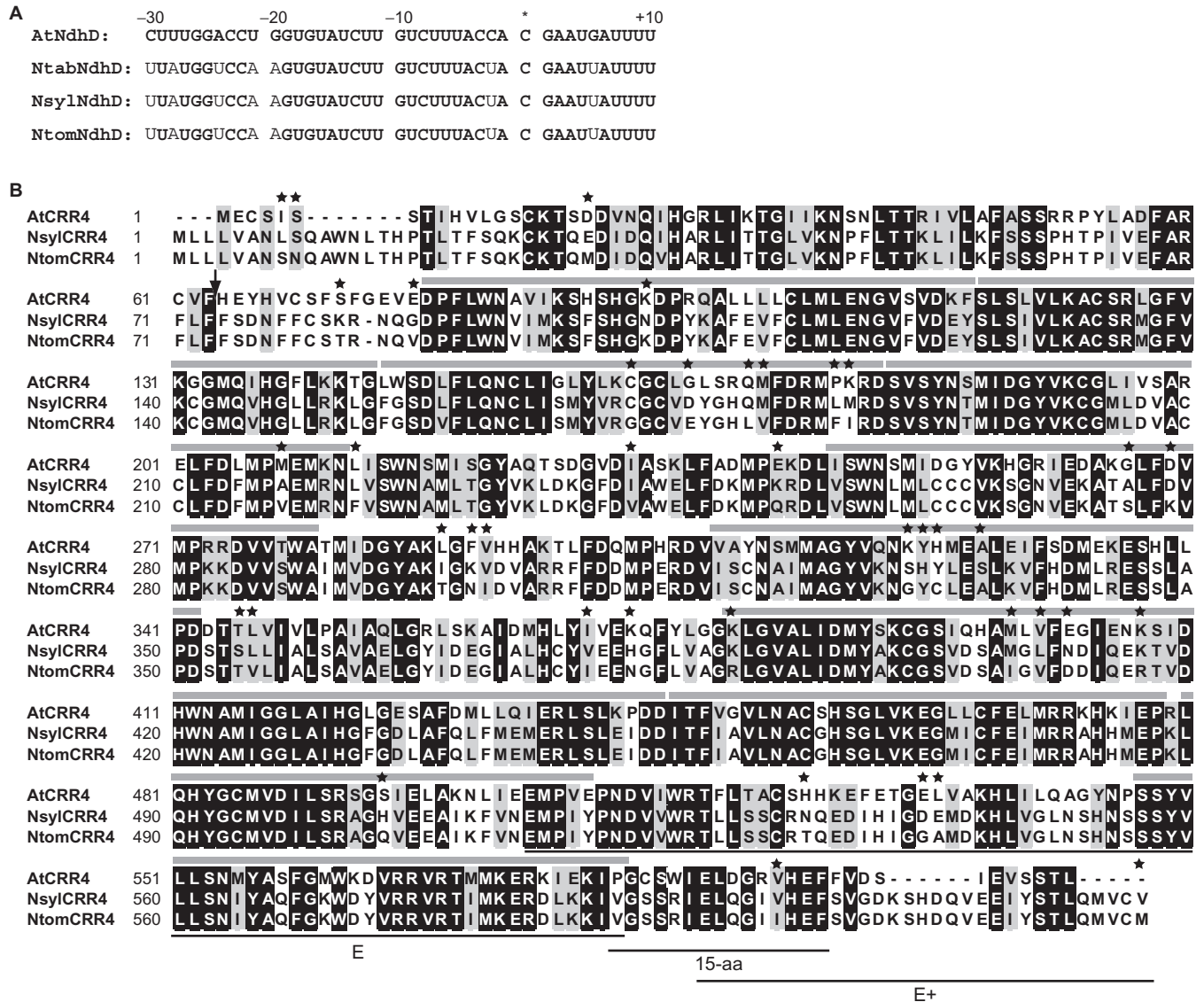


Figure 4. Comparison of the *cis*-elements and *trans*-factors of *Arabidopsis* and *Nicotiana* species. (A) Comparison of the nucleotide sequences in the regions (–30 to +10) surrounding the *ndhD*-1 site. Nucleotides that are identical to those in the *cis*-element of the *ndhD*-1 site in *Arabidopsis* are shown in bold. The target C is shown by an asterisk. *Arabidopsis thaliana* (AtNdhD), *N. tabacum* (NtabNdhD), *N. sylvestris* (NsylvNdhD) and *N. tomentosiformis* (NtomNdhD). (B) Multiple sequence alignment of *Arabidopsis* CRR4 (AtCRR4) with its *Nicotiana* orthologous proteins (NsylvCRR4 and NtomCRR4). Identical residues are shaded in black, and similar residues are shaded in gray. Gray bars above the sequences indicate the PPR motifs. Lines beneath the sequences indicate the E, the E+ and the 15-amino acid motifs. The transit peptide cleavage site predicted by the ChloroP 1.1 program is shown by an arrow. Numbers on the left indicate amino acid positions in protein. The positions of amino acid variation detected between NsylvCRR4 and NtomCRR4 are indicated by stars above the sequences.

RNA editing. In *ndhD*-1 RNA editing in *Arabidopsis*, a PPR protein, CRR4, specifically recognizes the *cis*-element of the *ndhD*-1 site, which consists of at least the 25-nt upstream and 10-nt downstream sequences surrounding the editing site (37). It is possible that *N. tomentosiformis* does not have either an appropriate *cis*-element or *trans*-factor for the efficient editing of *ndhD*-1. We first compared the 35 nt surrounding the *ndhD*-1 site among *Nicotiana* species to show that the sequence is completely conserved (Figure 4A). Furthermore, the *cis*-element is also similar to that of *Arabidopsis thaliana*, suggesting that the element is

recognized by a *trans*-factor similar to AtCRR4 in *Nicotiana* species.

We next investigated the possibility that the activity or the expression level of the *trans*-factor for the *ndhD*-1 RNA editing is reduced in *N. tomentosiformis*. We surveyed the RIKEN Transcriptome Analysis of BY-2, EST search database and found the EST that exhibits a high nucleotide sequence identity for AtCRR4 in *N. tabacum*. Based on this EST, full-length cDNAs were isolated from both *N. sylvestris* (NsylvCRR4) and *N. tomentosiformis* (NtomCRR4). They contain an open reading frame (ORF) of 1878 nt that encode a putative CRR4

orthologous protein of 626 amino acids (Figure 4B). PCR amplification of the genomic genes indicated that these genes are not disrupted by any introns in either *Nicotiana* species, which is not the case with *Arabidopsis* (data not shown). The program ChloroP 1.1 predicted that the first 79 amino acids were the target signal to the plastids (Figure 4B). NsylCRR4 and NtomCRR4 show 60% and 57% identity to AtCRR4, respectively (Figure 4B). In contrast, a BLAST search revealed that NsylCRR4 and NtomCRR4 did not exhibit significant sequence identity to any other PPR proteins in *Arabidopsis* (about 25% identity or less).

On the basis of bioinformatic analysis, the PPR protein family is subdivided into the P and PLS subfamilies (27). The PLS subfamily exhibits a variable tandem repeat of a standard pattern of three PPR motifs (27). Based on the association or not of this repeat with three non-PPR motifs at their C-terminus, the PLS subfamily is subdivided into a further four subgroups; PLS, E, E + and DYW (27). Both NsylCRR4 and NtomCRR4 contain 11 characteristic PPR motifs and the E and E + motifs following a tandem array of PPR motifs, whose motif structures are very similar to AtCRR4 except that *Nicotiana* CRR4 has a longer E + motif than AtCRR4 (Figure 4B). The amino acid sequences of NsylCRR4 and NtomCRR4 were 93% identical (97% similar), indicating that 37 amino acids were divergent in the mature region between NsylCRR4 and NtomCRR4 (Figure 4B). Thirty-two amino acid alterations were detected in the N-terminal PPR motifs and the C-terminal E/E + motifs were highly conserved for NsylCRR4 and NtomCRR4 (Figure 4B).

Low editing efficiency at ndhD-1 site in *N. tomentosiformis* caused by low CRR4 activity

Given the high sequence similarity, it is likely that these *Nicotiana* proteins are *Arabidopsis* CRR4 orthologs. To confirm this possibility, NsylCRR4 and NtomCRR4 genes were expressed in *Arabidopsis* *crr4-3*, in which the RNA editing of the ndhD-1 site is completely impaired (22). NDH activity was detected in both transgenic lines (Figure 5A) and the NdhH level was also restored close to the wild-type level (Figure 5B). We also confirmed that the introduction of these genes restored the RNA editing of ndhD-1 in *crr4-3* (Figure 5C). We conclude that NsylCRR4 and NtomCRR4 are orthologs of AtCRR4 and function as *trans*-factors of ndhD-1 RNA editing in *Nicotiana* species. Consistent with the conservation of *cis*-elements among *Arabidopsis* and *Nicotiana* species (Figure 4A), the mechanism of the RNA editing of ndhD-1 is conserved.

We then investigated the possibility that the lower level of the editing efficiency of ndhD-1 in *N. tomentosiformis* is due to the low level of NtomCRR4 transcripts. To verify this possibility, the transcript level of endogenous CRR4 orthologs in two *Nicotiana* species was analyzed by quantitative RT-PCR. The transcript level in NtomCRR4 was about a half of that in NsylCRR4 (Supplementary Data 2).

Although the introduction of NtomCRR4 restored the accumulation of NDH complex in *crr4-3*, a quantitative analysis of the RNA editing efficiency using enzymatic digestion of PCR products from 100 independent clones clarified that the editing level was lower than those in the wild-type *Arabidopsis* and *crr4-3* complemented by the introduction of NsylCRR4 (Figure 5C). This result appears to reflect the difference between the editing efficiencies of *N. sylvestris* and *N. tomentosiformis* (Figure 2B). However, it is possible that the difference may simply reflect the expression of transgenes. To eliminate this possibility, we analyzed the transcript level of transgenes in 11 independent lines transformed with each gene using quantitative RT-PCR analysis (Figure 5D). The transcript levels of transgenes were similar between lines expressing the different *Nicotiana* CRR4 orthologs, although there are minor fluctuations in the transcript level between lines (Figure 5D). We also determined the efficiency of the RNA editing using the QIAxcel system, in which PCR products are quantitatively analyzed in the automated capillary-type electrophoresis. The average of editing efficiency in *crr4-3* complemented by NsylCRR4 and by NtomCRR4 were 39% and 19%, respectively (Figure 5D), consistent with the editing efficiency determined by cloning of PCR products (Figure 5C). We conclude that NtomCRR4 has slightly lower activity in editing of the ndhD-1 site in *Arabidopsis*, and the same story is probably true in *N. tomentosiformis*. We cannot eliminate the possibility that NtomCRR4 is slightly less stable than NsylCRR4 both in *N. tomentosiformis* and *Arabidopsis*.

DISCUSSION

In this study, we found that the ndhD-1 site is edited in *N. tomentosiformis*, although its efficiency is lower than those in *N. tabacum* and *N. sylvestris* (Figure 2). This observation is inconsistent with a previous report in which direct cDNA sequencing of this site could not detect any editing in *N. tomentosiformis* (12). A similar discrepancy was also reported in *Arabidopsis*. Although Lutz and Maliga (47) reported that the ndhD-1 site was not edited in *Arabidopsis* leaves, it was partially edited as detected in other plants (13,22). This disagreement would be explained in terms of the influences imposed by plant development, tissue type, age and environmental factors (48,49). Furthermore, the site is partially edited even in wild-type leaves, which makes it difficult to detect low levels of RNA editing under certain conditions using a low resolution method such as the direct sequencing of RT-PCR products.

Heterologous complementation by NtomCRR4 restored the *crr4-3* defect with lower efficiency (21%) compared with that realized by NsylCRR4 (40%) (Figure 5C and D). We believe that the difference reflects the variations in the CRR4 sequence. Since the efficiency was evaluated with an identical genetic background and both transgenes were driven by the AtCRR4 promoter, 5'- and 3'-UTRs and NOS terminator, we also believe that the difference in CRR4 activity reflected the RNA editing efficiency.

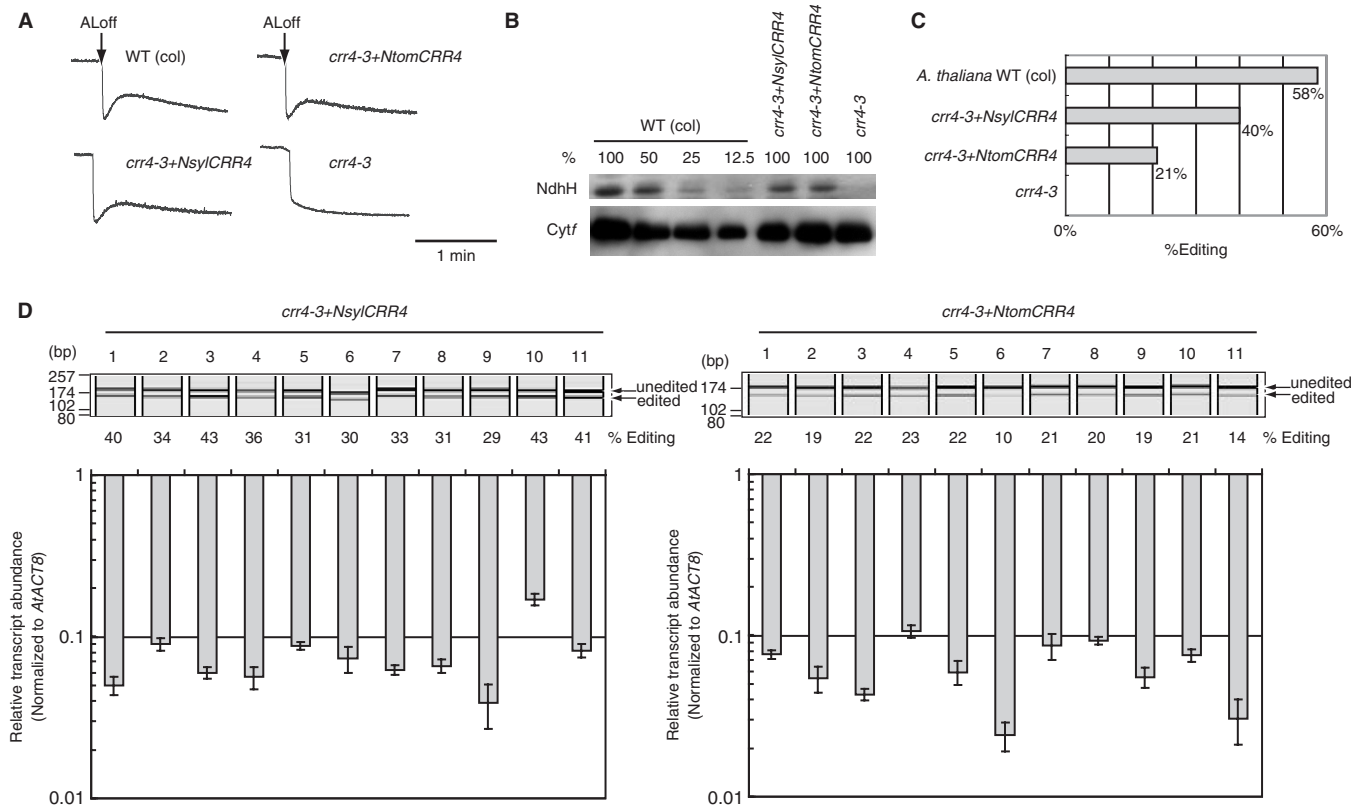


Figure 5. Heterologous complementation of the *Arabidopsis crr4-3* mutant by *Nicotiana CRR4* orthologs. (A) Analysis of the transient increase in chlorophyll fluorescence after turning off the AL. Leaves were exposed to AL ($50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 5 min. The AL was turned off and the subsequent change in chlorophyll fluorescence level was monitored. The fluorescence levels were normalized by the *Fm* levels. (B) Protein blot analysis of the NDH complex. Immunodetection of NDH subunits, NdhH and a subunit of the cytochrome *b₆f* complex, Cyt*f* (control). Proteins were extracted from the thylakoid membrane fractions. Lanes were loaded with protein samples corresponding to 0.5 μg chlorophyll for Cyt*f* and 5 μg chlorophyll for NdhH (100%) and the series of dilutions indicated. (C) Analysis of the extent of RNA editing in the *ndhD-1* site. The editing efficiency of the *ndhD-1* was analyzed as described in Materials and methods section. The ratio of the clones that originated from the edited RNA molecule is indicated with gray bars. Results are expressed as the total averages of clone analyzes using three independent transgenic lines in *crr4-3 + NsylCRR4* and two independent transgenic lines in *crr4-3 + NtomCRR4*. Approximately 100 clones were analyzed in each transgenic line. (D) The high-throughput quantitative analysis of the extent of RNA editing in the *ndhD-1* site and the transcript level of the transgene. Analysis of transgenic plants of *crr4-3 + NsylCRR4* and of *crr4-3 + NtomCRR4* is shown in the left and right panels, respectively. Upper panels are the electrophoregram by QIAxcel system. Eleven independent transgenic lines were analyzed for each genotype. The editing efficiency of the *ndhD-1* site is indicated under each lane. The positions of DNA size marker are indicated on the left of the panels. The quantitative real-time RT-PCR was carried out as described in Materials and methods section. The values are expressed as the mean \pm SD of three independent experiments. The transcript abundance of *NsylCRR4* and *NtomCRR4* normalized to the actin gene (*AtACT8*) was measured in 11 independent transgenic lines. *crr4-3 + NsylCRR4*, *crr4-3* complemented by introduction of the *CRR4* orthologous gene of *N. sylvestris*; *crr4-3 + NtomCRR4*, *crr4-3* complemented by introduction of the *CRR4* orthologous gene of *N. tomentosiformis*.

In fact, results from the quantitative RT-PCR analysis did not show the significant differences in transcript level between *NsylCRR4* and *NtomCRR4* lines (Figure 5D). We did not detect any tight correlations between the expression level of the transgene and the editing efficiency, except for two lines, #6 and #11, transformed with *NtomCRR4*, in which both transcript level and RNA editing efficiency were decreased (Figure 5D). We do not eliminate the possibility that the minor reduction in *NtomCRR4* transcript level in *N. tomentosiformis* somewhat influences the RNA editing efficiency. However, the RNA editing efficiency is significantly higher in *NsylCRR4* lines compared to that in *NtomCRR4* lines accumulating a similar level of transcripts (Figure 5D). Furthermore, the overexpression of *AtCRR4* under the

control of the cauliflower mosaic virus 35S promoter did not increase the efficiency of RNA editing, indicating that the efficiency of the RNA editing is not restricted by the *CRR4* transcript level (22,23). Thus, it is likely that difference in *CRR4* function determine the RNA editing efficiency between *N. sylvestris* and *N. tomentosiformis*, rather than the difference in gene expression. We do not eliminate the possibility that the amino acid variations of *CRR4* between *Nicotiana* species influence the protein stability and consequently the RNA editing efficiency. Due to the low accumulation level of *CRR4 in vivo*, we cannot evaluate the stability of *Nicotiana CRR4* (22,23). We conclude that the different efficiency of the RNA editing between lines complemented by *NsylCRR4* and *NtomCRR4* was reflected by different activity, and/or

stability possibly, of CRR4 in the heterogous system, and this idea provides a likely explanation of the lower RNA editing efficiency in *N. tomentosiformis*.

In flowering plants, molecules involved in plastid RNA editing have been identified only in *Arabidopsis*. This study first facilitated a direct comparison of the RNA editing machinery of different species, although indirect evidence also suggested the involvement of PPR protein in plastid RNA editing in tobacco (50). The discovery of AtCRR4 orthologs in *Nicotiana* species indicates that the RNA editing mechanism of the *ndhD-1* site is common at least in *Arabidopsis* and *Nicotiana* species. The translational initiation codon of the *ndhD* gene is generated by RNA editing in dicot plants (6). The *cis*-element of the *ndhD-1* site is highly conserved among dicot plants such as *Arabidopsis*, tobacco and tomato (Supplementary Data 3), implying that AtCRR4 orthologs also recognize the *ndhD-1 cis*-element in dicot plants. In contrast, the initiation codon of the *ndhD* gene is already encoded by ATG in the genome in monocots and angiosperm (6) and their sequences corresponding to the dicot *cis*-elements are not conserved (Supplementary Data 3). Bioinformatic analysis of the rice genome suggested that there are no CRR4 orthologous proteins in rice (private communication with Small I). These observations may suggest that the RNA editing system of the *ndhD-1* site was acquired during the divergence to monocots and dicot. This is the first molecular information on the coevolution of the RNA editing site and its cognate *trans*-factor.

Why does the editing efficiency of the *ndhD-1* site remain low in *N. tomentosiformis*? The *ndhD-1* site has a characteristic that distinguishes it from other editing sites. This RNA editing creates a translational initiation codon rather than altering the coding amino acid. This site is partially edited even in leaves where the *ndhD* gene is mainly expressed and its editing extent appears developmentally regulated (48), suggesting that the RNA editing may play a role in the regulation of *ndhD* translation. However, NDH activity and protein blot analysis suggested that 15% of the editing is sufficient for accumulating a level of NDH complex comparable to that observed in other species (Figure 3) and for activity detected in our chlorophyll fluorescence analysis (Figure 1). Thus, the low level of editing in the *ndhD-1* site may not be a result of regulation, although we cannot rule out the possibility that the RNA editing level limits translation under certain conditions. The population of *ndhD* mRNA with the translational initiation codon depends on the species, and the developmental and environmental conditions. This initiation codon is encoded by ATG in the genomes of monocots (6), suggesting that the regulation of RNA editing efficiency is not physiologically essential. The editing extents in tobacco, *Arabidopsis* and spinach are 45%, 61% and 41%, respectively (51). Taking account of our observation that 15% editing is sufficient for the function of the NDH complex in *N. tomentosiformis*, it may be more likely that the sites do not need to be edited completely. The RNA editing of *ndhD-1* in an etiolated seedling (25%) is lower than that in a green leaf (40%) (51), also suggesting that the translation of *ndhD* is regulated by RNA editing via light. However, the NdhH level of

N. tomentosiformis (15% editing) was comparable to those of *N. tabacum* (45% editing) and *N. sylvestris* (42% editing) (Figure 3). It is unclear whether the level of RNA editing (25%) limits the translation in etioplasts. Actually the NDH complex is already present in etioplasts and de-etiolation does not upregulate the accumulation (52). When we take account of all the information, the lower editing efficiency of *ndhD-1* in *N. tomentosiformis* might be a result of evolution permitting a reduction in editing efficiency simply because high editing efficiency was not required. PPR proteins appear to be evolutionarily highly flexible (53), and this fact might be essential to respond to newly occurring RNA editing sites in plant organelles.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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